EFFICACY AND SAFETY OF GLUCARPIDASE FOR ROUTINE USE AFTER HIGH DOSE METHOTREXATE IN PATIENTS WITH BONE SARCOMA

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DECLARATION

I, Martha Perisoglou confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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ABSTRACT

Background: High-dose methotrexate (HD-MTX) is an essential component of osteosarcoma treatment. Despite supportive measures MTX-related toxicity results in delays in subsequent chemotherapy administration and potentially reduced treatment efficacy. Alternative rescue regimens utilising glucarpidase in addition to folinic acid may be of benefit in reducing MTX-induced toxicity.

Patients and methods: A double blind randomised crossover clinical trial, GLU 1, was designed and activated to determine the efficacy and safety of routine use of glucarpidase after HD-MTX. To establish the frequency of MTX-induced toxicity so that bespoke study endpoints and sample size could be determined, the medical records of 56 patients with bone sarcoma treated with HD-MTX between 2004 and 2005 at University College Hospital (UCH) were studied. Data were collected on MTX-related toxicity and treatment delays. In a separate review, similar data from 17 patients aged ≥ 40 years, and 25 patients aged < 40 years treated with HD-MTX between 2002 and 2007 at UCH were compared. A high performance liquid chromatography (HPLC) assay was validated for the evaluation of plasma MTX and DAMPA concentrations for trial participants. In GLU 1, patients were randomised to receive two HD-MTX courses with folinic acid rescue (cycle FA) followed by two HD-MTX courses with folinic acid and glucarpidase (cycle glu/FA), or cycle glu/FA first followed by cycle FA. The data of 16 patients enrolled up to the interim analysis of the trial were analysed.

Results: MTX-related toxicity resulted in delays in half of subsequent chemotherapy cycles (58% in the ≥ 40 years group and 52% in the < 40 years group). In GLU 1, MTX toxicity resulted in delays in 43% of glu/FA cycles and 77% of FA cycles. The use of glucarpidase was not associated with a reduction in MTX AUC. The incidence and grade of MTX-induced toxicity were similar in glu/FA and FA although more

severe grades of mucositis were less frequent in glu/FA cycles. No glucarpidase toxicity was observed.

Conclusions: Glucarpidase offers a promising addition for rescue from MTX toxicity and continued clinical evaluation to determine its most effective use is warranted.

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1. INTRODUCTION

1.1. RATIONALE AND BACKGROUND

HD-MTX at a dose of 12 g/m^2, in combination with vigorous hydration and urinary alkalinisation along with a pharmacokinetically guided folinic acid “rescue” schedule, is an essential component of osteosarcoma treatment. Folinic acid replenishes the intracellular source of reduced active folates. Although folinic acid rescue may decrease the degree of MTX toxicity, patients will remain at risk as long as elevated MTX levels persist in the circulation. Moreover, if extracellular MTX concentration is very high, rescue with folinic acid may prove inadequate.

Despite current supportive measures, MTX-induced toxicity (myelosuppression, mucositis, hepatic and renal toxicity) still occurs and results in increased morbidity, patient discomfort, increased costs and potentially reduced treatment efficacy, due to suboptimal chemotherapy doses and/or delays in chemotherapy administration.

Several studies have shown that the fewer delays in MTX administration in osteosarcoma treatment, the better the outcome. Frei et al. (1980) reported that chemotherapy response in osteosarcoma improves by increasing MTX dose and worsens by increasing the time between MTX administrations. The French Tumour Study Group (1988) revealed that delay in MTX course administration is a negative prognostic factor in osteosarcoma. Moreover, Bacci et al (2001) showed that avoiding reductions in MTX doses and /or delays in chemotherapy is crucial in osteosarcoma outcome. A review of 30 studies by Delepine et al. (1996) demonstrated that the total planned dose and dose intensity of MTX (total MTX dose during treatment divided by total number of weeks), correlates significantly with disease free survival and proposed it to be a major factor in predicting the outcome

of patients with localised high grade osteosarcoma. These reports indicate that improving rescue from MTX toxicity is a worthwhile goal.

Glucarpidase (Voraxaze™, formerly known as Carboxypeptidase G_2) is an enzyme that cleaves the terminal glutamate from folate and folate analogues such as MTX. In the case of MTX, its action results in the production of the inactive metabolite DAMPA (4-deoxy-4-amino-N10-methylpteroic acid). It is currently used effectively to treat patients with MTX-induced renal dysfunction, in order to avoid potentially fatal MTX-related toxicity. A single intravenous dose of 50 units/kg of glucarpidase after MTX results in the reduction of plasma MTX levels to the non-toxic range within minutes without causing toxicity (Widemann and Adamson, 2006). Glucarpidase has much higher affinity for MTX than folinic acid so even high circulating folinic acid levels are unlikely to interfere with extracellular MTX inactivation. Moreover, glucarpidase is a high molecular weight protein and does not gain intracellular access. Therefore it is unlikely that it would counteract the anti-tumour effect of MTX trapped intracellularly in the form of polyglutamate. Glucarpidase seems to offer a promising opportunity for rescue from MTX toxicity.

Our aim is to establish the contribution of MTX-related toxicity to delays in delivering chemotherapy in patients with bone sarcoma and examine the role of glucarpidase in routine rescue after HD-MTX. Glucarpidase, if found to be effective and safe in maintaining the treatment intensity and reducing the incidence and severity of MTX-induced toxicity, could optimise treatment, improve patients' well-being, and reduce the use of health resources.

1.2. OSTEOSARCOMA AND TREATMENT OF OSTEOSARCOMA

1.2.1. OSTEOSARCOMA

Osteosarcoma is the most frequent primary malignant tumour of bone, deriving from primitive bone-forming mesenchyme and characterized by the production of osteoid tissue or immature bone by the malignant proliferating spindle cell stroma.

The approximate annual incidence of osteosarcoma is 2-3 per million in the general population; it is <1 per million in children under the age of 5 years, 2 per million at the age of 5-9 years, 7 per million at the age of 10-14 years and peaks at 8-11 per million at the age of 15-19 years (Bielack and Bernstein, 2005). There is a second smaller peak in older patients, which is due to osteosarcomas arising in abnormal bones, such as those affected by Paget's disease or previously treated with radiotherapy. Males are affected more frequently in most series (male: female ratio; 1.4:1) (Bielack and Bernstein, 2005). It occurs in any bone of the body. Among young patients, the most common site is the metaphysis of a long bone. Approximately half of all osteosarcomas originate in the region around the knee. The most frequent primary site is the distal femur, followed by the proximal tibia, followed by the proximal humerus. Other primary sites in descending order of frequency are pelvis, jaw, fibula and ribs (Bielack and Bernstein, 2005).

The World Health Organization's histologic classification of bone tumours separates osteosarcomas into central (medullary) and surface (peripheral) tumours and recognizes a number of subtypes within each group. The most common pathologic subtype is the conventional high-grade central osteosarcoma. It accounts for 80-90% of all osteosarcomas and is characterized by areas of necrosis, atypical mitoses and malignant cartilage. The most frequent subtypes of conventional high-

grade central osteosarcoma are osteoblastic, chondroblastic and fibroblastic osteosarcomas (Fletcher et al. 2002).

There is limited understanding of the aetiology of osteosarcoma. The peak incidence coincides with a period of rapid bone growth in young people, a feature that suggests a relationship between rapid bone growth and the development of this tumour (Marina N et al. 2004). Osteosarcomas occur at an earlier age in girls than in boys, corresponding to the more advanced skeletal age and earlier adolescent growth spurt of girls, whereas the increased risk for osteosarcoma among boys may results from the larger volume of bone formed during a longer growth period (Marina N et al. 2004). Osteosarcoma has a predilection for the metaphyseal portions of the most rapidly growing bones in adolescents and tumours of the humerus tend to occur at a younger age than do tumours of the femur and tibia, corresponding to the earlier growth spurt of the humerus. An explanation to that may be that rapidly proliferating cells might be particularly susceptible to oncogenic agents and mitotic errors which lead to neoplastic transformation (Jaffe et al. 2009). Nevertheless, it must be recognised that osteosarcoma arises in many patients well before and long after the adolescent growth spurt (Marina N et al. 2004).

Radiation is a well-documented aetiologic factor, being implicated in approximately 3% of osteosarcomas (Jaffe et al. 2009). An increased incidence is likely to be seen, as more patients survive long enough after primary irradiation to develop this complication. The interval between irradiation and appearance of osteosarcoma ranges from 4 to more than 40 years (median: 12-16 years) (Huvos 1991). Osteosarcomas have also been associated with the use of intravenous radium 224 and Thorotrast, a diagnostic radiocontrast agent (Loutit 1970; Harrist et al. 1979). Exposure to alkylating agents may also contribute to its development.

Approximately 2% of patients with Paget's disease develop osteosarcoma and cases of osteosarcoma in patients older than 40 years are often associated with this premalignant condition (Huvos 1991). Other conditions associated with an increased risk of development of osteosarcoma, are solitary or multiple osteochondroma, solitary enchondroma or enchondromatosis (Ollier's disease), multiple hereditary exostoses, fibrous dysplasia, chronic osteomyelitis, sites of bone infarcts and sites of metallic implants for benign conditions.

The incidence of osteosarcoma is increased in several well-defined hereditary disorders associated with germ-line alterations of tumour suppressor genes. However, these account for only a few percent of all osteosarcomas. Survivors of hereditary retinoblastoma with germline mutations of the retinoblastoma gene RB1 on chromosome 13q14 carry a risk which is 500 to 1000 times greater than that of the general population (Bielack and Bernstein, 2005). The Li-Fraumeni syndrome (germ-line mutations in the p53 gene) is associated with a 15-fold increase. Rothmund-Thomson, Bloom and Werner syndromes are also associated with an increase in osteosarcomas (Bielack and Bernstein, 2005).

The most common clinical presentation of osteosarcoma is pain in the involved region of bone, with or without a soft tissue mass. Pain is often attributed to trauma or vigorous physical exercise, both of which are common in the population at risk. Symptoms are usually present for several months before the diagnosis is made. In approximately 10%, the first sign of disease is a pathologic fracture (Bielack and Bernstein, 2005). 10-20% of patients with osteosarcoma present with radiographically detectable metastatic disease, but virtually all patients have sub clinical, microscopic metastases (Bielack and Bernstein, 2005). The most frequent site for metastatic presentation is the lung. Much less frequently, metastases at initial diagnosis occur in other bones and soft tissues.

1.2.2. TREATMENT OF NEWLY DIAGNOSED OSTEOSARCOMA

Almost all patients with high-grade osteosarcoma have at least microscopic metastatic disease at diagnosis. This necessitates the use of systemic chemotherapy in addition to surgery. In the UK, standard treatment for newly diagnosed osteosarcoma includes 10 weeks of neoadjuvant chemotherapy with Doxorubicin (**A**driamycin), Cis**p**latin and HD-**M**TX (**MAP**), followed by surgery, followed by 18 weeks of adjuvant chemotherapy with the same agents (Table 1).

Table 1: STANDARD OSTEOSARCOMA MANAGEMENT

CYCLE	WEEK	TREATMENT
1	1	Doxorubicin, 75 mg/m^2 + cisplatin, 120 mg/m^2
	2	
	3	
	4	HD-MTX, 12 g/m^2
	5	HD-MTX, 12 g/m^2
2	6	Doxorubicin, 75 mg/m^2 + cisplatin, 120 mg/m^2
	7	
	8	
	9	HD-MTX, 12 g/m^2
	10	HD-MTX, 12 g/m^2
	11	**SURGERY**
3	12	Doxorubicin, 75 mg/m^2 + cisplatin, 120 mg/m^2
	13	
	14	
	15	HD-MTX, 12 g/m^2
	16	HD-MTX, 12 g/m^2
4	17	Doxorubicin, 75 mg/m^2 + cisplatin, 120 mg/m^2
	18	
	19	
	20	HD-MTX, 12 g/m^2
	21	HD-MTX, 12 g/m^2
5	22	Doxorubicin, 75 mg/m^2
	23	
	24	HD-MTX, 12 g/m^2
	25	HD-MTX, 12 g/m^2
6	26	Doxorubicin, 75 mg/m^2
	27	
	28	HD-MTX, 12 g/m^2
	29	HD-MTX, 12 g/m^2

1.2.3. TREATMENT OF RELAPSED OSTEOSARCOMA

Surgery is the cornerstone of successful relapse therapy. However, chemotherapy should be offered to most patients who experience a relapse within the first three years after diagnosis and those with multiple metastases. The choice of drugs should be made on an individual basis. Patients, who have been previously treated with MAP, could potentially receive ifosfamide and etoposide. Nevertheless, there are patients with relapsed disease who have not received MTX at initial diagnosis, such as patients on the previous randomised MRC BO06 clinical trial (Lewis IJ et al. 2007), which compared standard and intensified doxorubicin and cisplatin regimens. In those patients HD-MTX would be recommended. In patients who relapsed, despite having received all five active chemotherapy agents (doxorubicin, cisplatin, MTX, ifosfamide and etoposide), but responded to MTX in the past, the use of further MTX can be considered.

1.3. MTX AND THE ROLE OF HD-MTX IN TREATMENT OF OSTEOSARCOMA

1.3.1. DESCRIPTION AND USE OF MTX

MTX (Amethopterin, 4-NH_2-4-deoxy-N^{10}-methyl-pteroyglutamic acid) is an analogue of folic acid. The molecular structure of MTX differs from folic acid only in that it has a 4-amino group in place of the hydroxyl group on the pteridine ring, and a methyl group at the N^{10} position. It was first used in the treatment of childhood acute lymphoblastic leukaemia in 1948. Since then it has been used in the treatment of various malignancies including osteosarcoma, non-Hodgkin's lymphoma, Hodgkin's disease, and breast cancer (Bleyer 1978; Jolivet et al. 1983; Bertino 1993). It is one of the few agents that can be given intrathecally and used for central nervous system involvement in leukaemia, lymphoma and solid tumours. Due to its

immunosuppressive effects, it is used in rheumatoid arthritis, psoriasis and the prevention of graft-versus-host disease after bone marrow transplantation.

1.3.2. MECHANISM OF ACTION OF MTX

MTX inhibits dihydrofolate reductase (DHFR), the enzyme responsible for converting folic acid to reduced folate cofactors. Reduced folates are necessary for the transfer of 1-carbon units in a variety of biochemical reactions, such as the biosynthesis of thymidylic acid, the nucleotide specific to DNA, and the biosynthesis of inosinic acid, the precursor of purines necessary for both DNA and RNA synthesis (Bleyer 1978).

1.3.3. HALF LIFE AND TRANSPORT OF MTX

After intravenous administration, the disappearance of MTX from plasma is triphasic (Huffman et al. 1973; Stoller et al. 1975). The initial half-life is 0.75 ± 0.11 h (Huffman et al. 1973). The second half-life has been reported as 2.06 ± 0.61 (Stoller et al. 1975), 3.49 ± 0.55 (Huffman et al. 1973) or 2.0-3.4 h (Pratt et al. 1975). The terminal half-life is 10.4 ± 1.8 h (Stoller et al. 1975) and begins as the plasma antifolate concentration approaches 10^{-7}M, approximately 30-48 hrs after high-dose therapy. The first half-life is probably that of distribution and the second half-life that of renal clearance. The prolonged terminal phase probably represents a combined effect of release from deep compartments, enterohepatic circulation and renal tubular re-absorption, and is responsible for the major portion of gastrointestinal and bone marrow toxicity (Bleyer 1978).

In human plasma, 50-70% of MTX is bound to protein, principally albumin. Alterations in plasma protein binding affect the amount of free extracellular MTX, which in turn influences the influx of MTX into cells and its rate of clearance by the kidneys (Bleyer 1978).

The transport of MTX and naturally occurring erduced folates across the cell membrane is mediated by three genetically dinstict and functionally diverse transport systems, the Reduced Folate Carrier (RFC), Folate Receptors (FR) and Proton-Coupled Folate Transporter (PCFT) (Desmoulin et al. 2012).

RFC is a secondary active anionic exchanger which transports reduced folates and antifolates, including MTX, in mammalian cells and tissues via counter-transport with organic anions (Matherly et al. 2007); it has a much lower (~50-100-fold) affinity for folic acid than that for reduced folates. Transport with RFC is charactarised by a neutral pH optimum and markedly decreased transport activity below pH 7 (Matherly et al. 2007; Zhao et al. 2007; Zhao et al. 2009).

Membrane-bound RFs mediate cellular uptake of folic acid, reduced folates and many antifolates, including MTX, via a non-classical endocytotic mechanism whereby folate ligands bind FRs at the cell membrane, followed by invagination and the formation of cytoplasmic vesicles (endosomes) (Rijnboutt et al. 1996; Sabharanjak et al. 2004). Release of bound ligands occurs upon endosomal acidification which facilitates dissociation of the ligand-FR complex, and exit of the folate ligand from the endosome to the cytoplasm by diffusion or a transport-mediated process that operates at acidic pH (Kamen et al. 1988).

PCFT is the third transport system; it is a proton-folate symporter that functions optimally at acidic pH (maximal transport at pH 5-55) by coupling the flow of protons down an electrochemical concentration gradient to the uptake of reduced folates, folic acid and antifolates, including MTX and pametrexed, into cells (Zhao et al. 2007; Qiu et al. 2006; Umapathy et al. 2007; Nakai et al. 2007).

At high extracellular concentrations (MTX serum levels >100 µmol/L), MTX also enters cells by passive diffusion (Hill et al. 1979). This appears to be the principal

means of drug accumulation by MTX resistant cells that are deficient in carrier-mediated transport.

Once inside the cell, MTX undergoes polymerisation of the glutamic acid chain, similar to endogenous folates, to form methotrexate polyglutamates (MTX-PG). While both MTX and MTX-PG competitively inhibit DHFR, MTX-PG has enhanced binding to and inhibition of the enzyme and serves to enhance the retention and potency of MTX against target enzymes. Formation of the MTX-PG is dependent upon intracellular MTX concentration and the duration of exposure.

1.3.4. METABOLISM AND EXCRETION OF MTX

Several metabolites have been found in human urine and plasma, particularly in patients receiving the highest doses of MTX (Bleyer, 1978). The metabolites account for <10% of the total dose administered if MTX is given intravenously at 30mg/m^2. If given orally at the same dose, as much as 35% of the absorbed dose may be excreted as metabolites. The higher amount of metabolites after oral administration than after intravenous injection is consistent with the hypothesis that MTX metabolism in man occurs primarily in the gastrointestinal tract or enterohepatic circuit.

Under conditions of normal renal function MTX clearance from plasma is 110 cc/min/m^2, 103 cc/min/m^2 of which is due to renal clearance (Liegler et al. 1969). Approximately 41% of an intravenously administered dose is excreted unchanged in the urine within 6 h after administration, 90% within 24 h, and 95% within 30h (Pratt et al. 1975; Henderson et al. 1965; Wang et al. 1976). At very low plasma concentrations, MTX appears to be reabsorbed by the kidney (Huffman et al. 1973). At higher concentrations, renal clearance of MTX is relatively constant (Huffman et al. 1973) and exceeds that of inulin (Liegler et al. 1969), suggesting that MTX is not

only filtered but also actively secreted by renal tubular cells. 1 to 2% of intravenously administered dose is excreted in the stool as the parent combound and metabolites (Bleyer 1978).

1.3.5. MTX RESISTANCE

Resistance to MTX may develop through a variety of mechanisms, including impaired transport of drug into the cell via the RFC (Sirotnak FM et al. 1981), alterations in the affinity of DHFR for MTX (Goldie et al. 1980; Flintoff et al. 1980), increase in DHFR due to gene amplification or increased transcription (Alt et al. 1978; Melera et al. 1980), and diminished intracellular retention secondary to decreased polyglutamation (Cowan et al. 1984).

In patients with osteosarcoma, therapy with conventional MTX doses is ineffective; thus HD-MTX is used. Several retrospective studies have suggested that a threshold peak MTX level needs to be achieved to obtain good histological response to chemotherapy. Guo et al. (1999) demonstrated that 65% of high grade OS samples at the time of initial biopsy were found to have decreased RFC expression, which suggests that impaired transport of MTX may be an important mechanism of intrinsic resistance in OS. This may partly explain why conventional dose of MTX is ineffective in the treatment of OS, as high doses may be needed to allow transport through alternatives means, such as passive diffusion. In the same study, although increased expression of DHFR was rare in the biopsy material, it was frequent in the recurrent pulmonary metastases and excision samples. Therefore it is possible that increased DHFR expression represents acquired MTX resistance, either through acquired alteration in tumour cells or through selection of a previously resistant clone. In addition, Meyer et al. (1990) reported that xenografts of osteogenic sarcoma cells form predominantly short chain MTX polyglutamates, suggesting

either relatively low FPGS activity, relatively high FPGH activity or a combination of these features.

Theoretically, all known mechanisms of MTX resistance could be overcome using HD-MTX. At high extracellular concentrations, passive diffusion of MTX may overcome resistance due to impaired membrane transport and the high intracellular levels achieved could overcome resistance due to the presence of increased DHFR levels or altered enzyme affinity. In addition, the availability of large amounts of free intracellular MTX might promote conversion to polyglutamate derivatives of the drug (Bleyer 1978).

1.3.6. ROLE OF HD-MTX IN OSTEOSARCOMA

Before the use of chemotherapy, 80-90% of patients with non-metastatic osteosarcoma died despite early radical surgery. With the use of multidrug chemotherapy, approximately two thirds of patients with non-metastatic resectable primary tumours can be cured (Link et al. 1986). The improved outcome has been attributed, in part, to the use of HD-MTX with folinic acid rescue as described by Jaffe (1972) and Jaffe et al. (1973; 1977) and emphasized by Rosen et al. (1974; 1975; 1979).

The first effective drugs to be introduced into the treatment of osteosarcoma were doxorubicin and HD-MTX (Jaffe et al. 1973 and 1977; Rosen et al. 1974, 1975 and 1979; Cortes et al. 1972). Rosen et al. (1975) combined these two drugs together with cyclophosphamide; bleomycin, cyclophosphamide and dactinomycin; and cisplatin. This innovative approach of aggressive multidrug chemotherapy provoked controversy and the role of HD-MTX was questioned in particular. Subsequently data from the study by Rosen et al. (1975) were analyzed by Meyers et al. (1992),

who convincingly showed a histologically proven 19% response rate of single-drug HD-MTX in 54 patients.

Since then, Delepine et al. (1996), in a review of 30 studies, revealed that the total planned dose and dose intensity of HD-MTX correlates significantly with disease free survival in patients with localised high grade osteosarcoma. However, there is still controversy regarding the optimal MTX plasma levels and/or the duration of exposure that must be achieved for optimum efficacy. Some studies suggest that MTX plasma levels of ≥ 700 μM (Bacci et al. 1996 and 1998) or ≥ 1000 μM (Delepine 1988; Graf et al. 1994) at the end of a 4-6 h infusion improves histologic response and event free survival. These levels are achievable at the end of a 4 h intravenous MTX infusion of 12 g/m^2 (Crews et al. 2004). However, Crews et al. (2004) reported that mean peak MTX levels >1500 μM is associated with lower event free survival, possibly because higher MTX levels are associated with more toxicity and therefore decreased dose intensity, or because increased folinic acid dosing in patients with very high MTX exposures may have compromised the antitumour effect of MTX. Others have observed significant differences in the disease free survival between patients whose mean area under the curve (AUC) was below or above 4000 μMh and recommended that the MTX dose should be increased such as to obtain an AUC >4000 μMh (Aquerreta et al. 2004).

1.3.7. HD-MTX INDUCED TOXICITY

Despite currently used supportive measures, MTX-induced toxicity still occurs, resulting in increased morbidity, suboptimal chemotherapy doses, delays in subsequent chemotherapy administration and possibly poorer outcome (Frei at al., 1980; French Tumour Study Group, 1988; Bacci et al., 2001; Delepine et al., 1996). There is wide inter- and intra-patient variability in relation to methotrexate tolerance (Bacci et al. 1996 and 1998; Zelcer et al. 2005; Ferrari et al. 1993; Delepine et al.

1995), the primary determinant of which appears to be variation in the pharmacokinetics of the drug.

Correlation of the toxic reactions with the drug's pharmacokinetics discloses certain time- and concentration-dependent relationships which appear to determine which target tissue is at risk of toxicity. For bone marrow and gastrointestinal epithelium, the plasma concentration- and time-threshold appear to be 2×10^{-8} M and about 42 hours, respectively (Levitt et al. 1973; Young et al. 1973). The severity of toxicity is positively associated with the duration of MTX exposure beyond the time-threshold, and relatively less dependent on the magnitude of MTX elevation above the extracellular concentration-threshold (Goldie et al. 1972).

The major focus of osteosarcoma research has been on maximising survival and there are almost no dedicated studies of complications of osteosarcoma therapy. In most publications of osteosarcoma clinical trials which included HD-MTX in their treatment regimen, data on chemotherapy-related toxicity were not documented or the information on toxicity was either not specific to HD-MTX or minimal (Rosen et al., 1982; Meyers et al., 1992; Meyers et al., 1998; Saeter et al.; 1991; Provisor et al., 1997; Goorin et al., 1987; Winkler et al., 1984; Winkler et al., 1988; Fuchs et al., 1998; Bacci et al., 1990; Bacci et al., 1993; Bramwell et al., 1992; Souhami et al., 1997; Meyers et al., 2005; and Ferrari et al., 2005).

Toxicity related to HD-MTX includes mucositis, hepatotoxicity, nephrotoxicity, myelosuppression, and less commonly dermatitis and encephalopathy. Available information in the literature on the incidence and severity of MTX-related toxicity is as follows:

a. MUCOSITIS

Sonis at al. (2004) reviewed three studies involving 132 patients receiving methotrexate chemotherapy, 23% developed grade 3-4 oral mucositis (Sonis et al., 2004). Saeter et al. (1991) reviewed 376 HD-MTX courses (8-12 g/m^2) given preoperatively in 97 patients with osteosarcoma (median age 16 years). Oral mucositis complicated 20% of those and was mild in the majority of the courses. In another review of 65 consecutive patients with osteosarcoma treated with 288 courses of HD-MTX courses at a dose of 12 g/m^2 (Holmboe et al., 2012), mucositis grade 1, 2, 3 and 4 (CTCAE v3.0) complicated 13%, 8%, 15% and 0% of those respectively.

b. HEPATOTOXICITY

Saeter et al. (1991) reported transient liver dysfunction in 80% of patients (mean ALT levels of 175 U/L), but almost all episodes were benign in consequence and reversible. In the review by Holmboe et al. (2012), hyperbilirubinaemia grade 1 and 2 (CTCAE v3.0) complicated 35% and 24% of methotrexate courses respectively whereas grade 3 and 4 (CTCAE v3.0) were not documented. Bilirubin was not affected in 42% MTX courses. Raised ALT grade 1, 2, 3 and 4 (CTCAE v3.0) complicated 14%, 25%, 42% and 17% of MTX courses respectively and only 2% of MTX were not associated with a rise in ALT. Zelcer et al. (2008) retrospectively reviewed the treatment of 82 osteosarcoma patients who received 708 MTX courses at the Memorial Sloan-Kettering Cancer Centre between 1996 and 2002 at a dose of 12 g/m^2. Most patients had transient elevation in ALT and bilirubin which were completely reversible.

c. RENAL DYSFUNCTION

The most comprehensive review on MTX-related nephrotoxicity was published by Widemann et al. (2004). In this review, 1.8% patients (68 of 3887 patients) developed MTX-related nephrotoxicity that was either ≥ grade 2 or significant enough to be reported and 23 patients (0.6%) developed grade 3 or 4 toxicity. Renal toxicity was graded using the World Health Organization criteria (Grade 1, serum creatinine levels < 1.5 x ULN; Grade 2, 1.5-3.0 x ULN; Grade 3, 3.1-6.0 x ULN; and Grade 4, > 6.0 x ULN, similar to CTCAE v3.0 grading). The mortality rate among those patients was 4.4% (3/68).

Holmboe et al. (2011) reviewed 65 consecutive patients with osteosarcoma (median age 18 years) treated with 288 courses of HD-MTX courses (at a dose of 12 g/m^2) on SSG VIII, ISG/SSG-I, ISG/SSG-II and ISG/SSG-XIV clinical trials between 1994 and 2003. Creatinine remained within normal range in 95% of MTX courses. Creatinine rise grade 1 and 2 (CTCAE v3.0) was noted in 3% and 2% of treatment courses respectively.

Zelcer et al. (2008) reviewed the treatment of 82 osteosarcoma patients who received 708 MTX courses (at a dose of 12 g/m^2) at the MSKCC between 1996 and 2002. The majority (98-99%) of the courses resulted in no significant elevation in creatinine levels (grade 0 and 1 as per NCI CTC v.2.0). Moderate to severe nephrotoxicity (grade 3 and 4) was not observed.

The above papers describe the incidence and severity of renal toxicity after treatment with HD-MTX. However, chemotherapy for osteosarcoma includes other nephrotoxic agents such as cisplatin and cumulative renal burden due to cisplatin should be taken into account.

d. MYELOSUPPRESSION

Saeter et al. (1991) reviewed 376 HD-MTX courses (8-12 g/m^2) given preoperatively in 97 patients with osteosarcoma (median age 16 years) on the SSG-II clinical trial between 1982 and 1989. Severe bone marrow toxicity (WHO III or IV) complicated 0.5% of courses whereas 19% of courses were associated with grade I bone marrow toxicity. Holmboe et al. (2012) reviewed 65 consecutive patients with osteosarcoma (median age 18 years) treated with 288 courses of HD -MTX courses at a dose of 12 g/m^2. Leucopenia grade 1, 2, 3 and 4 (CTCAE v3.0) complicated 5%, 30%, 27% and 6% of MTX courses respectively. MTX did not lead to leucopenia in 32% of the courses. Thrombocytopenia grade 1, 2, 3 and 4 (CTCAE v3.0) complicated 20%, 7%, 10% and 10% of MTX courses respectively although MTX did not lead to thrombocytopenia in 32% of the courses. Also, Zelcer et al. (2008) reviewed the treatment of 82 osteosarcoma patients who received 708 MTX courses at MSKCC between 1996 and 2002 at a dose of 12 g/m^2. 16%, 9% and 3% of the courses were associated with grade 3 and 4 neutropenia, leucopenia and anaemia/thrombocytopenia respectively.

e. NEUROTOXICITY

Acute transient neurological dysfunction following HD-MTX is reported in the literature to occur in 0.4-5% of children treated for osteosarcoma (Goorin et al. 2003, Walker et al. 1986, Packer et al. 1983, Saeter et al. 1991).

f. SKIN TOXICITY

Skin toxicity is reported to complicate 1.3% of MTX courses (Saeter et al. 1991)

1.3.8. ROLE OF FOLINIC ACID RESCUE AFTER HD-MTX

Folinic acid is a racemic mixture of the stereoisomers of N^5-formyl-FH_4. The D- and L-isomers differ significantly in their cellular and clinical pharmacology, with only the L-isomer having the capacity to rescue cells from MTX toxicity. Following oral or parenteral administration, folinic acid is readily converted to N^5-methyl-FH_4, the primary circulating folate in humans (Ackland et al. 1987).

The rationale for the use of folinic acid rescue is that provision of reduced folate to normal cells should circumvent the metabolic block produced by MTX and allow resumption of purine and pyrimidine synthesis. However, the selective rescue of normal tissues and not tumour cells has not been adequately explained, except perhaps when tumour cell resistance to MTX is caused by loss of the membrane transport system for reduced folates, thus excluding folinic acid from tumour cells (Ackland et al. 1987).

Folinic acid competes with MTX for entry into the cell because it is actively transported by the same cell transport system as MTX. This observation forms the basis for one of the hypotheses of selectivity, because tumour cells with a defect in the folate transport system would not be rescued since insufficient folate would enter the cell. This is in contrast to normal cells with intact folate transport, which could be more easily rescued.

Experimental observations have shown that folinic acid is able to competitively displace MTX from DHFR allowing its reactivation (Matherly et al. 1986). However, in the presence of MTX polyglutamates, such competitive displacement does not occur, and DHFR inhibition is sustained. The observation that most tumour cells synthesize much greater quantities of MTX polyglutamates than normal cells is central to understanding the selectivity of folinic acid rescue. In normal cells, such as

bone marrow precursors, which have few MTX polyglutamates, folinic acid administration promotes dissociation of MTX from DHFR, with consequent reactivation of the enzyme. Cellular levels of MTX polyglutamates then decrease rapidly, enabling reactivation of purine and thymidylate biosynthesis as reduced folate pools are restored. In tumour cells however, accumulation of MTX polyglutamates prevents competitive displacement from DHFR by folinic acid and DHFR inhibition is sustained. Thus cellular levels of MTX polyglutamates remain high and directly inhibit purine biosynthesis (Ackland et al. 1987).

An important issue to consider is that the concentration of folinic acid needed to rescue normal cells from MTX is dependent upon the concentration of MTX present. The concentration of folinic acid must be high enough to compete effectively for transport into the cells of normal tissue. Importantly, with high MTX concentrations, even ten-fold higher folinic acid concentrations (concentrations that theoretically are achieved with supra-pharmacologic dosing of folinic acid) are unable to rescue normal haematopoietic cells (Pinedo et al. 1976).

1.3.9. DELAYS IN OSTEOSARCOMA TREATMENT DUE TO MTX TOXICITY

Although several studies have shown that the fewer delays in MTX administration in osteosarcoma treatment, the better the outcome (Frei et al. 1980; French Bone Tumour Study Group 1988; Bacci et al. 2001; Delepine et al. 1996), there is currently no available literature on the incidence of delays in subsequent chemotherapy due to MTX toxicity in patients with osteosarcoma.

Information is only available on the incidence of delayed MTX elimination in patients with osteosarcoma which may then result in subsequent chemotherapy delays. Saeter et al. (1991) reviewed 376 HD-MTX courses (8-12 g/m^2) given preoperatively in 97 patients with osteosarcoma (median age 16 years) on the SSG-II clinical trial

between 1982 and 1989. Twenty-nine patients (30%) experienced delayed excretion constituting 15% of all HD-MTX courses. Winkler et al. (1988) reviewed the results of the COSS-82 clinical trial and reported that HD-MTX (12 g/m^2) toxicity led to delays in 36 of 495 observed cycles (7%). This is in keeping with Bacci et al. (2006) who reviewed 336 patients with osteosarcoma treated on three protocols of neoadjuvant chemotherapy including HD-MTX at Instituti Orthopedici Rizzoli between 1983 and 2004. Delayed MTX elimination and therefore delay to subsequent treatment complicated 3.1% of HD-MTX courses (8-12 g/m^2). This differed with different dose of MTX with 1.2%, 3.5% and 4.7% of MTX courses at a dose of 8, 10 and 12 g/m^2 respectively being complicated with delayed MTX excretion. In contrast, Zelcer et al. (2008) reviewed the treatment of 82 patients (median age 16 years) who received 708 MTX courses (12 g/m^2) at the MSKCC between 1996 and 2002 and reported that delayed MTX elimination complicated 38% of courses.

1.4. GLUCARPIDASE (VORAXAZE™) AND ITS RESCUE ROLE AFTER HD-MTX

Glucarpidase (formerly Carboxypeptidase G_2) is an enzyme originally isolated from *Pseudomonas sp* strain RV-308, cloned and now produced in *Escherichia coli*. it has a sub-unit molecular mass of 41,440 Da and dimeric molecular weight of approximately 83,000 Da (Minton et al. 1983). It is presented as a sterile, white lyophilized powder intended for single-use intravenous administration after reconstitution with 1 ml of sterile normal saline solution. Each vial of Voraxaze contains 1000 units of glucarpidase. One unit corresponds to the enzyme activity that cleaves 1 µmol/L MTX/min at 37°C. The product also contains approximately 10 mg of lactose as an inactive ingredient buffered to pH 7.0 to 8.0 (Voraxaze™ Investigator's Brochure, 2009).

Glucarpidase is currently used effectively to treat patients with MTX-induced renal dysfunction, in order to avoid potentially fatal MTX-related toxicity (Widemann BC, 2006). A single intravenous dose of 50 units/kg of glucarpidase after MTX results in the reduction of plasma MTX levels to the non-toxic range within minutes without causing toxicity.

1.4.1. MECHANISM OF ACTION OF GLUCARPIDASE

Glucarpidase hydrolyses the carboxyl terminal glutamate residue from folic acid and its analogues (e.g. MTX) (Sherwood et al. 1985). It follows Michaelis-Menten kinetics (Michaelis L et al. 2011) with K_m values of 4 µmol/L for folate, 8 µmol/L for MTX, 34 µmol/L for 5-methyl-FH4, and 120 µmol/L for 5-formyl-FH4 (folinic acid). Glucarpidase has >10-fold lower affinity for folinic acid than for MTX, which is of significance when considering the potential combined use of glucarpidase and folinic acid for HD-MTX rescue (Sherwood et al. 1985). Glucarpidase cleaves the MTX molecule into inactive metabolites, DAMPA and glutamate, which are metabolised by the liver, and thus provides an alternative route of MTX elimination (Donehower et al. 1979). This is particularly important in patients who develop renal dysfunction due to MTX nephrotoxicity (Adamson et al. 1991; Monty et al. 2000; Von Poblozki et al. 2000; Widemann et al. 2000) and would therefore not be able to renally excrete MTX.

1.4.2. CLINICAL STUDIES WITH GLUCARPIDASE

The safety and effectiveness of glucarpidase on systemic MTX concentrations and MTX toxicities has been assessed in three clinical studies, the Berlin Study, PR001-CLN-001 (Schwartz et al. 2004) the NCI Study, PR001-CLN-002 (Adamson et al. 2005; Buchen et al. 2005) and the PD Study, PR001-CLN-006 (Voraxaze™ Investigator's Brochure, 2009). A study to determine the pharmacokinetics of

glucarpidase in eight healthy normal subjects and four patients with severe renal impairment was also performed (PK Study: PR001-CLN-005, Voraxaze™ Investigator's Brochure, 2009). In addition, an interaction study between glucarpidase and folinic acid (LV Interaction Study: PR001-CLN-010) was performed since it is known that folinic acid is also a substrate of glucarpidase (Voraxaze™ Investigator's Brochure, 2009).

1.4.2.1. PK STUDY (VORAXAZE™ INVESTIGATOR'S BROCHURE, 2009).

The PK study was an open-label, single site, pharmacokinetic study of glucarpidase administered intravenously at a dose of 50 units/kg to eight subjects with normal renal function and four subjects with severe renal impairment. Two assay methods were used to quantify serum glucarpidase concentration, one measured glucarpidase enzyme activity and the other total glucarpidase. The pharmacokinetic data based on total glucarpidase indicated about 7% lower mean C_{max} in subjects with impaired renal function relative to those with normal renal function. However, the total glucarpidase exposure, as determined by $AUC_{0-\infty}$, was marginally higher, by about 5%, in subjects with impaired renal function relative to those with normal renal function. The median time to maximum serum concentration (T_{max}) was short for subjects with normal renal function, indicating a rapid equilibration of glucarpidase after completion of the short infusion. There is little effect in patients with renal dysfunction compared to healthy volunteers with mean half life of glucarpidase of 10 and 9 hours, respectively, after a dose of 50 units/kg of intravenous glucarpidase (Phillips et al. 2008). Large variability was noted especially for AUC_{0-t} and $AUC_{0-\infty}$, in the renally impaired subject group, with one subject having a higher exposure relative to the other three. Overall, the results of the PK Study showed little effect of renal impairment on the serum pharmacokinetics of glucarpidase.

1.4.2.2. LV INTERACTION STUDY (VORAXAZE™ INVESTIGATOR'S BROCHURE, 2009).

This study was a double-blind, placebo controlled, randomised, two-period crossover pharmacokinetic study. The primary objective was to assess the effect of glucarpidase on the pharmacokinetics of the active L-stereoisomer of folinic acid, (6)L/S-LV, following repeated doses of folinic acid. The secondary objectives of the study were to assess the effect of glucarpidase on the pharmacokinetics of 5-methyl-tetrafydrofolate, (6)L/S-LV-THF, the active metabolite of (6)L/S-LV. The study demonstrated that glucarpidase reduces the systemic availability of (6)L/S-LV, by up to 50%, and its active metabolite (6)L/S-LV-THF, but does not completely eliminate them. The reduced availability of (6)L/S-LV and its active metabolite could potentially lead to a reduction in the efficacy of folinic acid.

1.4.2.3. PD, NCI AND BERLIN STUDIES (SCHWARTZ ET AL. 2004; ADAMSON ET AL. 2005; BUCHEN ET AL. 2005; VORAXAZE™ INVESTIGATOR'S BROCHURE, 2009).

The above three studies were all compassionate use, multiple site, single arm, open label studies to provide access to the drug and assess the safety of 50 units/kg of glucarpidase given intravenously to patients with delayed elimination of MTX due to renal impairment. The majority of patients (65%) were enrolled in the NCI Study. The primary efficacy endpoint for all three studies was the proportion of patients who achieved a clinically important reduction (CIR) in plasma MTX concentration as measured by HPLC. A CIR is defined as a plasma or serum MTX concentration that has decreased to ≤ 1 µmol/L in all post glucarpidase samples, indicating a sustained reduction of MTX. The primary efficacy evaluation was performed on patients (the Eficacy Subset, ES) with valid primary efficacy data which included a pre-glucarpidase MTX concentration ≥ 1 µmol/L determined by HPLC and at least one

MTX concentration by HPLC after the last glucarpidase dose. A comparison across the three studies regarding demographics, diagnosis, renal function, MTX level at entry and glucarpidase doses, is shown in Table 2. Comparison of the Primary Efficacy Subsets across the studies is shown in Table 3.

Table 2: COMPARISONS ACROSS STUDIES - PD, NCI, BERLIN STUDIES

Demographics	**PD Study**		**NCI Study**		**Berlin Study**	
	No. (%[a]) of patients		**No. (%[a]) of patients**		**No. (%[a]) of patients**	
	Paediatric	**Adult**	**Paediatric**	**Adult**	**Paediatric**	**Adult**
Number of patients	68		227		42	
Age-Number of patients with data	67/68		216/227		42/42	
Age by paediatric or adult (≥ 18) [≥ 65]	31 (46%)	36 (54%) [7(10%)]	115 (53%)	101 (46%) [31(14%)]	1 (2%)	41 (98%) [7(17%)]
Age range (median)	2-84 (20)		0-82 (17)		10-78 (52)	
Gender – No. of patients with data	N=68/68		N=197/227		N=0/42	
Male	42 (62%)		127 (58%)		-	
Female	26 (38%)		70 (32%)		-	
Cancer diagnosis[b] No. patients	68/68 (67 with age)		188/216		42/42	
Osteosarcoma (102)	17/25	7/25	55/77	22/77	-	-
Leukaemia (61)	9/13	4/13	24/36	12/36	1/12	11/12
Lymphoma (122)	3/24	21/24	22/70	48/70	-	28/28
Other cancers (12)	2/6	4/6	-	4/4	-	2/2
Non Cancer (1)			-	1/1	-	-
Unknown Total (28)	-	-	14	14	-	-
Patients with serum creatinine > 2.2 mg/dL[c]	15/28 (54%)	30/34 (88%)	58/105 (55%)	70/86 (81%)	0/1 (0%)	22/41 (54%)

Demographics	PD Study		NCI Study		Berlin Study	
	No. (%[a]) of patients		No. (%[a]) of patients		No. (%[a]) of patients	
	Paediatric	Adult	Paediatric	Adult	Paediatric	Adult
MTX						
Dose range in g/m^2 (median)	1.0-20 (6.7)		0.4-19 (5.5)		0.9-12 (3)	
Pre-glucarpidase concentration in µmol/L (median)	3.47 - 708 (40.2) ES (23 patients)		1.1-849.10 (34.7) ES (70 patients)		1.1-166 (5.8) ES (23 patients)	
Glucarpidase dose range in Units/kg (median)	29.2-55 (50)		10.9-63.7 (49.8)		9.8-58 (50)	

Source: adapted from Voraxaze Investigator's Brochure edition number PR001-CLN-IB007

[a] Percentages have been rounded to the nearest whole number.

[b] One patient in the NCI Study did not have cancer and one patient in the PD Study had a diagnosis of osteosarcoma but age was not available.

[c] Patients with impairment equivalent to serum creatinine >1.5 x ULN post-MTX but pre-glucarpidase

Table 3: COMPARISONS OF THE PRIMARY EFFICACY SUBSETS ACROSS STUDIES - PD, NCI, BERLIN STUDIES

Demographic	PD Study		NCI Study		Berlin Study	
	No. (%[a]) of patients		No. (%[a]) of patients		No. (%[a]) of patients	
	Paediatric	Adult	Paediatric	Adult	Paediatric	Adult
Number of patients	23/68		70/227		23/42	
Age – number of patients with data	23/23		68/70		NA	
Age by paediatric or adult (≥18)	13 (57%)	10 (43%)	39 (57%)	29 (43%)	NA	NA
Gender – number of patients with data	23/23		61/70		0/23	
Male	14 (61%)		35 (50%)		-	
Female	9 (39%)		26 (37%)		-	
Cancer diagnosis number of patients	23/23		57/70		23/23	
Osteosarcoma (102)	11 (48%)		35 (61%)		-	

Demographic	PD Study		NCI Study		Berlin Study	
	No. (%[a]) of patients		No. (%[a]) of patients		No. (%[a]) of patients	
	Paediatric	Adult	Paediatric	Adult	Paediatric	Adult
Leukaemia (61)	3 (13%)		7 (12%)		8 (35%)	
Lymphoma (122)	8 (35%)		12 (21%)		13 (56%)	
Other cancers (12)	1 (4%)		3 (5%)		2 (9%)	
Non Cancer (1)	-		-		-	
Unknown Total (28)	-		13		-	
MTX						
Dose range in g/m^2 (median)	1.4-20 (8)		0.4-19 (8) (69 patients)		1.1-4.6 (2.9)	
Pre-glucarpidase concentration in µmol/L (median)	3.47 - 708 (40.2)		1.1-849.10 (34.7)		1.1-166 (5.8)	
Time between MTX & 1st dose glucarpidase (median)	1-6 (2) days		1-9 (3) days		1-4 (2) days	
Glucarpidase dose range in Units/kg (median)	39-52 (50)		15-55 (50)		11-58 (50)	

Source: adapted from Voraxaze Investigator's Brochure edition number PR001-CLN-IB007

[a] Patients with serum or plasma MTX concentration by HPLC prior to Voraxaze dosing, and in at least one sample after the last dose of Voraxaze and who had MTX ≥ 1 µmol/L prior to Voraxaze.

In all three studies the majority of patients treated with glucarpidase achieved CIR in MTX and thus met the primary endpoint. The reductions in MTX were consistently attained, rapid and generally sustained. The proportion of patients achieving CIR was 57% (13/23) for the PD Study, 57% (40/70) for the NCI Study and 83% (19/23) for Berlin Study. The primary analyses of CIR in all three studies were repeated for subgroups of patients based on pre-glucarpidase plasma/serum MTX concentrations, a diagnosis of osteosarcoma, gender and age (Table 4). In summary, a CIR was seen in 62% (72/116) of patients in the ES across studies and > 50% of patients in the ES of each study had a CIR. The incidence of CIR was

lower for the subgroups with higher pre-glucarpidase MTX concentrations, lower for patients with osteosarcoma, lower for female patients and higher for adult patients.

Table 4: EXPLORATORY SUB-GROUP ANALYSES OF CIR BASED ON PRE-VORAXAZE PLASMA/SERUM MTX CONCENTRATION, CANCER DIAGNOSIS AND GENDER – PD, NCI AND BERLIN STUDIES

	Proportion of patients with a clinically important reduction (CIR) in plasma MTX [percentage] (95% CI)[a]		
Pre-Voraxaze plasma/serum MTX concentration	**PD Study**	**NCI Study**	**Berlin Study**
<1 µmol/L	3/3 [100%] (44% to 100%)	4/4 [100%] (51% to 100%)	3/3 [100%] (44% to 100%)
≥1 µmol/L and <10 µmol/L	4/5 [80%] (38% to 96%)	20/22 [91%] (72% to 97%)	13/14 [93%] (69% to 99%)
≥10 µmol/L and <100 µmol/L	7/11 [64%] (35% to 85%)	13/27 [48%] (31% to 66%)	6/8 [75%] (41% to 93%)
≥100 µmol/L	2/7 [29%] (8% to 64%)	7/21 [33%] (17% to 55%)	0/1 [0%] (0% to 79%)
Cancer diagnosis of osteosarcoma?			
Yes	3/11 [27%] (10% to 57%)	17/35 [49%] (33% to 64%)	ND
No	10/12 [83%] (55% to 95%)	14/22 [64%] (43% to 80%)	ND
Gender			
Male	11/14 [79%] (52% to 92%)	23/35 [66%] (49% to 79%)	NK
Female	2/9 [22%] (6% to 55%)	12/26 [46%] (29% to 65%)	NK

Source: adapted from Voraxaze Investigator's Brochure edition number PR001-CLN-IB007

ND = Not done (no patient in the Berlin Study had osteosarcoma); NK = Not known (gender was not recorded in the Berlin Study), [a] Using the Newcombe & Altman statistical method

Secondary efficacy endpoints for the three clinical studies included the measurement of the reduction in plasma/serum MTX concentrations by HPLC immediately after the administration of glucarpidase (immediate reduction) and in all

samples taken post glucarpidase (sustained reduction). Immediately (10-50 minutes, median 15 minutes) after glucarpidase administration, plasma MTX concentrations for patients had fallen by >95% in the PD and NCI Studies and by > 97.8% in the Berlin Study. There was a gradual increase in MTX concentration following the immediate post-glucarpidase reduction, presumably due to some MTX being redistributed out of the cells. When expressed as a ratio of pre-glucarpidase value, the median sustained reduction in MTX was 98.7%, 97.8% and 96.5% for the PD, NCI and Berlin Studies, respectively.

For patients at risk of MTX toxicity due to delayed MTX elimination or inadvertent overdose, glucarpidase rescue has been shown to be a safe and effective addition to folinic acid rescue. Glucarpidase allows patients to then be managed with standard doses of folinic acid and, if administered early, greatly diminishes the risk of serious and/or life-threatening MTX toxicity. Moreover, patients avoid the risks associated with extracorporeal methods of MTX removal such as dialysis.

Glucarpidase rapidly converts MTX to the inactive metabolite DAMPA. DAMPA is normally a minor metabolite of MTX accounting for < 5% of the total dose of the drug that is excreted in urine (Donehower et al. 1979). It is presumably formed from MTX that is excreted via the bile into the intestinal tract, hydrolysed by bacterial carboxypeptidases, and then reabsorbed. In *vitro* experiments have shown that DAMPA has no cytotoxic potential and does not enhance MTX cytotoxicity (Donehower et al. 1979; Widemann et al 1999). DAMPA is approximately 10-fold less water soluble than MTX (aqueous solubility at pH 7.0: DAMPA 0.85 mg/ml, MTX 9.04 mg/ml), and persistently high concentrations of DAMPA could theoretically lead to further renal toxicity by precipitation in the renal tubules (Donehower et al. 1979; Widemann et al 1997). However, in patients treated for MTX-induced nephrotoxicity with glucarpidase, DAMPA plasma concentrations

declined more rapidly than MTX concentrations, which suggest a non-renal route of elimination for DAMPA. Data from the PD, NCI and Berlin Studies confirmed that DAMPA was effectively eliminated in patients with a median half-life of about 11 hours, even though almost all of them had renal insufficiency (Voraxaze™, Investigator's Brochure 2009).

Glucarpidase has >10-fold lower affinity for folinic acid than for MTX. However, folinic acid is a substrate for glucarpidase and thus may compete with MTX for glucarpidase binding sites. Hence, glucarpidase has the potential to reduce the efficacy of folinic acid. When glucarpidase was administered in healthy subjects (LV interaction study), their exposure to folinic acid was reduced by about 50%, in the absence of MTX; exposure to folinic acid was reduced for about 26 hours. Therefore, in order to minimize any potential interaction, it is recommended that folinic acid should not be administered in the 2-4 hours prior to or in the 2-4 hours following glucarpidase. In order to compensate for any reduced exposure to folinic acid caused by its potential interaction with glucarpidase, it is advised that the dose of folinic acid should be based upon the pre-glucarpidase MTX plasma levels and maintained at this dose for at least 48 hours after the dosing with glucarpidase.

1.4.3. ADVERSE EFFECTS RELATED TO GLUCARPIDASE

Glucarpidase is a pure enzyme (> 98% purity) with a specific activity to hydrolyse the N-carboxy terminal of folate-related molecules. It is administered specifically to hydrolyse MTX. It is not expected to have any adverse effects related to its pharmacological action when given as a single dose for the treatment of a single MTX course.

In the previously described PD, NCI and Berlin studies 52 glucarpidase-related adverse events (AEs) were reported in 8% (26/318) patients (Table 5); only two of

the glucarpidase-related AEs were reported as serious. No glucarpidase-related AEs were reported in the PK study. One treatment related AE was reported in the LV study (pain at injection site) although this event was also recorded by the same subject associated with the saline control injection.

Allergic reaction was the most common glucarpidase-related AE, occurring in 45 of patients treated. Allergic reactions consisted principally of temporally related symptoms of flushing, paraesthesia, and feeling hot/burning sensation. These reactions occurred primarily on the same day as dosing and all were transient. No anaphylactic reactions have been reported in any of the studies. The most common non-allergic AE considered related to treatment with glucarpidase was paraesthesia (0.6% of patients). Two serious adverse events (SAEs) hypertension and arrhythmia that may have been related to glucarpidase administration occurred on the NCI and PD Studies, respectively.

Table 5: ALL ADVERSE EVENTS CONSIDERED RELATED TO GLUCARPIDASE- PD, NCI AND BERLIN STUDIES

Body System/Adverse Event	**No. of patients with AE (N= 318[a])**	**Percentage of patients**
ALL	26	8.2%
Blood and lymphatic system disorders	1	0.3
Pancytopenia	1	0.3
Cardiac disorders	2	0.6
Arrhythmia	1	0.3
Tachycardia	1	0.3
Gastrointestinal disorders	4	1.3
Abdominal pain	1	0.3
Diarrhoea	1	0.3
Nausea	2	0.6
Oral discomfort	1	0.3
vomiting	1	0.3
General disorders and administration site conditions	4	1.3

Body System/Adverse Event	No. of patients with AE (N= 318[a])	Percentage of patients
ALL	26	8.2%
Feeling hot	3	0.9
Pyrexia	1	0.3
Immune system disorders	1	0.3
Hypersensitivity	1	0.3
Investigations	2	0.6
Aspartate aminotransferase increased	1	0.3
Blood creatinine increased	1	0.3
Blood urea increased	1	0.3
Metabolism and nutrition disorders	1	0.3
Hypokalaemia	1	0.3
Hyponatraemia	1	0.3
Nervous system disorders	11	3.5
Burning sensation	3	0.9
Headache	3	0.9
Paraesthesia	7	2.2
Tremor	2	0.6
Renal and urinary disorders	1	0.3
Oliguria	1	0.3
Respiratory, thoracic and mediastinal disorders	2	0.6
Dyspnoea	1	0.3
Throat irritation	1	0.3
Throat tightness	1	0.3
Skin and subcutaneous tissue disorders	2	0.6
Dermatitis allergic	1	0.3
Hyperhidrosis	1	0.3
Pruritus	1	0.3
Vascular disorders	11	3.5
Flushing	7	2.2
Hot flush	1	0.3
Hypertension	1	0.3
Hypotension	2	0.6

Source: Voraxaze Investigator's Brochure edition number PR001-CLN-IB007

[a] Although 329 patients were treated in these studies and included in the study reports, data from follow-up assessments after glucarpidase dosing is only available for 318.

Glucarpidase is a recombinant bacterial protein and it therefore has the potential to induce an immune response. This could produce an allergic reaction on subsequent administration of glucarpidase or reduce its efficacy by neutralizing the activity of glucarpidase.

Across the PD, NCI, Berlin and PK Studies 43% (26/61) of subjects developed anti-glucarpidase antibodies after administration of glucarpidase. Of the positive samples, 19% (5/26) subjects inhibited the activity of glucarpidase. The reduction in enzyme activity in the five patients who had samples which caused a reduction were 35%, 49% and 84% for the PD Study, 23% for the Berlin Study and 44% for the PK Study.

1.5. AIMS

Current literature suggests that the fewer MTX-related chemotherapy delays in osteosarcoma treatment, the better the outcome (Frei et al. 1980; French Bone Tumour Study Group 1988; Bacci et al. 2001; Delepine et al. 1996). However, there is no available data on the incidence chemotherapy delays due to MTX toxicity.

Our first aim was to establish the incidence of chemotherapy delays due to MTX-related toxicity in patients with osteosarcoma, treated with doxorubicin, cisplatin and HD-MTX (MAP).

Medical records of patients with osteosarcoma treated with MAP at the University College Hospital in London were reviewed, and data were collected on age, gender, chemotherapy dates, surgery dates, folinic acid rescue regimens, MTX toxicity and causes of any chemotherapy delays. In a separate review, the tolerance of HD-MTX (12 g/m^2) in patients aged ≥ 40 years was studied and compared with that of younger patients, aged < 40 years.

Our second aim was to examine the role of glucarpidase in routine rescue after HD-MTX in patients with bone sarcoma. Glucarpidase seemed to offer a promising opportunity for rescue from MTX toxicity and if found to be effective and safe in maintaining the treatment intensity and reducing the incidence and severity of MTX-induced toxicity, could optimize treatment, improve patients' well-being, and reduce the use of health resources.

In order to investigate the role of glucarpidase after HD-MTX and whether routine rescue with glucarpidase reduces delays to subsequent cycles of chemotherapy due to MTX toxicity, a randomised, cross-over, phase II clinical trial was set up. Trial patients were treated with 4 doses of HD-MTX (12 g/m^2); 2 of them were given with standard folinic acid rescue and the other 2 with glucarpidase and folinic acid rescue. Delays to subsequent chemotherapy were examined. Reduction of serum MTX levels after glucarpidase, MTX-related toxicity after glucarpidase with standard rescue and after standard rescue alone, glucarpidase-related adverse reactions and anti-glucarpidase antibody response were also examined.

DAMPA, the catabolic product of glucarpidase action on MTX, is known to cross-react with MTX in most commercial immunological MTX assays (Albertioni et al. 1996; Widemann et al. 1997). Therefore, a high performance liquid chromatography (HPLC) assay was validated as part of this project for the evaluation of plasma MTX and DAMPA levels and used for the trial patients.

2. MATERIALS AND METHODS

2.1. REVIEW ON MTX RELATED TOXICITY AND RESULTING DELAY IN SUBSEQUENT CHEMOTHERAPY ADMINISTRATION

In order to determine the incidence of delays in chemotherapy due to MTX toxicity in patients with osteosarcoma treated with doxorubicin, cisplatin and HD-MTX (MAP), we reviewed the medical records of 56 patients with osteosarcoma, treated with MAP in the University College Hospital, London, UK, between January 2004 and January 2005. Data were collected on age, gender, chemotherapy dates, surgery dates and folinic acid rescue regimens. Delayed chemotherapy courses were identified and further information was collected on delays due to MTX toxicity.

A further review was carried in order to study the tolerance of HD- MTX (12 g/m^2) in patients ≥ 40 years. 17 patients, aged ≥ 40 years and treated with MAP in the University College Hospital, London, UK, between December 2002 and October 2007, were identified. 25 patients, aged < 40 years and treated with MAP in the University College Hospital, London, UK, between December 2002 and October 2007, were also identified. Data were collected on age, gender, chemotherapy dates, surgery dates, and daily MTX plasma levels, number of received MTX courses, folinic acid doses, MTX-related toxicity and delays in subsequent chemotherapy due to MTX-related toxicity. Data from different age groups were compared.

2.2. GLU 1 CLINICAL STUDY: A BLIND RANDOMISED, CROSS-OVER, PHASE II CLINICAL TRIAL, TO INVESTIGATE THE EFFICACY AND SAFETY OF GLUCARPIDASE FOR ROUTINE USE AFTER HD-MTX IN PATIENTS WITH BONE SARCOMA

In order to investigate the role of routine glucarpidase rescue after HD-MTX, the GLU 1 clinical trial was set up. GLU 1 was a blind randomised, cross-over, phase II clinical trial, to investigate the efficacy and safety of glucarpidase for routine use after HD-MTX in patients with bone sarcoma (Appendix 1: GLU 1 clinical trial protocol; latest version). **My responsibilities included the following:** designing of the trial and writing of the trial protocol, submission to the Regulatory Authorities and Ethics Committee of all appropriate documentation and subsequent amendments to the trial protocol, validation of a high performance liquid chromatography (HPLC) assay for the measurement of MTX and DAMPA plasma levels, assessment of MTX and DAMPA plasma levels for trial patients with HPLC, informed consent and enrolment of trial participants, involvement in the clinical care of trial participants, nursing and medical staff training on the trial, data management and safety management of the trial.

2.2.1. STUDY OBJECTIVES AND ENDPOINTS

The primary objective of the GLU 1 study was to investigate whether glucarpidase rescue after HD-MTX reduces delay to subsequent cycles of chemotherapy due to MTX toxicity. In order to meet our primary objective trial participants were assessed for fitness to receive chemotherapy on day 15 of each cycle. Patients were considered fit to receive chemotherapy if ALL the following 8 criteria were fulfilled:

- Neutrophils ≥ $0.75x10^9$/L or WCC ≥ $2x10^9$/L
- Platelets ≥ $100x10^9$/L

- Bilirubin ≤ 1.5xULN
- GFR (estimated) ≥ 70 ml/min/1.73m^2
- Mucositis (clinical and functional): grade ≤ 1 (CTCAE v3.0)
- No clinical evidence of infection
- No pyrexia
- Good overall clinical condition

Secondary objectives included the following:

1. To investigate whether glucarpidase rescue after HD-MTX reduces the incidence, severity and duration of MTX associated adverse effects
2. To study the pharmacokinetics of MTX and DAMPA after glucarpidase administration
3. To evaluate any adverse effects associated with the use of glucarpidase
4. To investigate the economic impact of using glucarpidase versus standard rescue
5. To investigate the effect of glucarpidase on the quality of life of patients treated with HD-MTX
6. To assess the anti-glucarpidase antibody response

In order to meet our secondary objectives the following were evaluated:

- Incidence and grading of mucositis, renal toxicity, liver toxicity, neutropenia, thrombocytopenia and infections
- Plasma MTX and DAMPA concentrations
- Incidence of glucarpidase related adverse effects
- Number of days required in hospital per cycle and total dose of folinic acid required per cycle
- Quality of life in study participants

- Serum anti-glucarpidase IgG levels following glucarpidase administration

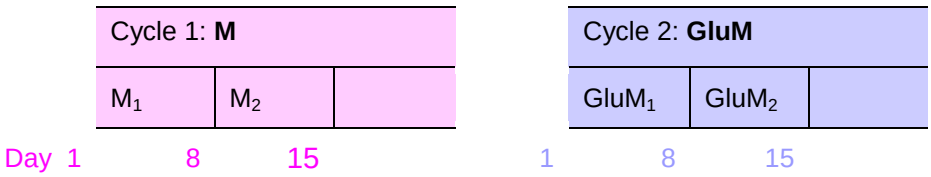
2.2.2. STUDY DESIGN

The GLU 1 clinical trial was conducted in compliance with the principles of ICH GCP, the Declaration of Helsinki, the Medicines for Human Use (Clinical Trials) Regulations 2004 and all applicable regulatory requirements.

Patients participating in the study received four consecutive MTX courses. Two of those were given with standard folinic acid rescue (**courses M**: MTX at a dose of 12 g/m^2 given intravenously with standard folinic acid rescue and placebo, 0.9% Normal Saline injection BP). The other two were given with a combination of folinic acid and glucarpidase rescue (**courses GluM**: MTX at a dose of 12 g/m^2 x 1 given intravenously with folinic acid and glucarpidase rescue at a dose of 50 units/kg given intravenously).

Study participants were randomised to **arm A** or **B** in a 1:1 randomisation. Each arm consisted of two cycles. In **arm A** patients received cycle M first followed by cycle GluM, whereas in **arm B**, they received cycle GluM first followed by cycle M.

Arm A:

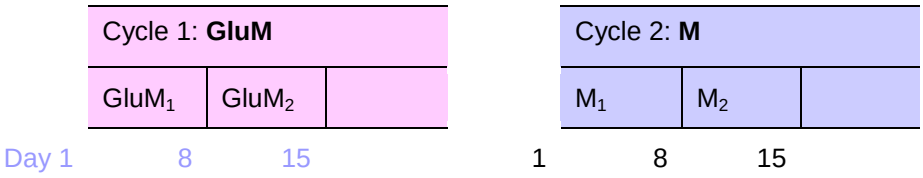


Cycle M started with course **M_1** on day 1 followed by course **M_2** planned for day 8.

Cycle GluM started with course **$GluM_1$** on day 1 followed by **$GluM_2$** planned for day 8. Cycle GluM was not planned to start for a minimum of 14 days from the beginning of course M_2, or until bone marrow, renal and hepatic functions completely recovered and study participants were clinically ready to receive further

chemotherapy (minimum requirements are described in Section 2.2.5). A minimum of 14 days from M_2 to the beginning of the second cycle seemed appropriate to allow complete recovery.

Arm B:



Cycle GluM started with course **$GluM_1$** on day 1 followed by **$GluM_2$** planned for day 8.

Cycle M started with course **M_1** on day 1 followed by course **M_2** planned for day 8. Cycle M was not planned to start for a minimum of 14 days from the beginning of course $GluM_2$, or until bone marrow, renal and hepatic function completely recovered and study participants were clinically ready to receive further chemotherapy (minimum requirements are described in Section 2.2.5.).

GLU 1 was undertaken at the University College Hospital (UCH), London, UK and Harley Street at UCH, London, UK. The London Sarcoma Service at UCH is the largest clinical practice treating bone sarcomas in the UK and one of the largest in Europe, treating 30-40 patients with osteosarcoma every year. Anticipated accrual for the trial was 10 patients per year.

Double-blinding (ie patients and investigators / assessors) was used to reduce any investigator's bias in describing subjective assessments, such as mucositis grading. Patients' responses to Quality of Life Questionnaires were not biased either. UCH Pharmacy Department held the master randomisation list for the study.

Individual treatment could be unblinded only if:

- Serum MTX concentration was ≥ 100 μmol/L at 24 hours or ≥ 10 μmol/L at 48 hours after MTX administration and there was a rise in serum creatinine of ≥ 100% within 24 hours of MTX administration
- Patient experienced a grade 4 allergic reaction after glucarpidase/placebo administration, although he/she remained on study.

In addition, treatment could be unblinded for SUSAR (Suspected Unexpected Serious Adverse Reaction) regulatory reporting by the trial Sponsor.

2.2.3. PATIENT RECRUITMENT

Eligible patients were registered following fully informed consent (Appendix 2: Patient and parent information sheets; Appendix 3, GP information sheets; Appendix 4, patient and parent consent form). There was no time limit between confirmation of diagnosis and registration. A registration form for each patient was completed for each enrolled patient and a unique study number was assigned to each participant (Appendix 5: GLU 1 clinical trial registration form). The registration form was emailed and faxed to UCH Pharmacy Department within 24 hours from registration. UCH Pharmacy assigned the next available treatment slot according to the randomisation list provided by the UCLH Medical Statistics Department. The randomisation list was computer generated and blocked to ensure approximately equal number of patients were allocated to the two sequences. For participants treated at Harley Street at UCH, UCH Pharmacy faxed the randomisation details to 020 7691 5867, for the attention of Aoife Shields, Pharmacy Departrment, Harley Street at UCH.

In order to minimize any cumulative dose effect which might have interfered with the pharmacokinetics of MTX and any MTX-related toxicity, half of the patients followed

arm A and half, arm B. The cross-over design allowed smaller sample size, since patients acted as their controls.

Based on our audit findings (sections 2.1 and 3), nearly 55% of the patients in the standard rescue group were expected to be treatable on day 15 of each cycle. The sample size calculation for the GLU 1 study was based on a McNemar's test with an assumption that the responses to glucarpidase and folinic acid rescue were independent (Machin D et al., 1997). With anticipated proportions of responses to folinic acid alone and glucarpidase with folinic acid of 55% and 90% respectively, GLU 1 required 28 patients to give 80% power at a significance level of 5% and to allow for up to 5% drop-out during the study.

The planned duration of the study was 6 weeks for each participant, 21 days after starting cycle 2. However, patients were followed up on day 30 after starting their second chemotherapy cycle, to investigate anti-glucarpidase antibody response. If anti-glucarpidase antibodies were present on that day, patients were followed up with a further blood test, at 3 and 6 months after starting cycle 2.

Inclusion Criteria

- Patients had to fulfill the following criteria for registration on the trial:
- Written informed consent from patient or parent/guardian
- Diagnosis of high grade osteosarcoma, localised or metastatic, or high grade osteosarcoma as a second malignancy, or spindle cell sarcoma of bone, or relapsed high grade osteosarcoma
- Age: 5-50 years at registration
- Ability to comply with study and follow up procedures (WHO performance scale 0-2) (Appendix 7: Performance status)

- No concomitant anti-cancer or investigational drugs during the study and complete resolution of toxicity related to previous treatment
- Life expectancy of at least 3 months
- Haematopoietic function: absolute neutrophil count ≥ 0.75 $x10^9$/L, platelets ≥ 100 $x10^9$/L
- Hepatic function: Bilirubin ≤ 1.5 x ULN, ALT ≤ 1.5 x ULN, Albumin > LLN
- Renal function: Glomerular Filtration Rate (radioisotope) ≥ 70 ml/min/1.73m^2

Exclusion Criteria

- Previous treatment with glucarpidase
- Pregnant or breast feeding women (patients with reproductive potential of either gender had to use contraception as per Table 6)
- Concomitant treatment with agents which interact with MTX metabolism or excretion (Appendix 8: Drug interactions with MTX)
- Serous effusions, including ascites and pleural effusions

Table 6: ADEQUATE MEANS OF CONTRACEPTION FOR THE GLU 1 PARTICIPANTS

Adequate means of contraception
• Combined oral contraceptive pill • Long acting progesterone only methods (such as the 3-monthly depo injection or the implant) • Intrauterine contraceptive device or intrauterine device • Contraceptive patch • Sterilisation

2.2.4. STUDY ASSESSMENTS

INVESTIGATIONS / PROCEDURES	CYCLE 1						CYCLE 2							
DAY OF CYCLE	-7 to 0	1	2	8	9	15	1	2	8	9	15	30	90	180
DAY OF STUDY	-7 to 0	1	2	8	9	15	22	23	29	30	36	51	111	201
Eligibility & Consent	X													
Medical history	X													
Physical examination, performance status, weight		X		X		X	X		X		X			
Methotrexate administration		X		X			X		X					
Glucarpidase / Placebo administration			X		X			X		X				
Haematology	X			Xa		X	X		Xa		X			
Biochemistry / LFTs	Xb			Xb		X	Xb		Xb		X			
Glomerular Filtration Rate	Xc			Xd		Xd	Xd		Xd		Xd			
Concomitant medication	X			X		X	X		X		X			
[MTX] & [DAMPA] by HPLC analysis		Xe		Xe			Xe		Xe					
[MTX] estimation by immunoassay		Xf		Xf			Xf		Xf					

INVESTIGATIONS / PROCEDURES	CYCLE 1						CYCLE 2							
Anti-glucarpidase antibodies and enzyme neutralisation assay	X			X		X	X		X		X	X	Xg	Xg
Glucarpidase adverse events			Xh		Xh			Xh		Xh				
MTX toxicity assessment		X		X		X	X		X		X			
Quality of Life questionnaires		X		X		X	X		X		X			

a: and at least every 3rd day until haematological recovery; **b:** and at least daily until completion of rescue; **c:** GFR (radio-isotopic assay); **d:** estimated GFR (see Appendix 7); **e:** at 0, 4, 24, 24:20*, 48, 72 hours after starting methotrexate and then daily until methotrexate plasma levels <0.2 µmol/L (*for glucarpidase cycles only); **f:** at 24, 48, 72 hours after starting methotrexate and then daily until methotrexate plasma levels <0.2 µmol/L; **g:** if positive antiglucarpidase antibody response on day 30 of cycle 2; **h:** daily until discharge from hospital

2.2.5. STUDY MEDICATION AND TREATMENT PLAN

2.2.5.1. AGENTS USED

The following agents were used in this study:

- MTX
- Folinic Acid
- Glucarpidase (Voraxaze™)

MTX and folinic acid were obtained from UCH Pharmacy or Harley Street at UCH Pharmacy. The supplier's recommendations regarding storage, stability, dilution, incompatibilities, and measures of caution were followed.

Glucarpidase (Voraxaze™) was the Investigational Medicinal Product in this trial. Labelled vials of Voraxaze™, containing sterile, lyophilised glucarpidase (1000 units/vial), were supplied. The drug substance was manufactured by Eurogentec S.A. Rue du Bois Saint Jean, 14 Liege Science Park B-4102 Seraing (Liege), Belgium. The sterile filling and freeze-drying was performed by Cangene Corporation, 104 Chancellor Matheson Road, Winnipeg, Manitoba, Canada R3T 5Y3. The unlabelled drug product was then released by a qualified person by Protherics UK Limited, Blaenwaun, Ffostrasol, Llandysul, Ceredigion, Wales, UK, SA44 5JT. The final labelled drug product was then supplied by Biotec Distribution Wales Ltd, 17 St Theodore's Way, Brynmenyn Industrial Estate, Bridgend, CF32 9TZ, UK. An approved copy of the label was submitted to the Regulatory Authorities. Voraxaze™ was supplied together with batch numbers and a certificate of release authorised by Protherics.

UCH Pharmacy and Harley Street at UCH Pharmacy were responsible for appropriate storage of Voraxaze™ vials according to the supplier's

recommendations, to ensure stability and integrity. UCH Pharmacy and Harley Street at UCH Pharmacy were also responsible for maintaining a careful record of receipt, use and disposition of unused supplies of Voraxaze™. Samples of the batch of Voraxaze™ used in the study were to be retained by Protherics for 2 years after completion of the study. Following completion of the study and sponsor review of accountability, all unused supplies were to be destroyed and certificates of destruction provided to the sponsor and Protherics.

Normal saline (0.9% Sodium Chloride injection BP) intravenously was used as placebo. The volume of placebo was the same as if calculating the volume for glucarpidase.

2.2.5.2. PRESCRIPTION OF MTX, FOLINIC ACID AND GLUCARPIDASE/PLACEBO

MTX and glucarpidase/placebo were prescribed on ChemoCare® by qualified medical staff. Folinic acid was prescribed on ChemoCare® by qualified medical staff, and also on standard UCH and Harley Street at UCH drug charts. All prescriptions were kept within the participants' notes.

2.2.5.3. MANDATORY ASSESSMENTS PRIOR TO EACH CHEMOTHERAPY COURSE

- Height, weight and surface area
- Clinical examination and concomitant medication
- Full blood count and differential white cell count
- Blood chemistry (creatinine, urea, sodium, potassium, magnesium, phosphate, albumin, alanine transaminase, bilirubin)
- Measurement of glomerular filtration rate (GFR):

- by radio-isotopic method prior to the first course of cycle 1 for all patients
- by estimation prior to all other courses (Appendix 9: Measuring renal function)

- Mucositis assessment (Section 2.2.9)
- Quality of life assessment (Appendix 10)
- Performance status (Appendix 7)
- Blood sample for anti-glucarpidase antibodies and enzyme neutralisation assay

2.2.5.4. MINIMUM REQUIREMENTS PRIOR TO COURSE M_1 AND $GLUM_1$ IN BOTH ARMS

- General clinical condition permitting chemotherapy including no evidence of infection, no pyrexia, mucositis or diarrhoea
- No serous effusions including ascites and pleural effusions
- Neutrophils ≥ 0.75 x 10^9/L and platelets ≥ 100 x 10^9/L
- GFR ≥ 70 ml/min/1.73m^2
- Bilirubin ≤ 1.5 x ULN, ALT ≤ 1.5 x ULN, Albumin > LLN
- Urinary pH >7.0 immediately prior to MTX and good urine output

2.2.5.5. MINIMUM REQUIREMENTS PRIOR TO COURSE M_2 AND $GLUM_2$ IN BOTH ARMS

- General clinical condition permitting chemotherapy including resolving mucositis ≤ grade 1 but no evidence of infection, no pyrexia
- No serous effusions including ascites and pleural effusions
- Neutrophils ≥ 0.25 x 10^9 /L and platelets ≥ 50 x 10^9/L
- Bilirubin ≤ 1.5 x ULN
- Transaminases: any value in the absence of other causes of liver dysfunction
- GFR ≥ 70 ml/min/1.73m^2

- Urinary pH >7.0 immediately prior to MTX and good urine output

2.2.5.6. ADMINISTRATION OF MTX ($GLUM_1$, $GLUM_2$, M_1 AND M_2 COURSES)

Administration of $GluM_1$, $GluM_2$, M_1 and M_2 courses was as follows:

- At -6 hours: patients were hydrated with 800 ml/m^2 4% glucose + 0.18% sodium chloride intravenously (iv) over 6 hours, with potassium chloride 20 mmol/L and sodium bicarbonate 50 mmol/L. Prehydration was continued for at least 6 hours and until urinary pH >7.
- At 0 hours: blood sample for plasma MTX levels was taken immediately prior to starting MTX (HPLC analysis). Extra sodium bicarbonate at a dose of 50-100 mmol, intravenously or orally, was occasionally necessary to maintain alkaline urine (pH 7-8). Provided that urinary pH >7, MTX 12 g/m^2 was infused in 1000 ml 5% glucose over 4 hours along with iv hydration 4% glucose + 0.18% sodium chloride with potassium chloride 20 mmol/L and sodium bicarbonate 50 mmol/L, maintaining a total rate of 125 ml/m^2/h. At the end of MTX infusion, blood sample was taken for plasma MTX levels (HPLC analysis).
- At +4 hours: post hydration was initiated, maintaining a combined oral/iv (4% glucose + 0.18% sodium chloride with potassium chloride 20 mmol/L and sodium bicarbonate 50 mmol/L) fluid intake at 3 L/m^2/day. Post hydration was continued until plasma MTX levels were < 0.2 µmol/L and for a minimum of 72 hours from the beginning of MTX infusion.
- At +24 hours: blood sample was taken for plasma MTX levels for immunoassay and HPLC analysis (pre-glucarpidase plasma MTX levels). Glucarpidase/Placebo was given as a slow intravenous injection over 5 min. Patients were observed for 1 hour after completion of glucarpidase/placebo infusion for hypersensitivity reactions.

- At +24:20 hours: blood sample was taken for plasma MTX and DAMPA levels for HPLC analysis.
- At +26:00 hours: folinic acid rescue was started and continued every 6 hours. Apart from the first dose of folinic acid which was standard (15 mg/m^2 orally), all other folinic acid doses were adjusted according to plasma MTX levels. For the first 48 hours after glucarpidase/placebo, folinic acid dose was adjusted based upon the pre-glucarpidase/placebo plasma MTX levels. After that, folinic acid dose was adjusted according to MTX plasma levels measured by HPLC. In patients who did not tolerate folinic acid orally (due to vomiting, nausea etc), the same dose was given intravenously.
- At +48 hours: blood sample was taken for MTX and DAMPA levels for immunoassay and HPLC analysis.
- At +72 hours: blood sample was taken for MTX and DAMPA levels for immunoassay and HPLC analysis. Folinic acid dose was adjusted according to plasma MTX levels measured by HPLC.
- Plasma MTX and DAMPA levels were evaluated every 24 hours by immunoassay and HPLC analysis until plasma MTX levels were < 0.2 µmol/L and for a minimum of 72 hours from the start of MTX infusion. Folinic acid rescue, hydration and urinary alkalinisation were continued until plasma MTX levels were < 0.2 µmol/L, for a minimum of 72 hours from the start of MTX infusion. In order to compensate for any reduced potential exposure to folinic acid caused by its potential interaction with glucarpidase, the dose of folinic acid was based upon the pre-glucarpidase/placebo MTX plasma levels and maintained for at least 48 hours after the dosing with glucarpidase. After that, folinic acid dose was adjusted according to plasma MTX levels measured by HPLC. Urinary output and urinary pH were maintained at all times at >1 ml/kg/h and pH >7, respectively.

2.2.5.7. GLUCARPIDASE DOSE AND ADMINISTRATION

The Pharmacy Department of the appropriate centre (UCH Pharmacy or Harley Street at UCH Pharmacy) was informed the day prior to any elective admissions of the GLU 1 participants for HD-MTX. Each vial of Voraxaze™ was reconstituted in with 1 ml sodium chloride 0.9%. The vials were stored in the fridge (at 2° to 8°C) and once reconstituted were kept in room temperature and used within 8 hours.

Glucarpidase was administered by qualified nursing staff, at a dose of 50 units/kg, by a slow intravenous injection over 5 minutes, 24 hours after the start of MTX infusion. Date and time of administration were clearly signed and documented in the patient's ChemoCare® chart. The ChemoCare® chart was subsequently filed in the patient's notes.

Patients were monitored for allergic reaction (rash, wheezing, pyrexia) and have their vital signs monitored (heart rate, blood pressure and temperature) prior to glucarpidase and every 15 min minutes until 1 hour after glucarpidase.

If a trial participant suffered with grade 1 (CTCAE v3.0) anaphylactic reaction after glucarpidase, hydrocortisone (≤ 5 years: 50mg, ≥ 6 years: 100mg) was administered, by slow intravenous injection 30 min prior to any subsequent injection of glucarpidase/placebo. If a trial participant experienced grade 2-4 anaphylaxis, he/she was removed from the study.

2.2.5.8. FOLINIC ACID DOSE CALCULATION

$$\text{Total daily dose of folinic acid (mg)} = \frac{\text{Patient's serum MTX levels x standard daily dose of folinic acid*}}{\text{Upper limit of plasma MTX levels for day and time**}}$$

* Standard daily dose is 60 mg/m^2
** Upper limits for plasma MTX levels were:
at 24 h: 20 µmol/L
at 48 h: 2 µmol/L
at 72 h: 0.2 µmol/L

Folinic acid at a dose of ≥ 45 mg was given intravenously in view of its decreased gastric absorption at higher doses.

2.2.5.9. MTX INDUCED RENAL FAILURE AND DELAYED MTX EXCRETION

In case that plasma MTX concentration was ≥ 100 µmol/L at 24 hours or ≥ 10 µmol/L at 48 hours after MTX administration and there was a rise in serum creatinine of ≥ 100% within 24 hours of MTX administration, there was a risk that MTX elimination would be delayed. In such case patients' treatment was unblinded, although they remained on study. If they were found to have received placebo, intervention with glucarpidase was considered.

2.2.6. MTX AND DAMPA PHARMACOKINETIC ASSESSMENTS

DAMPA is known to cross-react with MTX in most commercial immunological MTX assays (Albertioni et al. 1996; Widemann et al. 1997). Consequently MTX levels determined by commercial laboratories are unreliable following treatment with glucarpidase. In the GLU 1 clinical trial plasma MTX levels were measured by both immunoassay and high performance liquid chromatography (HPLC). Plasma DAMPA levels were measured by HPLC.

In order to minimise any potential for *ex vivo* conversion of MTX to DAMPA, all samples taken for HPLC analysis were put on ice as soon as they were taken and spun to separate the plasma as soon as practical. The plasma was then immediately transferred to tubes containing hydrochloric acid at a volume ratio of 10 parts plasma to 1 part acid.

2.2.6.1. ANALYSIS OF MTX PLASMA LEVELS BY IMMUNOASSAY

The TDx/TDxFLx Methotrexate II assay (Abbott Laboratories), which utilizes Fluorescence Polarization Immunoassay (FPIA) technology, was used to measure plasma MTX levels.

Following each of the four HD-MTX doses (on days 1 & 8 of cycles 1 and 2), blood samples were collected at the following time points: at 24 hours (prior to glucarpidase/placebo administration), 48 hours and 72 hours after starting MTX and then daily until plasma MTX levels were < 0.2 µmol/L. At each time point, 5 mls of blood in a plain (red top) bottle was sent for analysis to Clinical Biochemistry UCL Hospitals, 60 Whitfield St, London, W1T 4EU where they were analysed by UCLH biochemistry staff.

2.2.6.2. ANALYSIS OF MTX AND DAMPA PLASMA LEVELS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

A HPLC assay was validated for the GLU 1 study and used for the analysis of MTX and DAMPA plasma levels for trial participants. The validation of the HPLC assay is described in section 4.

Following each of the four HD-MTX doses (on days 1 & 8 of cycles 1 and 2), blood samples were collected for MTX and DAMPA HPLC analysis at the following time points: at 0 hours (prior to starting the MTX infusion), 4 hours (immediately prior to the end of the infusion), 24 hours (prior to glucarpidase/placebo administration), 24:20 hours (15 minutes after glucarpidase/placebo administration), 48 hours and 72 hours after starting MTX infusion and then daily until plasma MTX levels were <0.2 µmol/L (as measured by HPLC).

At each time point, 6 mls of blood were collected in an EDTA bottle. Patient's trial number, hospital number and date of birth were documented on the sample label as

well as the date and time of sample collection. All samples were immediately placed on ice and taken to the University College London Cancer Institute, Paul O'Gorman Building, 72 Huntley Street, London, WC1E 6BT. All samples were then centrifuged at 2000 rpm for 10 minutes and the plasma aliquoted into 3x1 ml freezer vials containing hydrochloric acid at a volume ratio of 10 parts plasma to 1 part acid. Samples which were not analysed immediately were labelled and frozen at -80°C. A sample log was kept for all stored samples.

All HPLC assessments for the GLU 1 study were carried out by me at the University College London, Cancer Institute, the Paul O'Gorman Building, 72 Huntley Street, London, WC1E 6BT.

2.2.7. ASSESSMENT OF ANTI-GLUCARPIDASE ANTIBODY RESPONSE

2.2.7.1. BACKGROUND

Since two doses of glucarpidase were planned for each GLU 1 participant, it seemed important to study the anti-glucarpidase antibody response.

The presence of antiglucarpidase IgG antibodies was determined by a validated qualitative ELISA assay. In addition, serum samples containing anti-glucarpidase antibodies were evaluated for their ability to reduce the enzyme activity of glucarpidase by the use of a validated enzyme inhibition assay.

2.2.7.2. BLOOD SAMPLING SCHEDULE

Blood samples for anti-glucarpidase antibody assessment and enzyme neutralisation assay were collected on days 0, 1, 8 and 15 of cycle 1 and days 1, 8, 15 and 30 of cycle 2. If there was positive anti-glucarpidase antibody response on day 30 of cycle 2, two further blood samples were collected at 3 and 6 months after starting cycle 2.

2.2.7.3. SAMPLING PROCEDURES

At each time point, 5 mls of blood was collected in plain tube (red top) and sent to: University College London Cancer Institute, Paul O'Gorman Building, 72 Huntley Street, London, WC1E 6BT. Samples were then centrifuged at 2000 rpm for 10 minutes and 1 ml of serum put in a 1 ml freezer vial, labelled and frozen at -70°C. Patients' trial number, hospital number and date of birth were documented on the sample label as well as the date and time of sample collection. Samples were subsequently sent on dry ice to Covance Laboratories Limited, Biotechnology Division, Otley Road, Harrogate, North Yorkshire, HG3 1PY. Following receipt at Covance, one serum aliquot was stored appropriately for anti-glucarpidase antibody neutralisation analysis and the second aliquot was shipped to BioAnaLab Limited, Florey House, 3 Robert Robinson Avenue, the Oxford Science Park, Oxford OX4 4GP for anti-glucarpidase antibody analysis. Following analysis of samples by BioAnaLab for anti-glucarpidase antibody, all positive samples were identified to Covance who then performed the anti-glucarpidase antibody neutralisation analysis using the second aliquot.

2.2.8. ASSESSMENT OF GLUCARPIDASE AND MTX RELATED TOXICITY

2.2.8.1. DEFINITIONS

ADVERSE EVENT

An adverse event (AE) is any untoward medical occurrence in a clinical trial subject administered a medicinal product, including occurrences which are not necessarily caused by or related to the medicinal product.

ADVERSE REACTION

An adverse reaction (AR) is any untoward and unintended response in a subject to an investigational medicinal product which is related to any dose administered to that subject.

SERIOUS ADVERSE EVENT OR REACTION

A serious adverse event (SAE) or serious adverse reaction (SAR) is an adverse event or adverse reaction that:

- results in death regardless of its cause
- is life-threatening
- requires hospitalisation or prolongation of an existing hospitalisation. Not every hospitalisation constitutes a reportable serious adverse event (exceptions in “Clarifications and exceptions for GLU 1”).
- results in persistent or significant disability or incapacity (exceptions in “Clarifications and exceptions for GLU 1”)
- is a congenital anomaly or birth defect
- any other medically important condition such as abnormal biological or vital signs, pregnancy and secondary malignancies (exceptions in “Clarifications and exceptions for GLU 1”).

SUSPECTED UNEXPECTED SERIOUS ADVERSE REACTION

A suspected unexpected serious adverse reaction (SUSAR) is a SAR, the nature or severity of which is not consistent with the applicable product information.

Examples include:

- an expected SAR with an unexpected outcome (e.g. fatal outcome)
- “acute renal failure” is an expected AR, a subsequent new report of “interstitial nephritis” is more specific and, therefore, unexpected

- an increase in the rate of occurrence of an expected AR which is judged to be clinically important is considered unexpected.

CLARIFICATIONS AND EXCEPTIONS FOR GLU 1

- Any death during the study was to be reported as an SAE, including death due to disease progression.
- The term “life-threatening” referred to an event where the patient was at immediate risk of death at the time of the event (e.g. required immediate intensive care treatment).
- Hospitalisation was defined as at least one unplanned overnight admission.
- Hospitalisation for HD-MTX was not reported as an SAE. In addition, expected methotrexate side-effects, listed in the product information (section 2.2.8.2 Expected Adverse Events), were not reported on an SAE form, unless they unexpectedly prolonged hospitalisation or required intensive care therapy.
- Hospitalisation due to signs and symptoms associated with disease progression were not considered an SAE, unless outcome leads to death during the study.
- Elective hospitalization for pre-existing condition that had not worsened did not constitute a SAE.
- Disability was defined as a substantial disruption in a person’s ability to conduct normal life functions. Disability directly due to osteosarcoma did not constitute a SAE.
- Other medically important conditions were important medical events which in the opinion of the investigator might not have been immediately life-threatening or resulted in death or hospitalisation but might have jeopardised the patient or might have required intervention to prevent one of the other outcomes listed in the definition above (e.g. anaphylaxis). Expected serious adverse reactions (SAR) such as haematological toxicity or increase in liver enzymes after

methotrexate which resolved were examples of SARs which were not considered reportable as SAE.

2.2.8.2. EXPECTED ADVERSE EVENTS

Expected adverse events associated with glucarpidase are described in Section 1.4.3.

Expected adverse events associated with methotrexate are shown on Table 7.

Table 7: EXPECTED ADVERSE EVENTS RELATED TO MTX

Expected adverse events related to methotrexate			
	Happen to 21-100 patients out of every 100	Happen to 5-20 patients out of every 100	Happen to <5 patients out of every 100
Immediate: Within 1-2 days of receiving drug	_	Nausea, vomiting, anorexia	Dizziness, malaise, blurred vision, allergic reaction, peeling, redness, and tenderness of the skin, especially the soles and palms
Prompt: Within 2-3 weeks, prior to the next course	Transaminase elevations	Diarrhoea, myelosuppression, stomatitis, photosensitivity	Alopecia, folliculitis, renal toxicity, leukoencephalopathy (L), seizures, acute neurotoxicity
Delayed: Any time later during therapy, excluding the above conditions	–	–	Lung damage (L), hyperpigmentation, liver damage (L), osteoporosis (L) Learning disability (L)
Late: Any time after completion of treatment	–	–	Progressive CNS deterioration
Unknown Frequency and Timing: **Foetal and teratogenic toxicities			

Source: extract from EURAMOS 1 clinical trial protocol

**Methotrexate crosses the placenta to the foetus. Foetal toxicities and teratogenic effects of methotrexate (either alone or in combination with other antineoplastic agents) have been noted in humans. The toxicities include: congenital defects, chromosome abnormalities, malformation, severe newborn myelosuppression, pancytopenia, and low birth weight.

**Methotrexate is excreted into breast milk in low concentrations. However, because the drug may accumulate in neonatal tissues, breast feeding is not recommended. Methotrexate is considered to be contraindicated during breast feeding because of several potential problems, including immune suppression, neutropenia, adverse effects on growth, and carcinogenesis.
(L): Toxicity may also occur later

2.2.8.3. REPORTING ADVERSE EVENTS IN THE GLU 1 CLINICAL TRIAL

All AEs related to the study participants were documented in the individual's Case Record Form (CRF) (Appendix 6: GLU 1 clinical trial, Case Record Form) and graded for severity according to Common Terminology for Adverse Events v.3.0 (CTCAE).

AEs related to glucarpidase were recorded in the Glucarpidase Adverse Event form (part of the CRF) and followed-up until their resolution. AEs not related to glucarpidase were followed until the end of study, i.e. day 21 of cycle 2.

2.2.8.4. REPORTING SERIOUS ADVERSE EVENTS IN THE GLU 1 CLINICAL TRIAL

All SAEs regardless of causal relationship were notified within 24 hours to the Joint UCLH/UCL Biomedical Research Unit (JBRU). Notification was promptly followed by a detailed, written report using the Joint UCLH/UCL Serious Adverse Report Form which was faxed to the JBRU on 020 7380 9937. An email was sent to the Lead trial co-ordinator in the JBRU. All SAE forms were also emailed to Dr Karen Maubach, Clinical Development Executive, Protherics at karen.maubach@btgplc.com. In addition, the UCLH NHS Foundation Trust incident form was completed.

If the SAE was classified as a SUSAR the report was sent to the MHRA within the required timeframe by the JBRU (7 days if the event was fatal or life-threatening with a further follow-up in 8 days or 15 days for all other events categorised as SUSARs). The report was also sent by the JBRU to the appropriate research ethics committee.

In addition, the report was sent to Dr Karen Maubach at karen.maubach@btgplc.com who forwarded the information to Protherics Inc. (Nashville, USA). Protherics ensured that the necessary reporting of SAEs was undertaken in the USA.

2.2.9. ASSESSMENT OF MUCOSITIS IN STUDY PARTICIPANTS

No single severity scale meets all requirements of a mucositis assessment tool or is universally accepted. Therefore, in this study four different assessment tools were used:

- World Health Organisation Toxicity Criteria for Oral Mucositis (World Health Organization 1979)
- National Cancer Institute Common Terminology criteria for Adverse Events, version 3.0 (NCI CTCAE v 3.0) (NCI 2003)
- Oral Assessment Guide (Eilers et al. 1988)
- Oral Mucositis Weekly Questionnaire (Stiff et al. 2006)

All four mucositis assessment tools are described in Appendix 11.

The **NCI CTCAE v3.0** and **WHO** scoring scales were chosen as assessment tools in view of their widespread use in studies examining mucositis. Both these scales measure anatomical, symptomatic and functional components of oral mucositis. In addition, the NCI CTCAE v3.0 scale also grades enteritis, colitis, diarrhoea and abdominal distension /bloating.

The **Oral Assessment Guide** is a validated instrument which has been extensively used in adults with cancer. It is designed to objectively assess the physiological changes of the oral cavity following the administration of chemotherapy and

radiotherapy. An adapted version has now been validated for paediatric use (Glenny et al. 2010).

The **Oral Mucositis Weekly Questionnaire** was adapted from the Oral Mucositis Daily Questionnaire, which is developed as a mucositis specific questionnaire to assess patient-reported outcomes (Bellm et al. 2002). It is designed to assess the severity and impact of oral mucositis by evaluating mouth and throat soreness and the degree to which these interfere with activities of daily life such as eating, swallowing, drinking, talking and sleeping. It also assesses the overall health of the patient and the severity of diarrhoea. It has been shown to be a feasible, reliable, valid and responsive patient-reported measure of oral mucositis toxicity (Stiff et al. 2006).

2.2.10. ASSESSMENT OF QUALITY OF LIFE IN STUDY PARTICIPANTS

For study participants aged 15 and under, Quality of Life was assessed using the "PedsQL 3.0 Cancer Module Acute Version questionnaire" (Appendix 10: Quality of Life questionnaires). It encompasses 8 scales: pain and hurt (2 items), nausea (5 items), procedural anxiety (3 items), treatment anxiety (3 items), worry (3 items), cognitive problems (5 items), perceived physical appearance (3 items), and communication (3 items). PedsQL (Varni et al. 2002) has questionnaires for different age groups including 5-7 years, 8-12 years and 13-18 years. There are self-report questionnaires and parent proxy reports. Both the self-reporting and the parent proxy questionnaire had to be completed.

For study participants aged 16 and over at registration, quality of life was assessed using the EORTC (European Organisation for Research and Treatment of Cancer) QLQ-C30 version 3 (Aaronson et al. 1993; Fayers et al 2001) and the FACT-G

(Functional Assessment of Cancer Therapy Scale-General) (Cella et al. 1993) questionnaires (Appendix 10: Quality of Life questionnaires).

The QLQ-C30 questionnaire is a reliable and valid measure of the quality of life of cancer patients in multicultural clinical research settings. It incorporates nine multi-item scales: five functional scales (physical, role, cognitive, emotional and social); three symptom scales (fatigue, pain, nausea and vomiting); and a global health and quality of life scale. The average time required to complete the questionnaire is 11 minutes, and most patients do not require assistance (Aaronson et al. 1993).

The FACT-G is a 33-item questionnaire that can be easily completed in 5 minutes, usually without assistance (Cella et al. 1993). It meets all requirements for use in an oncology clinical trial including ease of administration, brevity, reliability, validity and responsiveness to clinical change. It includes questions on physical, social/family, emotional and functional well-being along with questions on relationship with the doctor.

2.2.11. INTERIM ANALYSIS

An interim analysis was planned after treatment data for half of the planned number of patients was obtained. The Independent Data Monitoring Committee (IDMC) of the GLU 1 study was asked to make a recommendation about the continuation of the trial as instructed by the trial protocol (Appendix 1). In particular, termination of the trial would have been considered if review of the primary outcome showed a statistically significant benefit of glucarpidase, treatment with glucarpidase and folinic acid was significantly worse than standard treatment, glucarpidase associated adverse events outweighed its benefit and recruitment rate appeared inadequate to achieve the required sample size for the trial. In examining efficacy based on the primary outcome, the O'Brien-Fleming method for judging significance of results

from a McNemar's test was used (O'Brien and Fleming TR, 1979). A significance level of 0.005 was used for the interim analysis. To assess whether glucarpidase may be delaying further chemotherapy, the McNemar's test with a one sided significance level of 5% was used.

3. CHEMOTHERAPY DELAYS DUE TO MTX TOXICITY

In order to determine the incidence of delays in chemotherapy due to MTX toxicity, medical records of patients with osteosarcoma treated with MAP at the University College Hospital in London were reviewed, and data were collected on age, gender, chemotherapy dates, surgery dates, folinic acid rescue regimens, MTX toxicity and causes of chemotherapy delays (review A). In a separate review, the tolerance of HD-MTX (12 g/m^2) in patients aged ≥ 40 years was studied and compared with that of younger patients, aged < 40 years (review B).

3.1. REVIEW A: MAP REGIMEN AND CHEMOTHERAPY DELAYS DUE TO MTX TOXICITY

The medical records of 56 patients with osteosarcoma, treated with MAP in University College Hospital, London, UK, between January 2004 and January 2005 were reviewed. Data were collected on age, gender, chemotherapy dates, surgery dates and folinic acid rescue regimens. Delayed chemotherapy courses were identified and further information was collected on delays due to MTX toxicity.

Of the 56 patients studied, 35 were males and 21 were females, with a male to female ratio of 1.6:1 and a median age of 20 years. A total of 235 MAP chemotherapy cycles were reviewed. The median number of cycles received per patient was 5. 175 cycles were “applicable” for analysis of the incidence of subsequent delays in chemotherapy administration due to MTX toxicity. Treatment cycles were not applicable for analysis if there was not enough data in patients’ notes related to the toxicity these cycles resulted in. For example, last chemo cycles were often not applicable as there were no toxicity data in patients’ notes after the last inpatient admission. Similarly cycles preceding surgery were usually not applicable; surgery did not take place at UCH and patients were not reviewed at

UCH prior to surgery in order to have their toxicity documented. The median number of “applicable cycles” received per patient was 4 (Table 8).

52% of chemotherapy cycles (92/175) were delayed due to MTX toxicity by a median of seven days (range 1-28 days), (tables 8 and 9, figure 2). The median number of delayed cycles per patient was 1.5. Causes of delay in starting subsequent chemotherapy included mucositis in 51% of cycles, bone marrow suppression in 28%, infection (12%), nephrotoxicity (8%) and elevated liver enzymes (1%), (figure 3). There were no deaths associated with MTX toxicity in the studied set of patients.

Out of 350 planned MTX courses, 5% (18/350) were omitted due to previous MTX toxicity. Treatment with MTX was discontinued early in 10% (6/56) of patients due to MTX toxicity (Table 9).

Two folinic acid rescue regimens were used in the period studied. In **regimen A**, folinic acid rescue was started at 24 hours after starting the infusion of MTX at a dose of 15 mg/m^2, every six hours, orally or intravenously. MTX plasma levels were measured at 48 hours after starting MTX and then daily until they were < 0.2 µmol/L. The dose of folinic acid was adjusted according to the **48 hour MTX plasma levels** and subsequent daily levels thereafter. The following formula was used to adjust the dose of folinic acid.

$$\text{Total daily dose of folinic acid (mg)} = \frac{\text{Patient's serum MTX levels x standard daily dose of folinic acid*}}{\text{Upper limit of plasma MTX levels for day and time**}}$$

* Standard daily dose is 60 mg/m^2, given in 4 divided doses
** Upper limits for plasma MTX levels:
- at 24 h: 20 µmol/L
- at 48 h: 2 µmol/L
- at 72 h: 0.2 µmol/L

In **regimen B**, which replaced regimen A towards the end of 2004, folinic acid rescue was also started at 24 hours after starting the infusion of MTX at a dose of 15 mg/m^2, every six hours, orally or intravenously. MTX plasma levels were measured at 24 hours after starting methotrexate and then daily until <0.2 µmol/L. The dose of folinic acid was adjusted according to the **24 hour MTX plasma level** and subsequent daily levels thereafter. The above described formula was used to adjust the dose of folinic acid.

Regimen A was used in 98 cycles of which 57% (56/98) were delayed due to MTX toxicity (median 7 days, range 1-28 days), (Table 9 and Table 10, Figure 3). Regimen B was used in 77 cycles of which 47% (36/77) were delayed due to MTX toxicity (median 7 days, range 3-27 days), (Table 9 and Table 10, Figure 2). Early folinic acid dose adjustment, at 24 hours versus 48 hours after starting the infusion of MTX resulted in a decrease in MTX-induced chemotherapy delays by 20%. Despite early folinic acid dose adjustment almost half of chemotherapy cycles (47%) were not given on time due to MTX toxicity.

Table 8: PATIENTS' CHARACTERISTICS, DELAYED CYCLES/APPLICABLE TO REVIEW CYCLES, DISCONTINUATION OF TREATMENT WITH MTX DUE TO MTX TOXICITY

Age	Sex	Total no. of cycles	Number of delayed cycles/applicable to review cycles	Cycle 2 no. of days delayed by:	Cycle 3 no. of days delayed by:	Cycle 4 no. of days delayed by:	Cycle 5 no. of days delayed by:	Cycle 6 no. of days delayed by:	Omitted courses of MTX due to MTX toxicity	MTX discontinuation due to MTX toxicity
12	M	6	5/5	10	N/A	7	9	>7	5c*	NO
12	M	6	2/4	0	0	N/A	17	14	NO	NO
10	M	6	1/4	0	N/A	0	0	7	NO	NO
6	M	6	1/4	>7	N/A	0	0	0	1c*	NO
11	F	6	0/4	0	N/A	0	0	0	NO	NO
8	M	6	2/4	0	N/A	0	7	16	NO	NO
12	F	6	2/3	12	N/A	6	N/A	0	NO	NO
10	F	3	0/2	0	0	N/A	changed to different regimen	changed to different regimen	NO	NO
6	F	6	2/4	0	N/A	0	8	7	NO	NO
8	M	1	1/1	13	not received yet	not received yet	not received yet	not received yet	NO	NO
12	F	1	0/1	0	not received yet	not received yet	not received yet	not received yet	NO	NO
37	M	1	0/1	0	not received yet	not received yet	not received yet	not received yet	NO	NO

Table 8: PATIENTS' CHARACTERISTICS, DELAYED CYCLES/APPLICABLE TO REVIEW CYCLES, DISCONTINUATION OF TREATMENT WITH MTX DUE TO MTX TOXICITY

Age	Sex	Total no. of cycles	Number of delayed cycles/applicable to review cycles	Cycle 2 no. of days delayed by:	Cycle 3 no. of days delayed by:	Cycle 4 no. of days delayed by:	Cycle 5 no. of days delayed by:	Cycle 6 no. of days delayed by:	Omitted courses of MTX due to MTX toxicity	MTX discontinuation due to MTX toxicity
47	M	2	1/2	0	28	changed to different regimen	changed to different regimen	changed to different regimen	2c*	YES
17	F	1	0/1	0	not received yet	not received yet	not received yet	not received yet	NO	NO
17	M	4	0/3	0	N/A	0	0	not received yet	NO	NO
15	M	5	3/4	0	N/A	9	6	3	NO	NO
17	F	4	0/3	0	N/A	0	0	not received yet	NO	NO
16	M	3	0/2	0	N/A	0	not received yet	not received yet	NO	NO
23	F	1	1/1	4	not received yet	not received yet	not received yet	not received yet	NO	NO
20	M	1	1/1	>7	changed to different regimen	changed to different regimen	changed to different regimen	changed to different regimen	1c*	YES
24	M	6	2/4	8	N/A	0	13	0	NO	NO

Table 8: PATIENTS' CHARACTERISTICS, DELAYED CYCLES/APPLICABLE TO REVIEW CYCLES, DISCONTINUATION OF TREATMENT WITH MTX DUE TO MTX TOXICITY

Age	Sex	Total no. of cycles	Number of delayed cycles/applicable to review cycles	Cycle 2 no. of days delayed by:	Cycle 3 no. of days delayed by:	Cycle 4 no. of days delayed by:	Cycle 5 no. of days delayed by:	Cycle 6 no. of days delayed by:	Omitted courses of MTX due to MTX toxicity	MTX discontinuation due to MTX toxicity
23	M	1	0/1	0	changed to different regimen	changed to different regimen	changed to different regimen	changed to different regimen	NO	NO
22	M	2	1/2	0	>7	changed to different regimen	changed to different regimen	changed to different regimen	NO	NO
40	M	3	1/3	5	0	0	not received yet	not received yet	NO	NO
32	F	2	2/2	>7	>7	changed to different regimen	changed to different regimen	changed to different regimen	1c and 2c*	YES
26	M	4	1/3	0	1	0	0	not received yet	NO	NO
34	M	1	1/1	25	not received yet	not received yet	not received yet	not received yet	NO	NO
20	M	6	2/4	11	N/A	0	2	not received yet	NO	NO
40	M	5	1/4	0	0	N/A	0	3	NO	NO

Table 8: PATIENTS' CHARACTERISTICS, DELAYED CYCLES/APPLICABLE TO REVIEW CYCLES, DISCONTINUATION OF TREATMENT WITH MTX DUE TO MTX TOXICITY

Age	Sex	Total no. of cycles	Number of delayed cycles/applicable to review cycles	Cycle 2 no. of days delayed by:	Cycle 3 no. of days delayed by:	Cycle 4 no. of days delayed by:	Cycle 5 no. of days delayed by:	Cycle 6 no. of days delayed by:	Omitted courses of MTX due to MTX toxicity	MTX discontinuation due to MTX toxicity
20	M	3	0/2	0	N/A	0	not received yet	not received yet	NO	NO
18	M	6	1/4	>7	N/A	0	0	0	1c*	NO
17	M	6	3/4	3	N/A	5	>7	>7	4c*	NO
43	M	4	2/3	0	10	7	N/A	changed to different regimen	NO	NO
21	F	6	3/5	>7	3	0	12	0	1c*	NO
37	F	5	1/4	0	N/A	0	0	>7	NO	NO
14	M	6	4/5	9	>7	15	0	7	2c*	NO
20	F	6	3/4	11	N/A	17	7	0	NO	NO
24	M	4	2/4	5	0	0	4	changed to different regimen	NO	NO
18	M	2	2/2	>7	>7	changed to different regimen	changed to different regimen	changed to different regimen	1c and 2c*	YES

Table 8: PATIENTS' CHARACTERISTICS, DELAYED CYCLES/APPLICABLE TO REVIEW CYCLES, DISCONTINUATION OF TREATMENT WITH MTX DUE TO MTX TOXICITY

Age	Sex	Total no. of cycles	Number of delayed cycles/applicable to review cycles	Cycle 2 no. of days delayed by:	Cycle 3 no. of days delayed by:	Cycle 4 no. of days delayed by:	Cycle 5 no. of days delayed by:	Cycle 6 no. of days delayed by:	Omitted courses of MTX due to MTX toxicity	MTX discontinuation due to MTX toxicity
16	M	6	1/4	>7	N/A	0	0	0	1c*	NO
17	M	6	2/4	4	N/A	0	0	7	NO	NO
22	M	3	2/2	5	N/A	10	changed to different regimen	changed to different regimen	NO	NO
30	F	5	2/4	0	N/A	0	4	4	NO	NO
14	M	6	4/4	5	>7	7	N/A	6	2c*	NO
31	M	2	1/2	0	9	changed to different regimen	changed to different regimen	changed to different regimen	NO	NO
21	F	6	5/5	10	>7	3	10	10	2c*	NO
31	F	6	3/3	8	>7	>7	N/A	N/A	2c and 3c*	NO
19	F	6	1/4	0	N/A	0	14	0	NO	NO
14	M	6	0/4	0	N/A	0	0	0	NO	NO
14	F	6	2/4	0	N/A	8	27	0	NO	NO
34	M	6	2/4	5	N/A	0	0	15	NO	NO

Table 8: PATIENTS' CHARACTERISTICS, DELAYED CYCLES/APPLICABLE TO REVIEW CYCLES, DISCONTINUATION OF TREATMENT WITH MTX DUE TO MTX TOXICITY

Age	Sex	Total no. of cycles	Number of delayed cycles/applicable to review cycles	Cycle 2 no. of days delayed by:	Cycle 3 no. of days delayed by:	Cycle 4 no. of days delayed by:	Cycle 5 no. of days delayed by:	Cycle 6 no. of days delayed by:	Omitted courses of MTX due to MTX toxicity	MTX discontinuation due to MTX toxicity
22	F	2	1/1	11	N/A	changed to different regimen	changed to different regimen	changed to different regimen	NO	NO
24	F	3	2/3	0	13	10	changed to different regimen	changed to different regimen	NO	YES
20	M	6	2/4	13	0	0	N/A	13	NO	NO
23	F	4	3/4	>7	0	10	15	changed to different regimen	1c*	YES
13	F	6	5/5	9	4	6	7	6	NO	NO

FOLINIC ACID RESCUE REGIMENS

	Regimen A
	Regimen B

*1a, 1b, 1c, 2a, 2b, 2c etc.: numbers refer to the cycle of chemotherapy and letters refer to treatment course; a=doxorubicin & cisplatin, b=1st MTX in each cycle, c=2nd MTX in each cycle; for example 4a means doxorubicin &cisplatin in cycle 4.

Table 9: INCIDENCE OF DELAYED CHEMOTHERAPY CYCLES DUE TO MTX TOXICITY

	Total number of cycles	Delayed cycles	Incidence of delay	Median delay, days (range)
Regimen A (Late rescue)	98	56	57%	7(1-28)
Regimen B (Early rescue)	77	36	47%	7(3-27)
Both regimens	175	92	52%	7(1-28)

Figure 1: INCIDENCE OF DELAYED CHEMOTHERAPY CYCLES DUE TO MTX TOXICITY

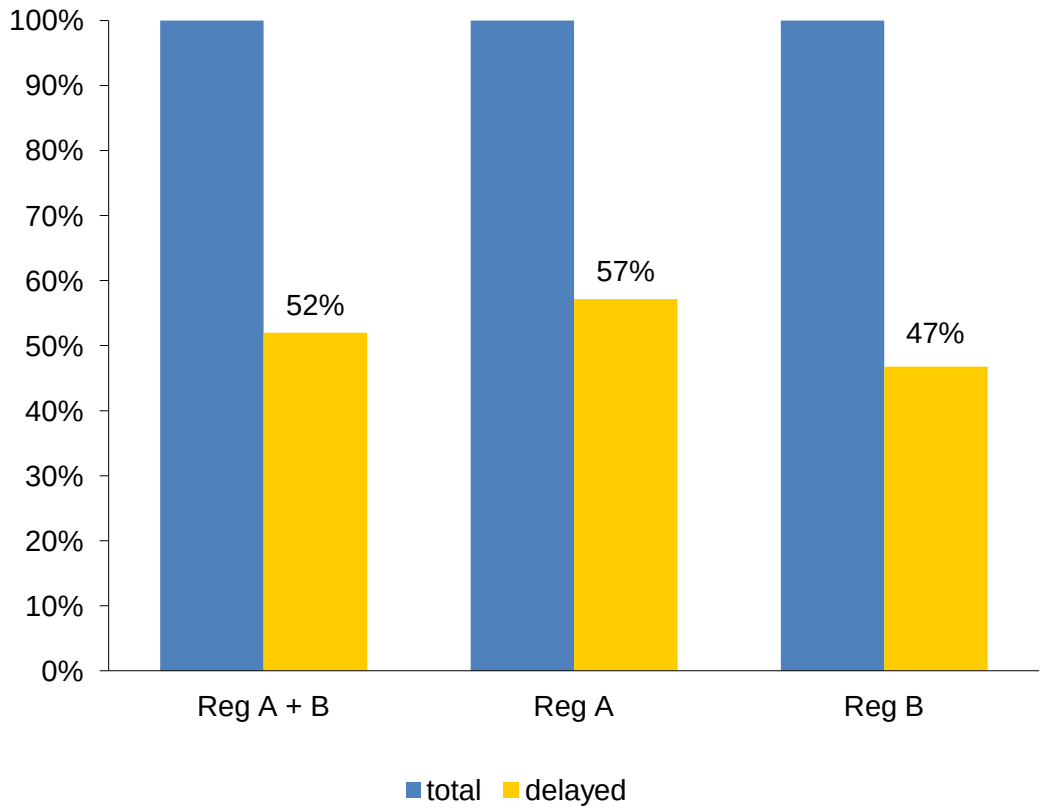


Figure 2: CAUSES OF DELAY IN STARTING SUBSEQUENT CHEMOTHERAPY

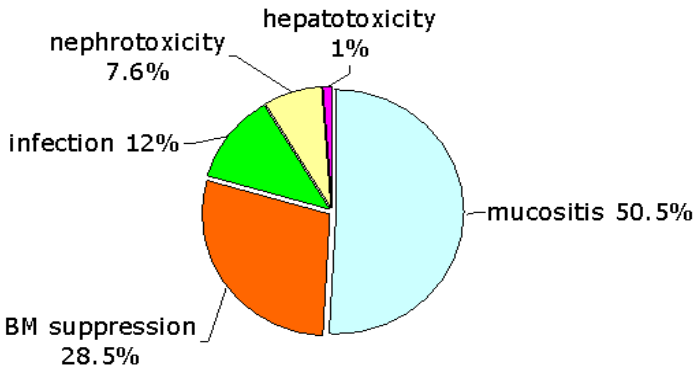
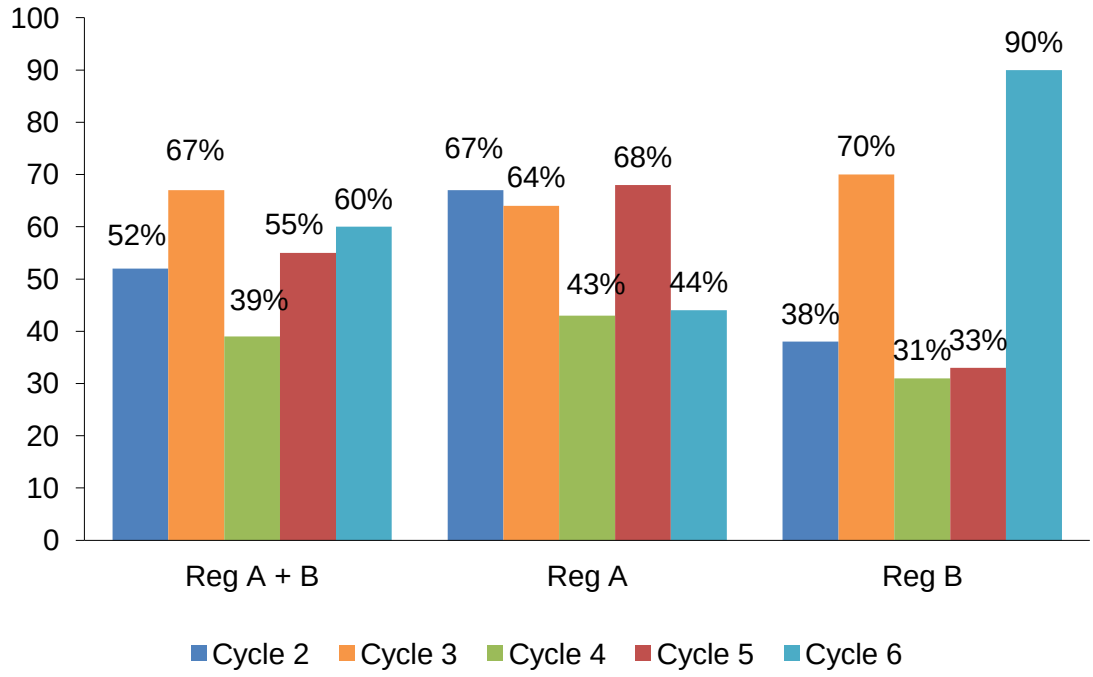


Table 10: INCIDENCE OF CHEMOTHERAPY DELAY PER CYCLE FOR BOTH FOLINIC ACID REGIMENS AND FOR EACH FOLINIC ACID REGIMEN INDIVIDUALLY

	Both FA regimens		**Regimen A**		**Regimen B**	
Cycles	**Incidence of delay**	**Median delay, days (range)**	**Incidence of delay**	**Median delay, days (range)**	**Incidence of delay**	**Median delay, days (range)**
Cycle 2	52% (29/56)	7 (3-25)	66.6% (18/27)	7 (3-13)	38% (11/29)	7 (5-25)
Cycle 3	66% (14/21)	7 (1-28)	63.6% (7/11)	7 (3-10)	70% (7/10)	7 (1-28)
Cycle 4	38.5% (15/39)	7 (3-17)	43.5% (10/23)	7 (3-17)	31% (5/16)	9 (6-15)
Cycle 5	55% (17/31)	8 (2-27)	68.5% (13/19)	9 (4-27)	33% (4/12)	6.5 (2-17)
Cycle 6	60.7% (17/28)	7 (3-16)	44.5% (8/18)	7 (4-13)	90% (9/10)	7 (3-16)

Figure 3: INCIDENCE OF CHEMOTHERAPY DELAY PER CYCLE FOR BOTH FOLINIC ACID REGIMENS AND FOR EACH FOLINIC ACID REGIMEN INDIVIDUALLY



3.2. REVIEW B: TOLERANCE OF HD-MTX (12 g/m^2) IN PATIENTS ≥ 40 YEARS

A further review was carried in order to study the tolerance of HD-MTX (12 g/m^2) in patients ≥ 40 years. 17 patients, aged ≥ 40 years who were treated with MAP regimen in the University College Hospital, London, UK, between December 2002 and October 2007, were identified. 25 patients, aged < 40 years who were treated with MAP regimen in the University College Hospital, London, UK, between June 2003 and September 2006, were also identified. Data were collected on age, gender, chemotherapy dates, surgery dates, and daily MTX plasma levels, number of received MTX courses, folinic acid doses, MTX-related toxicity and delays in subsequent chemotherapy due to MTX-related toxicity. Data from different age groups were compared.

PATIENTS ≥ 40 YEARS

17 eligible patients were identified, although medical notes of 16 patients were retrieved. The age of the patients ranged between 40-50 years with a median age of 44.5 years. 9 of those were males and 7 were females. On average each patient received 4.9 courses of MTX (range: 2-12). 55 chemotherapy cycles reviewed, and 41 of those were applicable for analysis of the incidence of subsequent delays in chemotherapy administration due to MTX toxicity. 24 out of the 41 applicable MAP cycles were delayed (58.5%) by 2-26 days (median 7.5 days) due to MTX related toxicity. Causes of delay in starting subsequent chemotherapy included: mucositis in 37.5% MAP cycles (9/24), renal impairment in 20.8% (5/24), mucositis and bone marrow suppression in 16.6% (4/24), mucositis and renal impairment in16.6% (4/24) and bone marrow toxicity in 8.3% (2/24).

In 15 out of a total of 16 reviewed patients, treatment with MTX was stopped early. Only one patient completed all planned 12 MTX courses. In 12 of those 15 patients, MTX treatment was stopped early due to MTX related toxicity. Reasons for stopping MTX treatment early included renal impairment in 50% (6/12) of the patients and mucositis in the other 50% (6/12) of the patients.

MTX plasma levels at 24 hours after starting MTX ranged from 2.95 to 115.66 µM (median: 25.89 µM, average 40.30 µM). Days required to clear MTX ranged from 2 to 9 (median: 3, average 3.7). With regards to folinic acid regimen used 10/16 of patients (72%) had 3 hourly doses for the first 24 hours and then 6 hourly and 4/16 of patients (28%) had 6 hourly doses from the start of their folinic acid rescue.

PATIENTS < 40 YEARS

The medical noted of 25 eligible patient were retrieved and reviewed. The age of the patients ranged between 7-39 years with a median age of 15 years. 17 of those

were males and 8 were females. On average each patient received 10.26 courses of MTX (range: 1-12). 135 chemotherapy cycles were reviewed, and 92 of those were applicable for analysis of the incidence of subsequent delays in chemotherapy administration due to MTX toxicity. 48 out of the 92 applicable MAP cycles were delayed (52.2%) due to MTX related toxicity. Causes of delay in starting subsequent chemotherapy included: mucositis in 29% MAP cycles (14/48), bone marrow toxicity in 25% (12/48), infection in 23% (11/48), mucositis and bone marrow suppression in 12.5% (6/48), mucositis and renal impairment in 8% (4/48), elevated liver enzymes in 4% (2/48) and renal impairment in 4% (2/48).

In 5 out of a total of 25 reviewed patients, treatment with MTX was stopped early. In 3 of those 5 patients, MTX treatment was stopped early due to MTX related toxicity. Reasons for stopping MTX treatment early included renal impairment in 2/3 of patients and patient's request in 1/3 of patients. Fifteen patients completed all planned 12 MTX courses.

MTX plasma levels at 24 hours for this group of patients ranged from 1.61 to 160.63 µM (median: 12.13 µM, average 17.5 µM). Days required to clear MTX ranged from 2 to more than 10 (median: 3, average 3.11).

Data from the two different age groups are shown on Table 11.

Table 11: TOLERANCE OF HD-MTX (12 g/m^2) IN PATIENTS ≥ 40 YEARS AND < 40 YEARS

	≥ 40 years	**< 40 years**
Number of patients	16	25
Age	40-50 years, median 44.5 years	7-39 years, median 15 years
Delayed/applicable cycles	24 / 41 cycles were delayed (58.5%)	48 / 92 cycles were delayed (52.2%)
MTX courses received	average 4.9 courses of MTX (2-12)	average 10.26 courses of MTX (1-12)

	≥ 40 years	< 40 years
Reason for delay	Mucositis 37.5% (9/24) Renal impairment 20.8% (5/24) Mucositis & BM suppression 16.6% (4/24) Mucositis & renal impairment 16.6% (4/24) BM suppression 8.3% (2/24)	Mucositis 29% (14/48) BM toxicity 25% (12/48) Infection 23% (11/48) Mucositis & BM suppression 12.5% (6/48) Mucositis & renal impairment 8% (4/48) Elevated liver enzymes 4% (2/48) Renal impairment 4% (2/48)
Reason for early termination of MTX treatment	Renal impairment 50% (6/12) Mucositis 50% (6/12)	Renal impairment 2/3 Patient's request 1/3
Days to clear MTX	2-9 (median: 3, average 3.7)	2->10 (median: 3, average 3.11)
24 hr MTX plasma level	2.95 -115.66 µM (median: 25.89 µM, average 40.30 µM)	1.61 -160.63 µM (median: 12.13 µM, average 17.5 µM)

CONCLUSIONS

- There was only a small difference in the number of subsequent chemotherapy delays due to MTX toxicity in the studied patient groups. In the ≥ 40 year-group, 58.5% (24/41) of MAP cycles were delayed due to MTX related toxicity. In the < 40 year-group, 52.2% (48/92) of MAP cycles were delayed due to MTX related toxicity.
- There was a large difference in the number of received MTX courses. In the ≥ 40 year-group, treatment with MTX was stopped early in 15/16 patients and only 1/6 of patients completed all planned 12 MTX courses. In 12/15 of patients, MTX treatment was stopped early due to MTX related toxicity. In the < 40 year-group, treatment with MTX was stopped early in 5/25 of patients. In 3/5 of patients treatment with MTX was stopped early due to MTX related toxicity. 15/25 of patients completed all planned 12 MTX courses.

4. VALIDATION OF MEASUREMENT OF MTX AND DAMPA IN HUMAN PLASMA WITH HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

4.1. INTRODUCTION

DAMPA, the catabolic product of glucarpidase action on MTX, is known to cross-react with MTX in most commercial immunological MTX assays (Albertioni et al. 1996; Widemann et al. 1997). Therefore, a HPLC assay for the evaluation of plasma MTX and DAMPA levels for trial patients was validated. The bioanalytical method is based on a published HPLC method (Albertioni et al. 1995).

In a previous validation study carried out by Protherics plc / Huntington Life Sciences Limited, fluorescence detection was used (personal correspondence; Study PTU/018: “Validation of a high performance liquid chromatographic method for measurement of methotrexate and DAMPA in human plasma”). However, in our laboratory UV detection at 313 nm was shown to produce a better linear standard curve over the range of 0-50 µmol/L for both MTX and DAMPA. MTX had a lower limit of detection at 0.2 µmol/L compared with 0.5 – 1 µmol/L for DAMPA (Figure 4a, 5b and 5c).

The method involved the extraction of MTX and DAMPA from aliquots of human plasma (1 mL) mixed with hydrochloric acid (111 µL, 1 M) using C18 Strata™ (500 mg, 3 mL) extraction cartridges. The extraction cartridges were conditioned with methanol (3 mL), water (3 mL) and sodium phosphate buffer (2 X 3 mL, 0.1 M, pH 6.5). The acidified plasma sample was applied to the cartridge which was then washed with sodium phosphate buffer (3 mL, 0.1 M, Ph 6.5). MTX and DAMPA were eluted from the extraction cartridges with methanol (3 mL) and the eluates were evaporated to dryness under a stream of nitrogen at nominally +50°C. The residues

were reconstituted in mobile phase (200 µL) and aliquots (10 µL) were injected onto a Phenomenex Luna 5 µ, C18 (2) 100A, (150 X 4.6 mm) analytical column. MTX and DAMPA were chromatographed isocratically and detected by UV at 313 nm. A comprehensive description of the method is described in Section 4.4. The assay was validated producing standard curves for MTX and DAMPA in both buffer and plasma and running the analysis to test linearity, precision, accuracy, sensitivity, recovery, specificity, dilution and stability.

Figure 4: REPRESENTATIVE STANDARD CURVES WITH UV AND FLUORESCENCE DETECTION

MTX and DAMPA standards were prepared in phosphate buffer at concentrations of 0.2, 0.5, 1, 5, 10, 20, and 50 µM. These were run on the standard HPLC method and processed both with UV (313 nm) and fluorescence (excitation 350 nm, emission 435 nm) detection. Figures 5b and 5c show that UV detection gives linear response from 0-50 µM, whereas the fluorescence detection shows linearity from 0-20 µM only (Figure 4a). Therefore UV detection (313 nm) has been used throughout this validation report.

Figure 4a: FLU CURVES

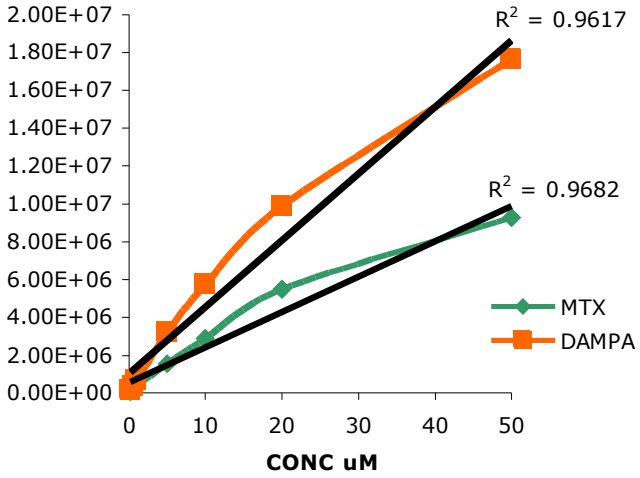


Figure 4b: DAMPA UV ANALYSIS

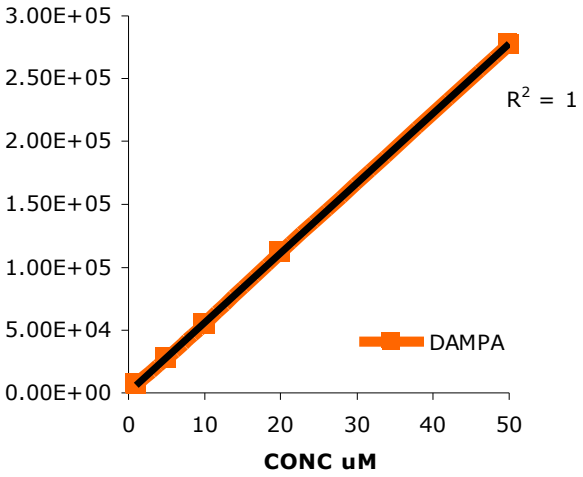
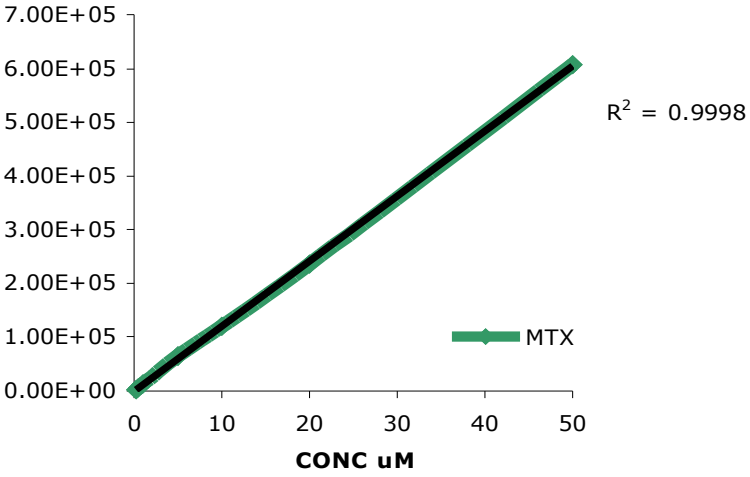


Figure 4c: MTX UV ANALYSIS



4.2. STUDY SCHEDULE

Experimental start date: 10 October 2006

Experimental completion date: 30 March 2007

4.3. TEST SUBSTANCES

MTX

Lot/batch no. : 044K07351

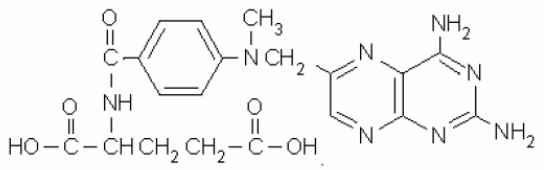
Grade/purity : 99%

Supplied by : SIGMA-ALDRICH

Storage : Room temperature

Molecular weight : 454.44

Chemical structure :



DAMPA

Lot/batch no. : 11502KC

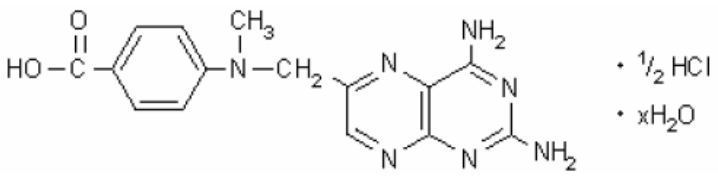
Grade/purity : 95%

Supplied by : SIGMA-ALDRICH

Storage : Room temperature

Molecular weight : 343.56

Chemical structure :



4.4. BIOANALYTICAL METHOD

a. Instrumentation

Waters 2695 Separations Module

Waters 2996 Photodiode Array Detector

Empower Pro software

b. Equipment

Analytical balance: Sartorius Research, R160P

Automatic pipettes: FinnPipette®, various sizes

Centrifuge: Heraeus Megafuge 1.0 and Eppendorf Centrifuge 5415D

Filtration apparatus: Millipore (UK) Ltd, Watford, UK, 0.2 µ

Micro test tubes: Ependorf® tubes, VWR, International, Poole, UK

pH meter: Hanna Instruments, pH 210, VWR, International, Poole, UK

Sample concentrator: SC-2, DB3, Techne Ltd, Cambridge, UK

Solid phase cartridges: Strata™ C18 (500 mg, 3 ml), Phenomenex, Macclesfield

SPE apparatus: Spe-ed Mate™-30, Applied Separations, Lehigh Valley, PA, USA

Top-loading balance: METTLER PJ400

Vortex mixer: AUTOVORTEX MIXER SA2, STUART SCIENTIFIC, UK

Water purification: USF ELGASTAT, Elga Ltd, High Wycombe, UK

c. Test Substances

Methotrexate was stored in a closed container, in the dark, with a desiccant at nominally -20°C. DAMPA was stored in a closed container, in the dark, with a desiccant at room temperature.

d. Materials

Acetonitrile: HPLC grade, Rathburn Chemicals, RH1016

Blank human plasma: Plasma pool from UCLH Blood Bank

Dimethylsulfoxide (DMSO): BDH AnalaR, VWR, International, Poole, UK

Hydrogen Peroxide (H_20_2): 30% solution in water (w/w), SIGMA ALDRICH

Methanol: HPLC grade, Rathburn Chemicals, RH1019

Sodium dihydrogen orthophosphate dihydrate: BDH AnalaR, VWR, International, Poole, UK

Disodium hydrogen orthophosphate dihydrate: BDH AnalaR, VWR, International, Poole, UK

Water: Ultra High purity (UHP) grade obtained from Elgastat water purification system

e. Reagents

Sodium dihydrogen orthophosphate dihydrate (0.1M)

This was prepared by dissolving sodium dihydrogen orthophosphate dihydrate (15.601 g) in water and making up to a final volume (1000 ml) with water.

Disodium hydrogen orthophosphate dihydrate (0.1M)

This was prepared by dissolving disodium hydrogen orthophosphate dihydrate (17.789 g) in water and making up to a final volume (1000 ml) with water.

Phosphate buffer (0.1M, pH 6.5)

This was prepared by mixing sodium dihydrogen orthophosphate dehydrate solution (0.1 M) and disodium hydrogen orthophosphate dihydrate solution (0.1 M) in a ratio 2:1 and checking the pH.

Mobile Phase

The Solvent Manager System mixed the mobile phase 90:10 of a:b where a = phoshate buffer (900 ml, 0.1 M, pH 6.5) plus 2 ml 30% w/w hydrogen peroxide, and b = acetonitrile. Buffer a was filtered and degassed by vacuum filtration through glass fibre filter paper. The final mobile phase was degassed by on-line vacuum degassing.

Column wash

100% water wash, followed by 50% acetonitrile & 50% water wash. Additionally, after every 5 assays 100% water wash, followed by 50% acetonitrile & 50% water wash, followed by 100% acetonitrile wash, followed by 50% acetonitrile & 50% water wash. Each stage run for 15 min.

Needle wash

This was prepared by mixing 50% methanol and 50% water.

All reagents were stored at room temperature (nominally +22°C), unless stated otherwise, and used for up to one month, except for the buffer phosphate which was

mixed with hydrogen peroxide (part of the mobile phase) which was used for up to 15 days. Different volumes of reagents were prepared on a *pro rata* basis.

f. Standard Solutions

MTX primary standard solution (10 mmol/L)

A primary standard solution of MTX was prepared at concentration of 10 mmol/L by accurately weighing methotrexate and dissolving it in DMSO to a concentration of 4.54 mg/ml (~10 mmol/L).

DAMPA primary standard solution (10 mmol/L)

A primary standard solution of DAMPA was prepared at concentration of 10 mmol/L by accurately weighing DAMPA and dissolving it in DMSO to a concentration of 3.43 mg/ml (~10 mmol/L).

MTX and DAMPA secondary standard solution (1 mmol/L)

The MTX primary standard solution (1 mmol/L) and DAMPA primary standard solution (1mmol/L) were prepared by 10-fold dilution with phosphate buffer (0.1 mmol/L, pH 6.5).

MTX and DAMPA working standard solutions

Aliquots of appropriate MTX and DAMPA standard solutions were transferred to Eppendorf® tubes and diluted with phosphate buffer (0.1 mmol/L, pH 6.5) to produce working standard solutions at concentrations of 0.4-40 µmol/L of MTX and DAMPA as detailed below:

Concentration of standard solution (µmol/L)	Volume of standard solution (µL)	Final volume (µL)	Concentration of working standard (µmol/L)
1000	40	1000	40

Concentration of standard solution (µmol/L)	Volume of standard solution (µL)	Final volume (µL)	Concentration of working standard (µmol/L)
1000	20	1000	20
20	500	1000	10
40	100	1000	4
20	100	1000	2
10	100	1000	1
4	100	1000	0.4

All standard solutions were stored in the dark at nominally +4°C in Eppendorf® tubes. Different volumes of standards were prepared on a *pro rata* basis.

g. Calibration Standards

Equal aliquots of MTX and DAMPA working standard solutions (100 µL) were mixed together to produce buffer calibration standards at concentrations of 0.2-20 µmol/L.

h. Quality Control Samples

QC samples were prepared independently for use during method validation, and to monitor the performance of the method during routine sample analysis. These were prepared in bulk by the addition of MTX and DAMPA standards in phosphate buffer (0.1 M, pH 6.5) to Eppendorf® tubes and made up to volume with blank plasma to give concentrations of 0.1, 0.2, 0.5, 10 and 50 µmol/L as detailed below:

Concentration of standard solution (µmol/L)	Volume of standard solution (µL)	Final volume (mL)	Concentration of QC (µmol/L)
2	500	10	0.1
4	500	10	0.2
10	500	10	0.5
200	500	10	10
1000	500	10	50

The bulk QC samples were divided into 1 mL aliquots and stored in Eppendorf® tubes at nominally -20°C until taken for analysis.

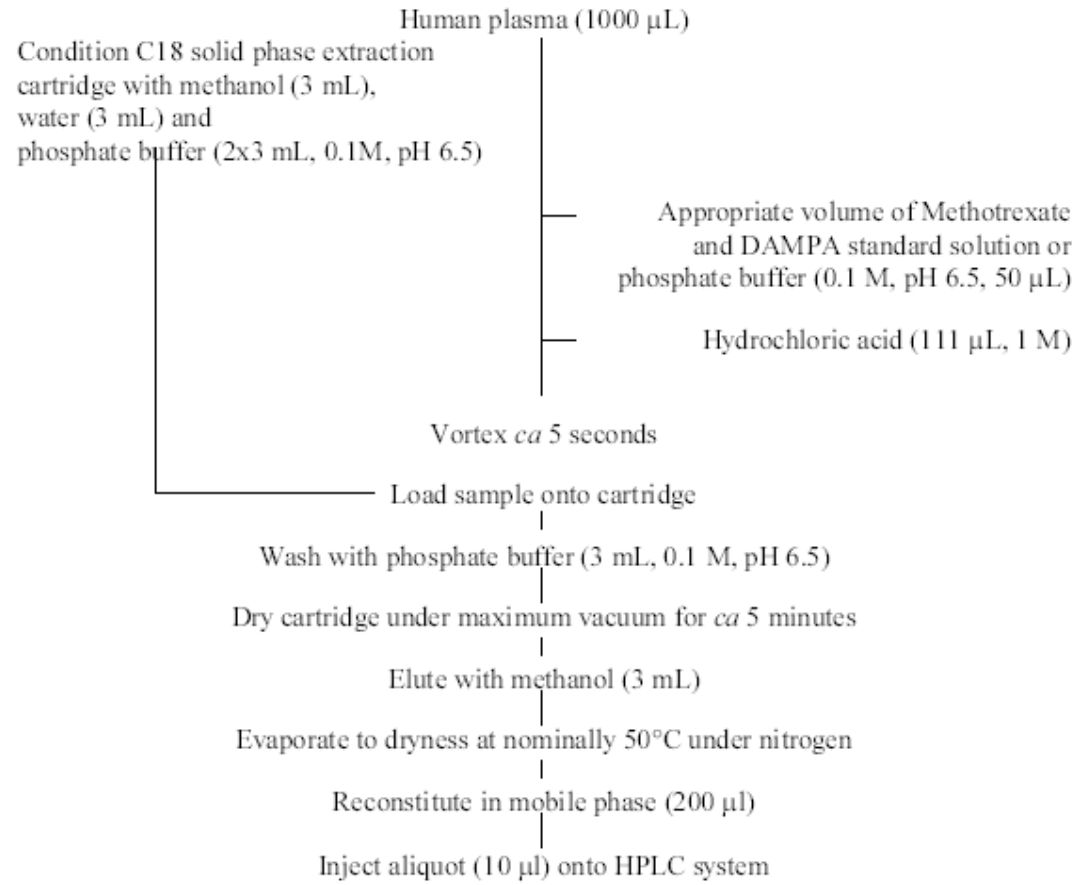
i. Dilution Samples

In many circumstances, it was necessary to dilute test samples with expected analyte concentrations greater than the highest calibration standard in order to be able to measure the analyte in the validated calibration range. For example, a 1 in 10 dilution factor was validated by dilution of the highest QC standard (50 μmol/L) with blank plasma before being extracted and analysed.

j. System Suitability

Prior to analysis of a batch of samples, a standard mixture containing MTX and DAMPA in phosphate buffer (0.1mmol/L, pH 6.5) was injected to check the performance of the chromatographic system in terms of retention times, peak shapes, resolution and instrument response.

k. Sample Preparation Procedure



l. Chromatography Conditions

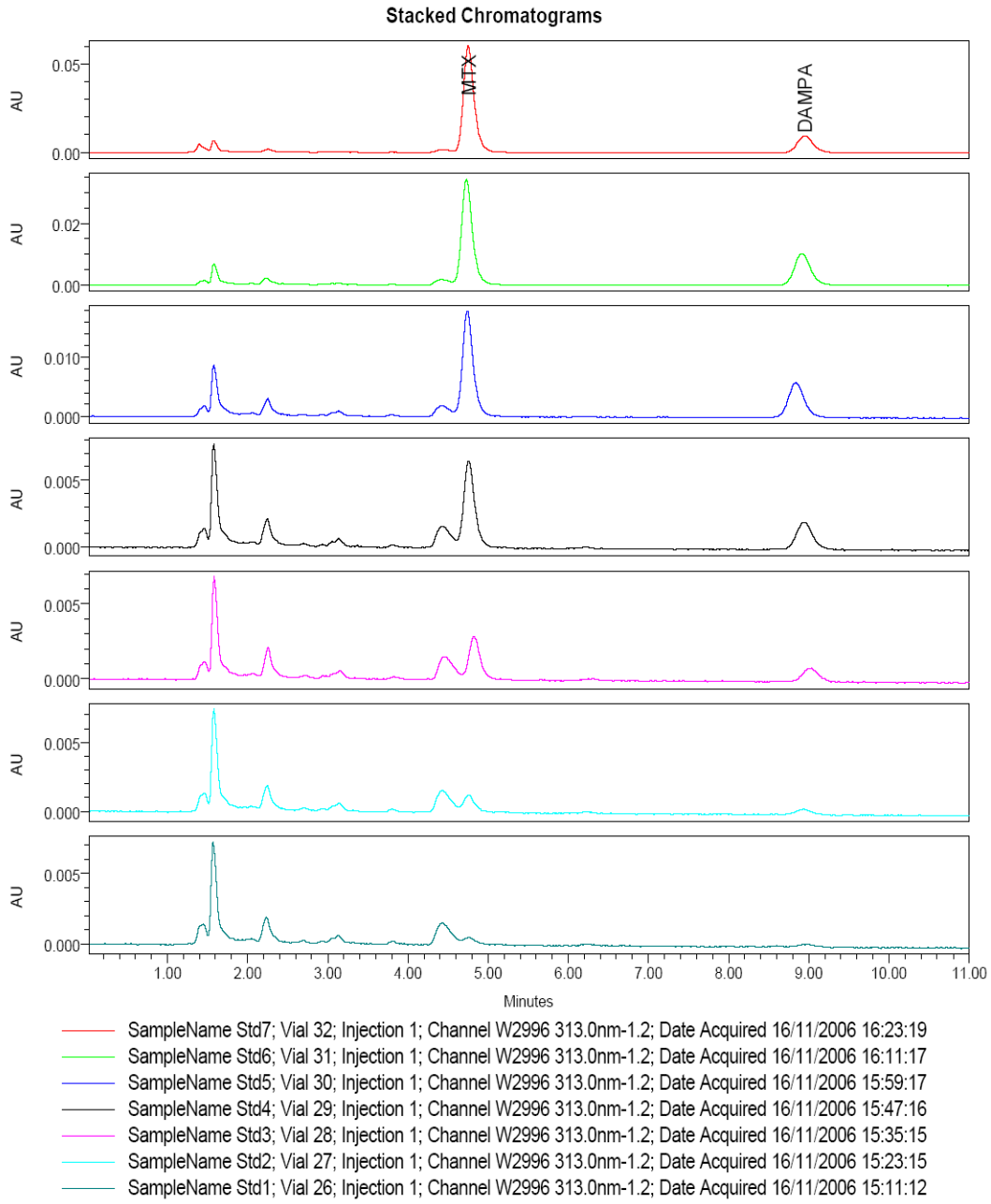
Auto sampler	Injection volume: 10 µL
	Analysis time: 11 min
	Sample temperature: ambient (nominally +22°C)
Pump	Flow rate: 1 mL/minute
Detector	Waters 2996 Photodiode Array Detector monitoring at 313 nm
Analytical column	Luna 5u C18 (2) 100A, Phenomenex
Guard column	C18 (ODS, Octadecyl, 4 mm L x 3.0 mm ID), Phenomenex.

Mobile phase Phosphate buffer (0.1 M, pH 6.5): acetonitrile: hydrogen peroxide (2 ml, 30% w/w) (90:10:0.2, v/v/v)

Software Empower Pro

Under these conditions the retention times of MTX and DAMPA were expected to be *ca* 4-5 minutes and *ca* 9 minutes respectively (Figure 6).

Figure 5: REPRESENTATIVE CHROMATOGRAMS OF CALIBRATION STANDARDS EXTRACTS MADE UP IN PLASMA



Std 1: 0.2 µmol/L of MTX and DAMPA
Std 2: 0.5 µmol/L of MTX and DAMPA
Std 3: 1 µmol/L of MTX and DAMPA
Std 4: 2 µmol/L of MTX and DAMPA
Std 5: 5 µmol/L of MTX and DAMPA
Std 6: 10 µmol/L of MTX and DAMPA
Std 7: 20 µmol/L of MTX and DAMPA

m. Data Processing

All data was processed using Empower Pro, Waters, 2002 software.

4.5. DATA FORMAT AND STATISTICS

All plasma concentrations of methotrexate and DAMPA measured as part of the study were reported to 3 significant figures. All statistics (e.g. mean, SD, CV (%) and RE (%)) presented in this report were based upon the rounded numbers from the original database.

4.6. UNEXPECTED EVENTS

Incorrect preparation of DAMPA standards on 23 January 2007

4.7. LOCATION OF STUDY RECORDS

This report was compiled from original data generated in the Department of Oncology, University College London, 91 Riding House Street, London, W1W 7BS. All the original data has been stored on password controlled COMPAC PC initially at the Department of Oncology, University College London, 91 Riding House Street, London, W1W 7BS and subsequently at the Department of Oncology, University College London, the Paul O'Gorman Building, 72 Huntley Street, London, WC1E 6BT. The data were backed up to a CD and stored in fireproof safe on the 4th floor of the Paul O'Gorman Building, 72 Huntley Street, London, WC1E 6BT.

4.8. RESULTS

a. Linearity

- **MTX** gave a linear standard curve over the range 0-50 μM with UV detection at 313nm. Mean R squared value was 0.997 (Table 12 and Figure 4c).

- **DAMPA** gave a linear standard curve over the range 0-50 μM with UV detection at 313 nm. Mean R squared value was 0.996 (Table 12 and Figure 4b).

Representative chromatograms of extracts of plasma spiked with calibration standards are shown in Figure 6. Daily calibration measurements are summarised in Table 13.

Table 12: BUFFER STANDARDS PROCESSED WITH UV AND FLUORESCENCE DETECTION

Conc μM	MTX PK Area FLU	DAMPA PK Area FLU	MTX PK Area UV	DAMPA PK Area UV
0.2	77404	140999	2058	
0.5	191446	340869	6236	
1	362716	690589	13223	6437
5	1541751	3208191	64877	27762
10	2906894	5727359	119932	55122
20	5507770	9887589	236241	111971
50	9272649	17648658	607423	277641
R^2	0.968	0.961	0.999	1.000

Table 13: DAILY CALIBRATION MEASUREMENTS

MTX: Calibration parameters			
Date of analysis	**Slope**	**Intercept**	**R Squared**
14-Feb-07	10700	0	0.998
19-Feb-07	10000	0	0.997
22-Feb-07	11000	0	0.994
15-March-07	10000	0	0.999
Mean	10425	0	0.997
SD	438		
CV (%)	4.2		

MTX: Measured values of peak area							
Concentration (μmol/L)	**14 Feb 07**	**19 Feb 07**	**22 Feb 07**	**15 Mar 07**	**Mean**	**SD**	**CV (%)**

Table 13: DAILY CALIBRATION MEASUREMENTS

0.2	1926	1678	2103	2578	2071.25	380.152	18
0.5	4684	4678	5563	5746	5167.75	566.999	10
1	10289	10024	10469	9861	10160.75	270.811	2.7
2	22150	20518	22629	22022	21829.75	912.690	4.1
5	54738	52325	49279	49470	51453	2595.520	5.0
10	94972	88858	89414		91081	3380.865	3.7
20	208316	205559	223931	225786	215898	10435.249	4.8

MTX: Back-calculated values of concentration

Concentration (µmol/L)	14 Feb 07	19 Feb 07	22 Feb 07	15 Mar 07	Mean	SD	CV (%)
0.2	0.19	0.17	0.19	0.23	0.195	0.02516	12
0.5	0.46	0.47	0.5	0.51	0.485	0.02380	4.9
1	1	1		0.88	0.960	0.06928	7.2
2	2.15	2	2	1.96	2.030	0.08381	4.1
5	5.3	5.22	4.4	4.4	4.830	0.49759	10
10	9.24	8.9	8.7		8.970	0.27300	3.0
20	20.3	20.5	20.8	20	20.400	0.33665	1.6

DAMPA: Calibration parameters

Date of analysis	Slope	Intercept	R Squared
14-Feb-07	4640	0	0.998
19-Feb-07	4690	0	0.996
22-Feb-07	5200	0	0.996
15-March-07	4710	0	0.996
Mean	4810	0	0.9965
SD	226		
CV (%)	4.7		

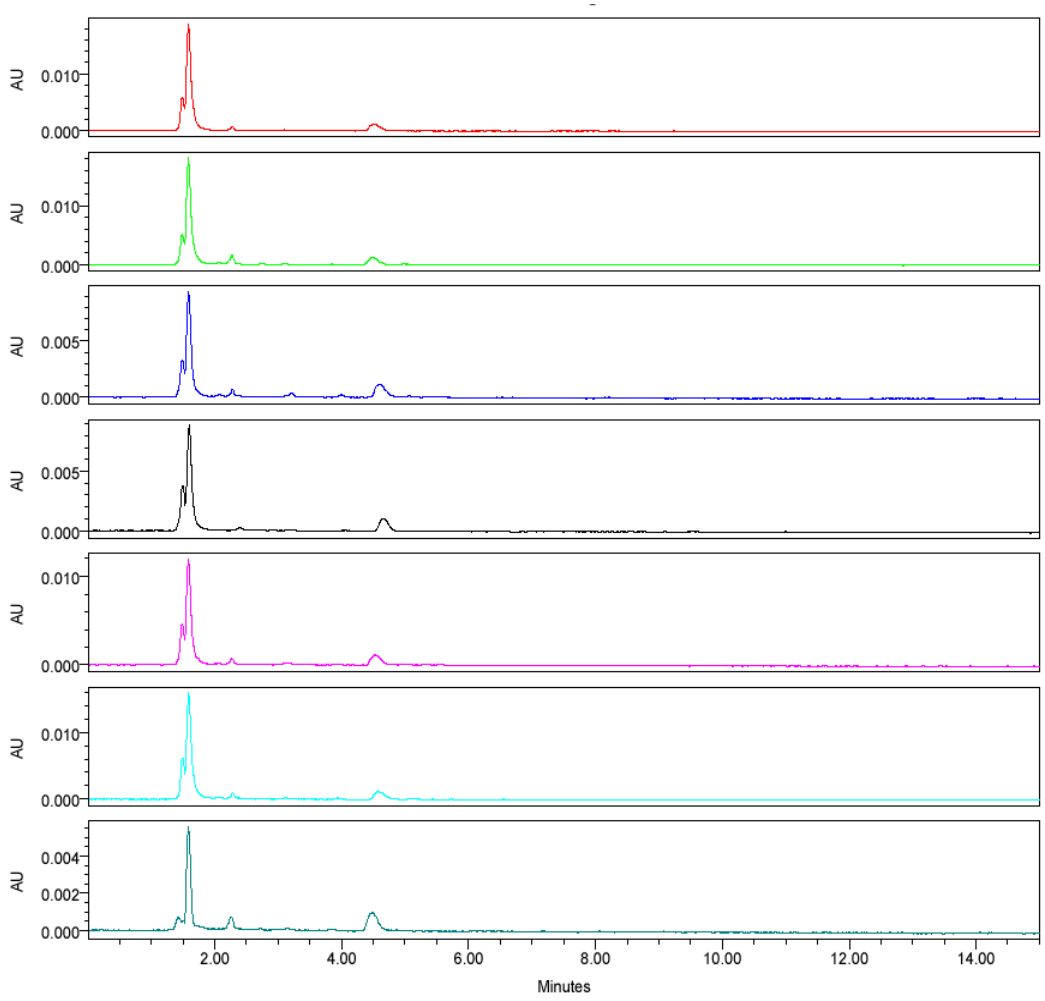
DAMPA: Measured values of peak area

Concentration (µmol/L)	14 Feb 07	19 Feb 07	22 Feb 07	15 Mar 07	Mean	SD	CV (%)
0.5	1714	1541	2622	3563	2360	931.652	39
1	3147		4968	5461	4525.33	1218.857	26
2	7469	8203	9569	9279	8630	971.797	11
5	24820	23274	21922	23238	23313	1185.056	5.0
10	42659	41179	46421	51296	45388	4514.224	9.9

Table 13: DAILY CALIBRATION MEASUREMENTS

20	94500	96796	108014	100699	100002	5922.510	5.9
DAMPA: Back-calculated values of concentration							
Concentration (µmol/L)	**14 Feb 07**	**19 Feb 07**	**22 Feb 07**	**15 Mar 07**	**Mean**	**SD**	**CV (%)**
0.5	0.37	0.33	0.5	0.7	0.475	0.16663	35
1	0.68		0.95	1.08	0.903	0.20404	22
2	1.6	1.7	1.8	1.8	1.725	0.09574	5.5
5	5.3	4.9	4.2	4.6	4.750	0.46547	9.7
10	9.2	8.8	8.9	10.2	9.270	0.63966	6.8
20	20.4	20.6	20.7	20	20.420	0.30956	1.5

Figure 6: REPRESENTATIVE CHROMATOGRAMS OF BLANK PLASMA EXTRACTS



Blank plasma sample 1
Blank plasma sample 2
Blank plasma sample 3
Blank plasma sample 4
Blank plasma sample 5
Blank plasma sample 6
Blank plasma pool (from UCLH Blood Bank)

b. Precision

- Within batches of extracted plasma samples spiked with 0.1-10 µmol/L MTX and DAMPA, prepared on three separate dates and each analysed 3-5 times, CV's of MTX analysis ranged from 2.3-10%, mean 5.2% (analysed 5 times between

15-21 Nov 2006), 7.0-11.2%, mean 8.5% (analysed 5 times between 4-8 Dec 2006) and 6.3-17.3%, mean 9.6% (analysed 3 times between 14-16 Feb 2007) (Table 14).

- Within batches of extracted plasma samples spiked with 0.1-10 μmol/L MTX and DAMPA, prepared on three separate dates and each analysed 3-5 times, CV's of DAMPA analysis ranged from 3.6-5.9%, mean 4.1% (analysed 5 times between 15-21 Nov 2006), 1.9-9.7%, mean 4.1% (analysed 5 times between Dec 4-8) and 4.3-11.7%, mean 4.4% (analysed 3 times between 14-16 Feb 2007) (Table 14).
- The overall mean CV's for the three batches were 6.2-13.6% for MTX and 4.2-6.5% for DAMPA (Table 15).
- Variations between extracted plasma samples, spiked with MTX and DAMPA (0.1-10 μmol/L) from three separate weighings of primary standards were low. CV values were 8.7-9.9% (mean 8.3%) and 1.5-4.6% (mean 3.6%) respectively refer to (Table 16).

Table 14: WITHIN-BATCH PRECISION AND ACCURACY MEASUREMENTS

MTX Batch 1 (extracted on 15 Nov 2006, analysed on 15,16,17,20 and 21 Nov 2006)									
Concentration (μmol/L)	**15 Nov**	**16 Nov**	**17 Nov**	**20 Nov**	**21 Nov**	**Mean**	**SD**	**CV (%)**	**RE (%)**
0.1	0.11	0.092	0.12	0.1	0.11	0.100	0.01071	10	6.4
0.2	0.19	0.19	0.22	0.19	0.2	0.200	0.01303	6.5	-1.0
0.5	0.54	0.52	0.55	0.492	0.51	0.520	0.02321	4.4	4.0
1	1.02	1.12	1.1	1.01	1.06	1.060	0.04816	4.5	2.6
2	2.1	2	2.2	2.06	2.2	2.110	0.08786	4.1	5.6
5	4.82	4.9	5.2	5.14	5.3	5.070	0.20376	4.0	1.4
10	10.5	10.5	11	10.7	11	10.700	0.25099	2.3	7.4
MTX Batch 2 (extracted on 4 Dec 2006, analysed on 4, 5, 6, 7 and 8 Dec 2006)									
Concentration (μmol/L)	**4 Dec**	**5 Dec**	**6 Dec**	**7 Dec**	**8 Dec**	**Mean**	**SD**	**CV (%)**	**RE (%)**
0.2	0.18	0.19	0.21		0.16	0.185	0.0208	11.2	-7.5

Table 14: WITHIN-BATCH PRECISION AND ACCURACY MEASUREMENTS

0.5	0.48	0.56	0.49	0.45	0.49	0.494	0.0404	8.2	-1.2
1	1	1.08	1.02	0.9		1.000	0.0748	7.5	0
2	1.96	2.18	1.99	1.75	1.9	1.956	0.1556	7.9	-2.2
5	5.18	5.3	5.3	4.5	4.8	5.016	0.3539	7.0	0.3
10	10.56	11.2	10.8	8.9	9.6	10.212	0.9406	9.2	2.0

MTX Batch 3 (extracted on 14 Feb 2007 and analysed on 14, 15 and 16 Feb 2007)

Concentration (µmol/L)	**14 Feb**	**15 Feb**	**16 Feb**	**Mean**	**SD**	**CV (%)**	**RE (%)**
0.1	0.11	0.11	0.08	0.1	0.01732	17	0
0.2	0.19	0.17	0.19	0.18	0.01154	6.2	-10
0.5	0.46	0.48	0.38	0.44	0.05291	12	-12
10	10	10.2	8.8	9.66	0.75718	7.8	-3.4

DAMPA Batch 1 (extracted on 15 Nov 2006, analysed on 15,16,17,20 and 21 Nov 2006)

Concentration (µmol/L)	**15 Nov**	**16 Nov**	**17 Nov**	**20 Nov**	**21 Nov**	**Mean**	**SD**	**CV (%)**	**RE (%)**
0.5	0.46	0.5	0.5	0.54	0.52	0.500	0.02966	5.8	0.8
1	1.06	1.05	1.06	1.14	1.06	1.070	0.03714	3.4	7.4
2	2.16	2.36	2.18	2.32	2.34	2.270	0.09444	4.1	13.6
5	5.04	5.52	5.1	5.2	5.3	5.230	0.18899	3.6	4.6
10	12	13.08		12.4	12.4	12.400	0.44825	3.5	24.7

DAMPA Batch 2 (extracted on 4 Dec 2006, analysed on 4, 5, 6, 7 and 8 Dec 2006)

Concentration (µmol/L)	**4 Dec**	**5 Dec**	**6 Dec**	**7 Dec**	**8 Dec**	**Mean**	**SD**	**CV (%)**	**RE (%)**
0.5	0.48	0.54	0.46	0.5	0.44	0.484	0.03847	7.9	-3.2
1	0.92	1.12	1.12	0.94	0.97	1.014	0.09838	9.7	1.4
2	1.92	1.78	1.94	1.96		1.900	0.08164	4.2	-5
5	5.5	4.6	5.04	5.5	4.74	5.076	0.41842	8.2	1.52
10		9.26			9.52	9.390	0.18384	1.9	-6.1

DAMPA Batch 3 (extracted on 14 Feb 2007 and analysed on 14, 15 and 16 Feb 2007)

Concentration (µmol/L)	**14 Feb**	**15 Feb**	**16 Feb**	**Mean**	**SD**	**CV (%)**	**RE (%)**
0.5	0.46	0.48	0.44	0.460	0.02	4.3	-8.0
5	4.9	4.9	5	4.930	0.05773	1.1	-1.4
10	10.7	9.22	10.3	10.070	0.76559	7.6	0.7

Table 15: OVERALL-BATCH PRECISION AND ACCURACY MEASUREMENTS

MTX concentration (µmol/L)	Mean	SD	CV (%)	RE (%)	n
Summary of combined measurements in batches 1 to 3 for MTX and DAMPA					
0.1	0.103	0.01401	13.6	0	8
0.2	0.189	0.01513	8.0	-5	12
0.5	0.485	0.03883	7.9	-4	13
1	1.031	0.06149	5.9	3	9
2	2.034	0.12176	5.9	1.5	10
5	5.044	0.27885	5.5	0.8	10
10	10.476	0.64959	6.2	4.8	13
DAMPA Concentration (µmol/L)	**Mean**	**SD**	**CV (%)**	**RE (%)**	**n**
0.5	0.483	0.02937	6.0	-4	13
1	1.044	0.06776	6.5	4	10
2	2.086	0.08804	4.2	4.5	9
5	5.080	0.22171	4.3	1.6	13
10	10.644	0.46589	4.3	6.4	9

Table 16: PRIMARY STANDARDS STABILITY IN PLASMA

MTX (µmol/L)	Spike A	Spike B	Spike C	Mean	SD	CV (%)
0.2	0.18	0.21	0.21	0.200	0.01732	8.6
0.5	0.46	0.55	0.52	0.510	0.04582	8.9
1	0.93	1.05	1.04	1.000	0.06658	6.6
2	1.83	2.18	2	2.000	0.17502	8.7
5	4.7	5.3	5.4	5.130	0.37859	7.3
10	9.12	10.9	10.9	10.300	1.02768	9.9
DAMPA (µmol/L)	**Spike A**	**Spike B**	**Spike C**	**Mean**	**SD**	**CV (%)**
5	4.99	4.6	5	4.860	0.22810	4.6
10	9.9	10.3	10.7	10.300	0.40000	3.8
20	20.1	20.6	20	20.230	0.32145	1.5
50	49.9	46.1	49.7	48.560	2.13853	4.4

c. Accuracy

Within batches of extracted plasma samples spiked with 0.1-10 μmol/L MTX and DAMPA, prepared on three separate dates and each analysed 3-5 times, RE's of MTX analysis ranged from -1 to 7.4% (15-21 Nov 2006), -7.5 to 2% (4-8 Dec 2006) and -12 to 0% (14-16 Feb 2007), (Table 14).

Within batches of extracted plasma samples spiked with 0.1-10 μmol/L MTX and DAMPA, prepared on three separate dates and each analysed 3-5 times, RE's of DAMPA analysis ranged from 0.8 to 24.7% (15-21 Nov 2006), -6.1 to 1.5% (4-8 Dec 2006) and -8 to 0.7% (14-16 Feb 2007), (Table 14).

The overall mean RE's for the three batches were -5 to 4.8 % for MTX and -4 to 6.4% for DAMPA, (Table 15).

d. Sensitivity (Lower limit of quantification)

At 0.2 μmol/L the QC precision for MTX is 3.4% and the inaccuracy of measurement is 15% (Table 17).

At 0.5 μmol/L the QC precision for DAMPA is 11% and the inaccuracy of measurement is 0% (Table 17).

Table 17: QC STABILITY IN PLASMA

MTX QC	Jan-23	Feb-13	Mar-21	Mean	SD	CV (%)
0.1	0.099	0.11	0.08	0.096	0.01517	15
0.2	0.16	0.17	0.17	0.166	0.00577	3.4
0.5		0.48	0.43	0.455	0.03535	7.7
10	9.6	10.2	9.6	9.800	0.34641	3.5
*50	45	46.5	55	48.833	5.39289	11

DAMPA QC	Jan-23	Feb-13	Mar-21	Mean	SD	CV (%)
0.2	**	0.14	0.21	0.175	0.04949	28
0.5	**	0.46	0.54	0.500	0.05656	11
10	**	10.7	9.4	10.050	0.91923	9.1
*50	**	49	54	51.500	3.53553	6.8

*Sample diluted 10 fold for analysis
****** Incorrect buffer standard prepared

e. Recovery

- Mean recovery of MTX from plasma samples spiked with MTX and DAMPA at 0.2 μmol/L, 0.5 μmol/L and 10 μmol/L, was 100.48%, CV 10.9% and 100.6%, CV 7.6% and 101.96%, CV 3.2% respectively (Table 18).
- Mean recovery of DAMPA from plasma samples spiked with MTX and DAMPA at 0.5 μmol/L and 10μmol/L, was 100.99%, CV 7.2% and 101.3%, CV 5.8% respectively (Table 18).

Table 18: RECOVERY

Analyte	Theoretical Concentration (μmol/L)	Mean recovery (%)	SD	CV (%)	n
MTX	0.2	100.48	11.03	10	5
	0.5	100.63	7.64	7.5	5
	10	101.96	3.30	3.2	5
DAMPA	0.5	100.99	7.28	7.2	5
	10	101.30	5.82	5.7	5

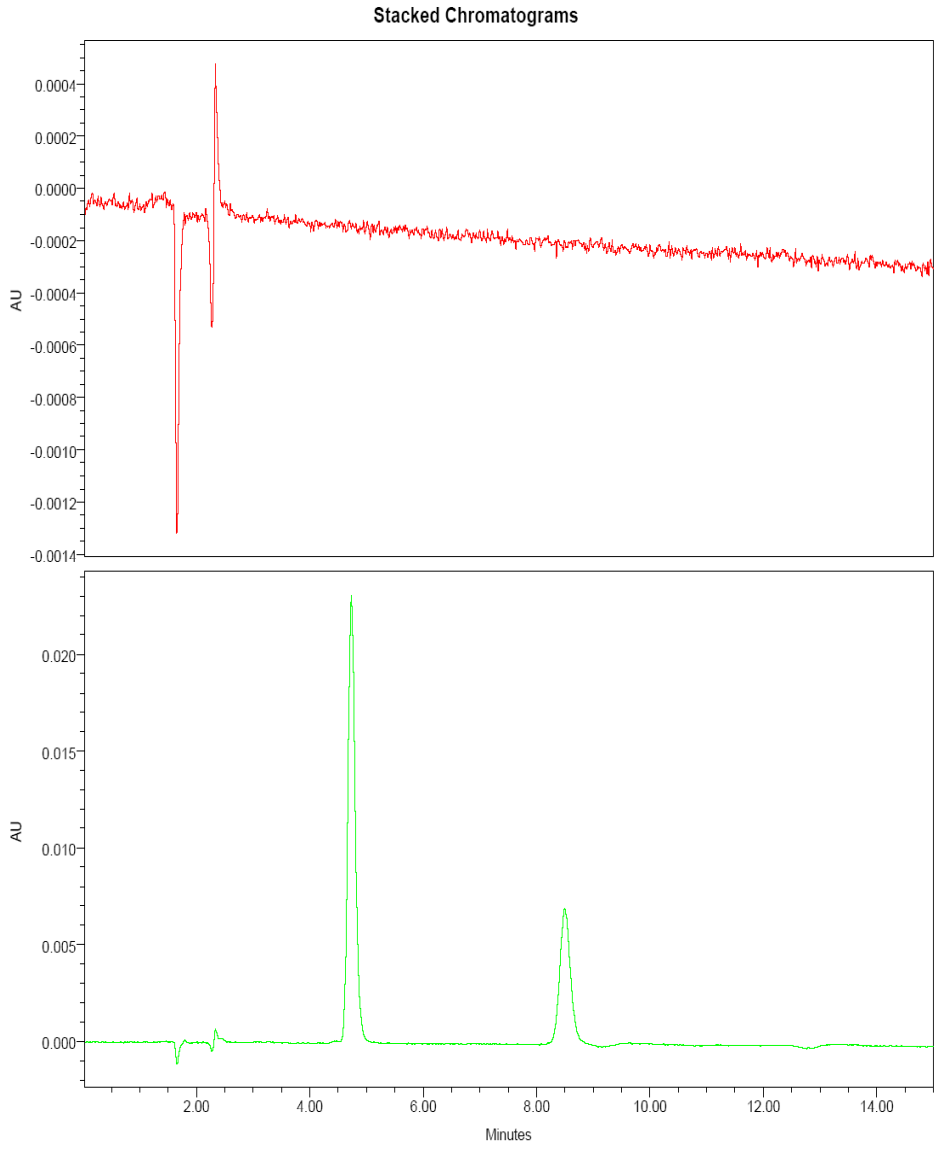
f. Specificity

HPLC analysis of plasma samples from 6 healthy volunteers showed no interfering peaks at the retention times for MTX and DAMPA (Figure 7).

There was an endogenous peak with a retention time of *ca* 1-2 minutes in the chromatograms of human plasma extracts, but it did not have any effect on the measurement of MTX and DAMPA.

Chromatograms of buffer and calibration standards demonstrate that there was no carry-over between injections as shown in Figure 8.

Figure 7: REPRESENTATIVE CHROMATOGRAMS DEMONSTRATING THE ABSENCE OF CARRY-OVER



_____ Buffer injection (date acquired: 14/02/2007 at 13.37)

_____ Calibration standard injection (date acquired: 14/02/2007 at 13.21)

g. Dilution

- A 10-fold dilution of plasma samples containing 50 or 100 μmol/L MTX and DAMPA gave good agreement with the theoretical concentration. RE values for

MTX were -8.4 at 50 μmol/L and 4.5 at 100 μmol/L. RE values for DAMPA were -3.2 at 50 μmol/L and 11.25 at 100 μmol/L (Table 19).

- A 100-fold dilution of plasma samples containing 1000 μmol/L MTX and DAMPA gave good agreement with the theoretical concentration. RE value for MTX was -8.0 and 2.5 for DAMPA (Table 19).

Table 19: PRECISION AND ACCURACY MEASUREMENTS IN PLASMA AFTER DILUTION

Theoretical MTX concentration (μmol/L), analysed on 29 March 2007						
Before dilution	**After dilution**	**Measured Concentration (μmol/L)**	**Mean Concentration (μmol/L)**	**SD**	**CV (%)**	**RE (%)**
50	5	4.6	4.58	0.14	3.0	-8.4
		4.74				
		4.4				
		4.6				
100	10	10.0	10.450	0.72	6.88	4.5
		10.6				
		9.8				
		11.4				
1000	10	9.8	9.20	0.40	4.3	-8.0
		9.0				
		9.0				
		9.0				
Theoretical DAMPA concentration (μmol/L), analysed on 29 March 2007						
Before dilution	**After dilution**	**Measured Concentration (μmol/L)**	**Mean Concentration (μmol/L)**	**SD**	**CV (%)**	**RE (%)**
50	5	4.92	4.840	0.24	4.97	-3.2
		4.6				
		5.14				
		4.7				
100	10	10.5	11.125	0.45	4.1	11.25
		11.2				
		11.2				

Table 19: PRECISION AND ACCURACY MEASUREMENTS IN PLASMA AFTER DILUTION

		11.6				
1000	10	9.6	10.250	0.55	5.3	2.5
		10.0				
		10.6				
		10.8				

h. Stability

Primary standards stability

Primary standards made in DMSO on 10 October 2006, 21 November 2006 and 7 December 2006, at a concentration of 10 mmol/L, were stable for at least 3 months at -20°C (Table 16). Spikes A, B and C were prepared in plasma and run on 5 January 2007, using primary standards made in DMSO on 10 October 2006, 21 November 2006 and 7 December 2006, respectively. Short and long term freeze-thaw stability (4 cycles) of primary standards was demonstrated (Table 20, Figure 8a, Figure 8b).

Table 20: STABILITY OF MTX AND DAMPA PRIMARY STANDARDS, 4x FREEZE-THAW CYCLES

MTX Concentration (µmol/L)	F/T 1 (peak area)	F/T 2 (peak area)	F/T 3 (peak area)	F/T 4 (peak area)	Mean (peak area)	SD	CV (%)
0.2	2058	2255	2630	2224	2291.75	241.517943	10.5
0.5	6236	5973	6617	5668	6123.5	402.628447	6.5
1	12223	12435	12133	10289	11770	995.416831	8.4
5	64877	60376	59049	59738	61010	2634.3342	4.3
10	119932	125719	118477		121376	3830.86061	3.1
20			239410	225399	232404.5	9907.27311	4.2

DAMPA Concentration (µmol/L)	F/T 1 (peak area)	F/T 2 (peak area)	F/T 3 (peak area)	F/T 4 (peak area)	Mean (peak area)	SD	CV (%)
1	3805	3945	3821	3742	3828.25	84.975977	2.2
5	24513	25730	25390	22352	24496.25	1518.66353	6.2
10	47970	49429	52689	49607	49923.75	1984.00729	3.9
20	100246	99047	104539	102976	101702	2506.03312	2.4
50	277641	256386	288355	276375	274689.25	13333.12	4.8

Figure 8: STABILITY OF MTX AND DAMPA PRIMARY STANDARDS, 4 x FREEZE-THAW CYCLES

Figure 8a: STABILITY OF PRIMARY MTX STANDARD

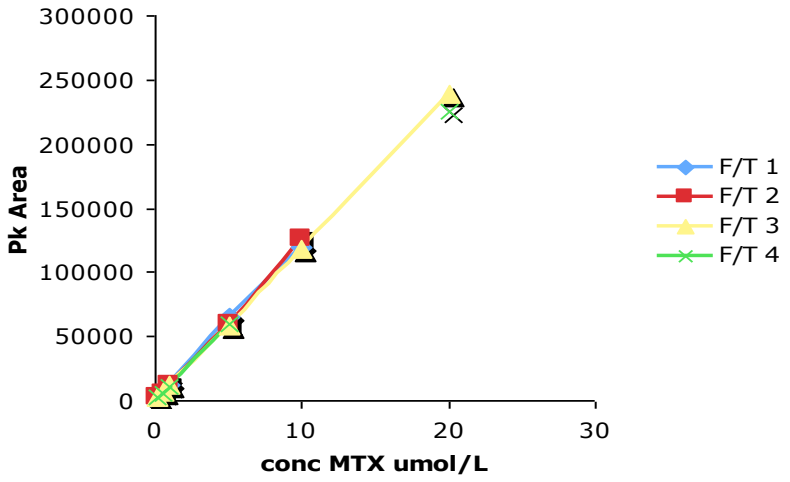
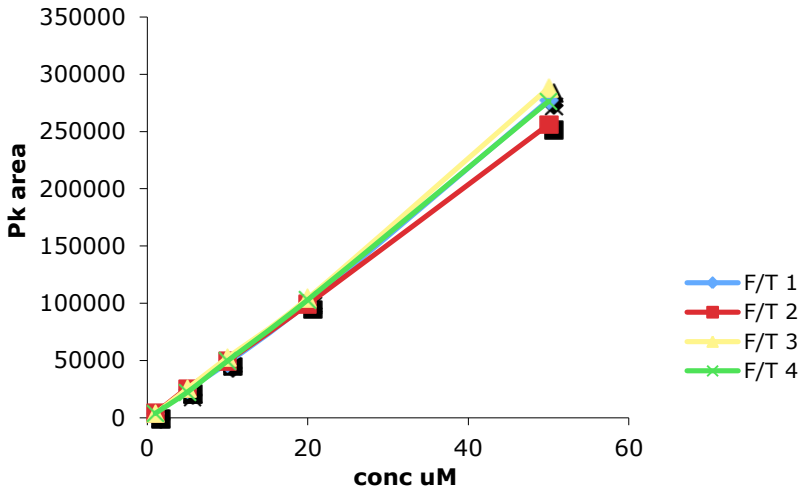


Figure 8b: STABILITY OF PRIMARY DAMPA STANDARD



Callibration standards stability

Calibration standards made from primary standards (prepared on 7 December 2006), prepared on 13 December and run on HPLC on 14 February 2007, 22 February 2007 and 15 March 2007, remained stable for 1 month at 4°C. However, the CVs from lower concentrations of DAMPA were high (Table 21).

Table 21: CALIBRATION STANDARDS STABILITY

MTX (buffer)	Feb-14 (peak area)	Feb-22 (peak area)	Mar-15 (peak area)	Mean (peak area)	SD	CV (%)
0.2	1926	2103	2578	2202.330	337.15921	15
0.5	4684	5563	5746	5331.000	567.74025	10
1	10289	10469	9861	10206.330	312.31607	3.0
2	22150	22629	22022	22267.000	319.96718	1.4
5	54738	49279	49470	51162.330	3098.0904	6.0
10	94972	89414		92193.000	3930.0994	4.2
20	208316	223931	225786	219344.330	9595.7468	4.3

DAMPA (buffer)	Feb-14 (peak area)	Feb-22 (peak area)	Mar-15 (peak area)	Mean (peak area)	SD	CV (%)
0.5	1714	2622	3563	2633.000	924.54907	35
1	3147	4968	5461	4525.330	1218.8577	26
2	7469	9569	9279	8772.330	1137.99531	12
5	24820	21922	23238	23326.660	1451.03319	6.2
10	42659	46421	51296	46792.000	4330.43565	9.2
20	94500	108014	100699	101071.000	6764.67567	6.6

Extracted samples stability

Extracted samples gave reproducible results stable for 1 week at 4°C. They were prepared on 15 November 2006 and run on 15, 16, 17 20, 21 November 2006 (Table 14).

QCs (frozen plasma samples)

QCs (frozen plasma samples) were stable for over a month at -20°C. They were all prepared on 22 January 2007 using primary standards weighed on 7 December 2006. Plasma samples were spiked, stored at -20°C and then extracted and analysed on 23 January 2007, 13 February 2007 and 21 March 2007 (Table 17).

i. System suitability

Prior to analysis of a batch of samples, a standard mixture containing methotrexate and DAMPA in phosphate buffer (0.1 mmol/L, pH 6.5) was injected to check the performance of the chromatographic system in terms of retention times, peak shapes, resolution and instrument response. To maintain consistency of these parameters remedial action was taken as required (e.g. guard column replaced, analytical column replaced or fresh mobile phase prepared).

4.9. CONCLUSIONS

In conclusion an HPLC-UV method for the measurement of MTX and DAMPA in human plasma has been successfully validated for use in the Department of Oncology, University College London, 91 Riding House Street, London, W1W 7BS and subsequently in the Department of Oncology, University College London, the Paul O'Gorman Building, 72 Huntley Street, London, WC1E 6BT, for the GLU 1 clinical trial.

Acceptable linearity, precision, accuracy and specificity were observed over the concentration ranges 0.2-50 μmol/L.

The lower limit of quantification was 0.2 μmolL for MTX and 0.5 μmolL for DAMPA.

The recovery of MTX and DAMPA from plasma was consistent over the calibration range.

There were no interfering peaks in human plasma that affected the measurement of MTX and DAMPA.

Plasma samples containing concentrations of MTX and DAMPA in excess of the validated range could be measured precisely and accurately after a 10- or 100-fold dilution with blank plasma, to enable measurement up to a maximum concentration of 2000 μmol/L using this method.

MTX and DAMPA were shown to be stable in plasma for over a month at -20°C. MTX and DAMPA were also stable in extracted samples for 1 week at 4°C. Primary standards made in DMSO were stable for at least 3 months at -20°. Calibration standards remained stable for 1 month at 4°C.

4.10. GLOSSARY

CV: Coefficient of variation

HPLC: High performance liquid chromatography

LLOQ: Lower limit of quantification

MTX: Methotrexate

DAMPA: 4-[[2,4-diamino-6-(pteridinyl)methyl]-methylamino] benzoic acid

n: Number of replicates

QC: Quality control

R: Correlation coefficient

RE: Relative error of measurement

SD: Standard deviation

UCLH: University College London Hospitals

5. GLU 1 CLINICAL TRIAL

5.1. INTRODUCTION

The two previously described reviews (Sections 3.1 and 3.2), highlighted that the incidence of chemotherapy delays due to MTX toxicity in patients with osteosarcoma is high. Early adjustment of folinic acid dose according to plasma MTX levels at 24 hours appears to somewhat reduce the incidence of chemotherapy delay. Nevertheless, improving rescue from MTX toxicity seemed a worthwhile goal.

Our second aim was to examine the role of glucarpidase in routine rescue after HD-MTX in patients with bone sarcoma. Glucarpidase seemed to offer a promising opportunity for rescue from MTX toxicity and if found to be effective and safe in maintaining the treatment intensity and reducing the incidence and severity of MTX-induced toxicity, could optimise treatment, improve patients' well-being, and reduce the use of health resources.

In order to investigate the role of glucarpidase in routine rescue after HD-MTX, the following needed to be evaluated:

- Reduction of plasma MTX levels after glucarpidase
- MTX related toxicity following glucarpidase
- Comparison of toxicity related to MTX when administered with normal supportive measures versus MTX related toxicity following glucarpidase
- Adverse reactions related to glucarpidase
- Anti-glucarpidase antibody response

The above objectives were studied in the GLU 1 trial, a randomised, cross-over, phase II clinical trial, to investigate the efficacy and safety of glucarpidase for routine

use after HD-MTX in patients with bone sarcoma, as described in details in Section 2.2.

The interim analysis of the GLU 1 clinical trial took place in May 2009. The study is still ongoing. The results of the study up to the point of the interim analysis are discussed here.

5.2. GLU 1 CLINICAL TRIAL: PATIENT CHARACTERISTICS

All enrolled patients received appropriate for age patient or parent/guardian information sheet and signed an informed consent form prior to trial entry as per trial protocol (Appendices 2 and 4). They all had glomerular filtration rate > 70 ml/min/1.73m^2 and were able to comply with the study and follow up procedures (WHO performance scale 0-2). None of the patients were on concomitant anti-cancer or investigational drugs during the study and they all had complete resolution of toxicity related to previous treatment. In addition, they all had life expectancy of at least 3 months at trial entry and complied with the GLU 1 clinical trial laboratory eligibility criteria. Moreover, none of the enrolled patients had previous treatment with glucarpidase, and no female participants were pregnant or lactating. No participants were on concomitant treatment with agents which interact with methotrexate metabolism or excretion at trial entry. One patient had a very small pleural effusion thought to be clinically unremarkable.

16 patients enrolled to the GLU 1 clinical trial up to the point of the interim analysis of the study, between 13 June 2007 and 29 January 2009. Among them, 13 (81%) were male and 3 (19%) were female, with male to female ratio of 4.3:1 (Table 22). Patient ages ranged from 13 to 47 years with a median age at trial entry of 19 years. 12 patients (12/16, 75%) had high grade conventional osteosarcoma, 2 patients (2/16, 12.5%) had high grade telangiectatic osteosarcoma and 2 patients (2/16,

12.5%) had high grade spindle cell sarcoma. The most frequent primary site was the proximal tibia in 3 patients (3/16, 18.75%), followed by the distal femur in 2 (2/16, 12.5%) patients, distal tibia in 2 (2/16, 12.5%), proximal humerous in 2 (2/16, 12.5%) and pelvis in 2 (2/16, 12.5%), followed by the ribs in 1 (1/16, 6.25%) patient, vertebrae in 1 (1/16, 6.25%) patient, maxilla in 1 (1/16, 6.25%) patient, metatarsal in 1 (1/16, 6.25%) patient and sphenoid bone in 1 (1/16, 6.25%) patient. 10 patients (10/16, 62.5%) had localised disease and 6 (6/16, 37.5%) had metastases at trial entry. One patient developed osteosarcoma which was likely secondary to radiation therapy for low grade glioma (Table 22).

13 patients (> 80%) received some chemotherapy prior to trial entry and only 3 (GLU1-03, GLU1-04 and GLU1-15) had no chemotherapy prior to trial entry. Seven patients GLU1 -05, GLU1 -08, GLU1 -09, GLU1 -10, GLU1 -11, GLU1 -13 and GLU1 -16) received adriamycin & cisplatin prior to trial entry. 4 patients (GLU1-02, GLU1-06, GLU1-07 and GLU1-14) received combination of adriamycin & cisplatin and HD-MTX. 1 patient (GLU1-12) received combination of adriamycin & cisplatin and ifosfamide & etoposide and 1 patient (GLU1-01) received combination of adriamycin & cisplatin, ifosfamide & etoposide and HD-MTX.

Demographics and treatment experience for each trial participant is described in details in Appendix 12.

Table 22: PATIENT CHARACTERISTICS

	Number of patients	**%**
Sex		
Male	13	81
Female	3	19
Diagnosis		
High grade intramedullary osteosarcoma	12	75
High grade telangiectatic osteosarcoma	2	12.5

	Number of patients	%
High grade spindle cell sarcoma	2	12.5
Primary tumour site		
Distal femur	2	12.5
Proximal tibia	3	18.75
Proximal humerus	2	12.5
Distal tibia	2	12.5
Pelvis	2	12.5
Vertebrae & ribs	2	12.5
Skull & face bones	2	12.5
Metatarsal bones	1	6.25
Localised/metastatic disease		
Localised disease	10	62.5
Metastatic disease	6	37.5
Prior chemotherapy		
No prior chemotherapy	3	18.75
AP	7	43.75
AP & MTX	4	25
AP & IE	1	6.25
AP & IE & MTX	1	6.25
Randomisation arm		
Arm B (glucarpidase in cycle 1)	7	43.75
Arm A (glucarpidase in cycle 2)	9	56.25

5.3. GLU 1 CLINICAL TRIAL: "DAY 15" ASSESSMENT ACCORDING TO RESCUE REGIMEN

7 patients (7/16, 44%) were randomised to treatment arm B and received glucarpidase in cycle 1 and 9 patients (9/16, 56%) were randomised to treatment arm A and received glucarpidase in cycle 2 (Table 23). 10 patients (10/16, 62.5%) received all four MTX courses, one patient received three MTX courses, three patients received two MTX courses and two patients only managed to received one MTX course whilst on the GLU 1 clinical trial. The median number of MTX courses received per participant was 4, whereas the mean number of MTX courses was 3.2.

In all cases the reason for not receiving the planned four MTX courses was impaired renal function (Table 23).

The primary objective of the GLU 1 study was to investigate whether glucarpidase rescue after HD-MTX reduces delay to subsequent cycle of chemotherapy due to MTX toxicity. In order to meet our primary objective trial participants were assessed for fitness to receive chemotherapy on day 15 of each cycle. Patients were considered fit to receive chemotherapy if eight criteria were fulfilled: neutrophils ≥ $0.75x10^9$/L or WCC ≥ $2x10^9$/L; platelets ≥ $100x10^9$/L; bilirubin ≤ 1.5xULN; GFR (estimated) ≥ 70 ml/min/1.73m^2; mucositis (clinical and functional): grade ≤ 1 (CTCAE v3.0); no clinical evidence of infection; no pyrexia; and good overall clinical condition.

27 treatment cycles were given within the GLU 1 clinical trial. Among them, 14 cycles (52%) were given with glucarpidase and folinic acid rescue and 13 cycles (48%) were given with folinic acid rescue (Table 23 and Table 24). Patient GLU1-05 required emergency management with glucarpidase following unblinding of his treatment due to his renal impairment and delayed methotrexate excretion after the first dose of MTX. This patient's data were analysed on intention to treat basis and his treatment was included in the group of cycles given with folinic acid rescue.

Among the cycles given with glucarpidase and folinic acid rescue, "day 15" criteria were met in 8/14 (57%) cycles and not met in 6/14 (43%) cycles. Among the cycles given with folinic acid rescue, "day 15" criteria were met in 3/13 (23%) cycles and not met in 10/13 (77%) cycles. Reasons for not meeting the "day 15" criteria in the glucarpidase and folinic acid rescue group included impaired renal function, delayed MTX elimination with or without deterioration in renal function and mucositis. Reasons for not meeting the "day 15" criteria in the folinic acid rescue group included impaired renal function, delayed MTX elimination with or without

deterioration in renal function, mucositis, bone marrow suppression and knee effusion (Table 24). Overall, “day 15” criteria were not met in 59% (16/27) of all given cycles.

Table 23: NUMBER OF MTX COURSES RECEIVED AND ASSESSMENT ON DAY 15 OF EACH CYCLE ACCORDING TO TREATMENT ARM

Trial patient	Treatment arm	Day 15 criteria met (YES, NO, N/A: not applicable) Treatment cycles with glucarpidase highlighted		Number of MTX courses received (Reason if fewer than 4 courses)
		Cycle 1 (reason if NO)	Cycle 2 (reason if NO)	
GLU1-01	B	YES	NO (mucositis, bone marrow suppression)	4
GLU1-02	A	NO (mucositis)	YES	4
GLU1-03	B	NO (mucositis)	NO (impaired renal function)	3 (impaired renal function)
GLU1-04	B	NO (delayed MTX elimination)	N/A	2 (impaired renal function)
GLU1-05	A	NO (impaired renal function)*	N/A	1 (impaired renal function)
GLU1-06	A	NO (mucositis)	YES	4
GLU1-07	B	NO (delayed MTX elimination)	NO (delayed MTX elimination)	4
GLU1-08	B	YES	YES	4
GLU1-09	A	YES	YES	4

Trial patient	Treatment arm	Day 15 criteria met (YES, NO, N/A: not applicable) Treatment cycles with glucarpidase highlighted		Number of MTX courses received (Reason if fewer than 4 courses)
		Cycle 1 (reason if NO)	Cycle 2 (reason if NO)	
GLU1-10	A	NO (mucositis)	YES	4
GLU1-11	A	YES	YES	4
GLU1-12	B	NO (impaired renal function, mucositis)	N/A	2 (impaired renal function)
GLU1-13	A	NO (impaired renal function, mucositis)	N/A	2 (impaired renal function)
GLU1-14	A	NO (knee effusion)	N/A	1 (knee effusion)
GLU1-15	B	NO (mucositis)	NO (mucositis)	4
GLU1-16	A	NO (mucositis)	YES	4

*Patient GLU 1-05 received glucarpidase outside the study as an emergency in view of nephrotoxicity and delayed MTX elimination

Table 24: RESCUE REGIMEN USED AND FITNESS TO PROCEED ON DAY 15 OF EACH CYCLE

Rescue regimen	Number of treatment cycles that "day 15" criteria WERE met (% out of total number of cycles on the same rescue)	Number of treatment cycles that "day 15" criteria WERE NOT met (% out of total number of cycles on the same rescue)	Reason for not meeting "day 15" criteria
Glucarpidase & folinic acid	8 (57%)	6 (43%)	Impaired renal function Delayed methotrexate elimination Mucositis
Folinic acid	3 (23%)	10 (77%)	Impaired renal function Delayed methotrexate elimination Mucositis Knee effusion

In the interim analysis, in examining efficacy based on patient's fitness to receive chemotherapy on day 15 of each cycle, the O'Brien-Fleming method for judging significance of results from a McNemar's test was used with a significance level of 0.005. There was no statistically significant benefit of glucarpidase with $P < 0.005$. However, this was expected as only 50% of intended trial sample size was studied.

To assess whether glucarpidase may be delaying further chemotherapy, the McNemar's test with a one sided significance level of 5% was used in the interim analysis. Treatment with glucarpidase and folinic acid was not found to be significantly worse than standard treatment using an one-sided test with $P < 0.05$.

5.4. INCIDENCE AND SEVERITY OF MTX RELATED TOXICITY

All AEs attributed to MTX related to study participants documented on the individual's Case Record Form (CRF) (Appendix 6) and graded for severity according to Common Terminology for Adverse Events (CTCAE) v.3.0 are shown in Table 25 and Table 26.

Table 25: MTX RELATED TOXICITY PER PATIENT, TREATMENT CYCLE AND TREATMENT COURSE

			CTCAE v3.0, grading scale (0-5) (max = worse documented toxicity per treatment cycle, cycles with glucarpidase rescue highlighted)										mls/min/1.73m^2
Patient ID	**Cycle**	**Course**	**Mucositis (clinical)**	**Mucositis (functional)**	**↑Creat**	**↑ALT**	**↑Bili**	**↓Phos**	**↓Mg**	**↓Hb**	**↓Neuts**	**↓Plts**	**GFR**
01	1	1	0	0	0	2	0	0	0	2	0	0	119
		2	0	0	0	3	0	0	0	2	0	0	
		Day 15	0	0	0	2	0	0	0	1	0	1	193
		max	0	0	0	3	0	0	0	2	0	1	
	2	1	2	1	0	3	2	0	0	1	0	1	176
		2	2	1	0	2	2	0	0	1	0	2	
		Day15	3	2	0	1	2	0	0	3	0	3	182
		max	3	2	0	3	2	0	0	3	0	3	
02	1	1	2	0	0	2	0	3	0	1	0	0	73
		2	2	0	0	2	0	3	0	1	0	0	
		Day 15	2	0	0	1	0	0	0	1	0	0	126
		max	2	0	0	2	0	3	0	1	0	0	
	2	1	0	0	0	1	0	3	0	1	0	0	156
		2	0	0	0	3	0	2	0	1	0	1	
		Day15	1	0	0	-	-	-	-	1	0	0	141
		max	1	0	0	3	0	3	0	1	0	0	
03	1	1	1	0	0	4	0	3	0	0	0	0	94
		2	1	0	0	3	2	3	0	0	0	0	
		Day15	2	0	0	3	0	0	0	0	0	0	79
		max	2	0	0	4	2	3	0	0	0	0	

Table 25: MTX RELATED TOXICITY PER PATIENT, TREATMENT CYCLE AND TREATMENT COURSE

			CTCAE v3.0, grading scale (0-5) (max = worse documented toxicity per treatment cycle, cycles with glucarpidase rescue highlighted)										mls/min/1.73m^2
Patient ID	**Cycle**	**Course**	**Mucositis (clinical)**	**Mucositis (functional)**	**↑Creat**	**↑ALT**	**↑Bili**	**↓Phos**	**↓Mg**	**↓Hb**	**↓Neuts**	**↓Plts**	**GFR**
	2	1	1	0	1	3	0	3	0	0	0	0	69
		2	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	
		Day 15	0	0	1	2	0	0	0	0	0	0	50
		max	1	0	1	3	0	3	0	0	0	0	
04	1	1	0	0	0	3	0	0	0	1	0	0	103
		2	0	0	1	3	0	0	0	2	1	1	
		Day 15	0	0	0	2	0	0	0	2	1	1	90
		max	0	0	1	3	0	0	0	2	1	1	
05	1	1	0	0	4	3	0	1	0	0	0	0	
		2	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	
		Day 15	0	0	2	0	0	0	0	0	0	0	44
		max	0	0	4	3	0	1	0	0	0	0	
06	1	1	2	0	0	3	0	2	0	0	0	0	116
		2	2	0	0	4	0	2	0	0	0	0	
		Day 15	3	3	0	4	0	0	0	0	0	0	199
		max	3	3	0	4	0	2	0	0	0	0	
	2	1	0	0	0	3	0	0	0	0	0	0	142
		2	0	0	0	3	0	0	0	0	0	0	
		Day 15	0	0	0	3	0	0	0	0	0	0	101
		max	0	0	0	3	0	0	0	0	0	0	

Table 25: MTX RELATED TOXICITY PER PATIENT, TREATMENT CYCLE AND TREATMENT COURSE

			CTCAE v3.0, grading scale (0-5) (max = worse documented toxicity per treatment cycle, cycles with glucarpidase rescue highlighted)										mls/min/1.73m^2
Patient ID	**Cycle**	**Course**	**Mucositis (clinical)**	**Mucositis (functional)**	**↑Creat**	**↑ALT**	**↑Bili**	**↓Phos**	**↓Mg**	**↓Hb**	**↓Neuts**	**↓Plts**	**GFR**
07	1	1	0	0	1	3	0	2	0	2	0	0	129
		2	0	0	1	4	0	2	0	2	0	0	
		Day 15	0	0	0	3	0	3	0	2	0	0	103
		max	0	0	1	4	0	3	0	2	0	0	
	2	1	0	0	1	4	0	3	0	2	0	0	115
		2	0	0	1	4	0	4	0	2	0	0	
		Day 15	0	0	0	2	0	0	0	2	0	0	115
		max	0	0	1	4	0	4	0	2	0	0	
08	1	1	0	0	0	3	0	0	0	3	0	0	106
		2	0	0	0	3	0	2	0	2	0	0	
		Day 15	0	0	0	1	0	0	0	2	0	0	187
		max	0	0	0	3	0	2	0	3	0	0	
	2	1	0	0	0	3	0	0	0	2	0	0	205
		2	0	0	0	3	0	2	0	2	0	0	
		Day 15	0	0	0	1	0	0	0	2	0	0	187
		max	0	0	0	3	0	2	0	2	0	0	
09	1	1	0	0	0	1	0	0	0	2	0	0	175
		2	0	0	0	1	0	0	0	2	0	0	
		Day 15	0	0	0	2	0	0	0	2	0	0	204
		max	0	0	0	2	0	0	0	2	0	0	

Table 25: MTX RELATED TOXICITY PER PATIENT, TREATMENT CYCLE AND TREATMENT COURSE

			CTCAE v3.0, grading scale (0-5) (max = worse documented toxicity per treatment cycle, cycles with glucarpidase rescue highlighted)										mls/min/1.73m^2
Patient ID	**Cycle**	**Course**	**Mucositis (clinical)**	**Mucositis (functional)**	**↑Creat**	**↑ALT**	**↑Bili**	**↓Phos**	**↓Mg**	**↓Hb**	**↓Neuts**	**↓Plts**	**GFR**
	2	1	0	0	0	3	0	0	0	2	0	0	117
		2	0	0	0	3	0	1	0	2	0	0	
		15	0	0	0	2	0	0	0	2	0	0	171
		max	0	0	0	3	0	1	0	2	0	0	
10	1	1	1	0	0	3	0	3	0	2	0	0	98
		2	3	2	1	3	0	0	0	2	0	1	
		Day 15	3	2	0	3	0	0	0	2	1	1	90
		max	3	2	1	3	0	3	0	2	1	1	
	2	1	0	0	0	3	2	2	0	2	2	1	95
		2	0	0	1	3	1	0	0	2	2	1	
		Day 15	0	0	0	3	0	0	0	1	2	1	72
		max	0	0	1	3	2	2	0	2	2	1	
11	1	1	0	0	0	4	0	3	0	1	0	0	111
		2	0	0	0	4	0	3	0	1	0	1	
		Day 15	0	0	0	2	0	-	0	1	0	1	215
		max	0	0	0	4	0	3	0	1	0	1	
	2	1	0	0	0	4	0	0	0	1	0	0	264
		2	0	0	0	3	0	1	0	1	0	1	
		Day 15	0	0	0	2	0	-	0	1	0	1	228
		max	0	0	0	4	0	1	0	1	0	1	

Table 25: MTX RELATED TOXICITY PER PATIENT, TREATMENT CYCLE AND TREATMENT COURSE

			CTCAE v3.0, grading scale (0-5) (max = worse documented toxicity per treatment cycle, cycles with glucarpidase rescue highlighted)										mls/min/1.73m^2
Patient ID	**Cycle**	**Course**	**Mucositis (clinical)**	**Mucositis (functional)**	**↑Creat**	**↑ALT**	**↑Bili**	**↓Phos**	**↓Mg**	**↓Hb**	**↓Neuts**	**↓Plts**	**GFR**
12	1	1	0	0	0	3	2	2	0	1	0	0	77
		2	2	2	2	3	0	2	0	2	0	0	
		Day 15	2	2	1	2	0	0	0	2	0	0	45
		Also on day 15 of cycle 1: enteritis grade 1, hypocalcaemia grade 3 and hypoalbuminaemia grade 2											
		max	2	2	2	3	2	2	0	2	0	0	
13	1	1	2	0	1	3	0	2	0	0	0	0	87
		2	1	0	1	4	0	3	0	0	0	0	
		Day 15	1	0	0	3	0	0	0	0	0	0	51
		max	2	0	1	4	0	3	0	0	0	0	
14	1	1	0	0	0	3	0	2	0	1	0	0	106
		2	0	0	0	2	0	0	0	1	0	0	136
		Day 15	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	
		max	0	0	0	3	0	2	0	1	0	0	
15	1	1	0	0	0	2	0	3	0	0	0	0	88
		2	0	0	0	1	0	2	0	0	0	0	
		Day 15	3	2	0	1	0	0	0	0	0	0	190
		max	3	2	0	2	0	3	0	0	0	0	
	2	1	0	0	0	2	0	1	0	0	0	0	154
		2	0	0	1	2	0	3	0	0	0	1	
		Day 15	2	1	0	1	0	0	0	0	0	1	118

Table 25: MTX RELATED TOXICITY PER PATIENT, TREATMENT CYCLE AND TREATMENT COURSE

			CTCAE v3.0, grading scale (0-5) (max = worse documented toxicity per treatment cycle, cycles with glucarpidase rescue highlighted)										mls/min/1.73m^2
Patient ID	**Cycle**	**Course**	**Mucositis (clinical)**	**Mucositis (functional)**	**↑Creat**	**↑ALT**	**↑Bili**	**↓Phos**	**↓Mg**	**↓Hb**	**↓Neuts**	**↓Plts**	**GFR**
15	2	max	2	1	1	2	0	3	0	0	0	1	
16	1	1	1	0	0	3	0	3	0	1	0	0	94
		2	1	0	0	3	0	3	0	1	0	0	
		Day 15	1	2	0	3	0	0	0	1	0	0	124
		max	1	2	0	3	0	3	0	1	0	0	
	2	1	0	0	0	3	0	3	0	1	0	0	145
		2	0	0	0	3	0	1	0	1	0	0	
		Day 15	0	0	0	3	0	0	0	1	0	0	136
		max	0	0	0	3	0	3	0	1	0	0	

Table 26: COMPARISON OF GRADING OF MTX RELATED TOXICITY IN CYCLES WITH GLUCARPIDASE AND FOLINIC ACID RESCUE (glu/FA) AND CYCLES WITH FOLINIC ACID RESCUE ALONE (FA)

CTCAE v3.0 grades	Mucositis clinical		Mucositis functional		↑ Creatinine		↑ ALT		↑ Bilirubin		↑ Phos-phate		↓ Mg		Haemo-globin		Neuts		Platelets	
	glu/FA	FA	glu/FA	FA	glu/FA	FA	glu/FA	FA	glu/FA	FA	glu/FA	FA	glu/FA	FA	glu/FA	FA	glu/FA	FA	glu/FA	FA
0	9/14 (64.3%)	6/13 (46.15%)	12/14 (85.7%)	8/13 (61.5%)	9/14 (64.3%)	8/13 (61.5%)	0	0	11/14 (78.6%)	12/13 (92.3%)	3/14 (21.4%)	2/13 (15.4%)	14/14 (100%)	13/13 (100%)	4/14 (18.6%)	4/13 (18.6%)	12/14 (85.7%)	12/13 (92.3%)	10/14 (71.4%)	9/13 (69.2%)
1	1/14 (7.14%)	2/13 (15.4%)	0	1/13 (7.7%)	4/14 (18.6%)	4/13 (30.8%)	0	0	0	0	2/14 (15.4%)	1/13 (7.7%)	0	0	3/14 (21.4%)	4/13 (18.6%)	1/14 (7.14%)	1/13 (7.7%)	4/14 (18.6%)	3/13 (23.1%)
2	3/14 (21.4%)	2/13 (15.4%)	2/14 (15.4%)	3/13 (23.1%)	1/14 (7.14%)	0	1/14 (7.14%)	3/13 (23.1%)	3/14 (21.4%)	1/13 (7.7%)	3/14 (21.4%)	3/13 (23.1%)	0	0	6/14 (43%)	4/13 (18.6%)	1/14 (7.14%)	0	0	1/13 (7.14%)
3	1/14 (7.14%)	3/13 (23.1%)	0	1/13 (7.7%)	0	0	9/14 (64.3%)	7/13 (53.8%)	0	0	6/14 (43%)	6/13 (46.15%)	0	0	1/14 (7.14%)	3/13 (23.1%)	0	0	0	0
4	0	0	0	0	0	1/13 (7.7%)	4/14 (18.6%)	3/13 (23.1%)	0	0	0	1/13 (7.7%)	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Fourteen cycles were given with glucarpidase and folinic acid rescue (glu/FA) and thirteen cycles were given with folinic acid rescue alone (FA). The most commonly observed adverse events were elevated alanine transaminase, mucositis, hypophosphataemia, renal impairment and mild bone marrow suppression.

a. HEPATOTOXICITY

All the GLU 1 treatment cycles were associated with acute transient ALT elevation. ALT CTCTAE v3.0 grade ≥ 3 complicated 93% (13/14) and 77% (10/13) of glu/FA and FA treatment cycles respectively. Mild hyperbilirubinaemia (CTCTAE v3.0 grades 1 and 2) complicated 21% (3/14) and 8% (1/13) of glu/FA and FA treatment cycles respectively. We did not document any severe hyperbilirubinaemia (CTCTAE v3.0 grades 3 and 4).

b. MUCOSITIS

Mucositis-clinical complicated 36% (5/14) and 54% (7/13) of glu/FA and FA treatment cycles respectively. More severe mucositis-clinical (CTCAE v3.0 clinical grades ≥ 3) complicated 7% (1/14) and 23% (3/13) of glu/FA and FA treatment cycles respectively. Mucositis-functional complicated 15% (2/14) and 38% (5/13) of glu/FA and FA treatment cycles respectively. More severe mucositis-functional (CTCAE v3.0 grades ≥ 3) complicated 8% (1/13) FA treatment cycles whereas severe mucositis-functional was not documented in any glu/FA treatment cycles.

c. NEPHROTOXICITY

Half (8/16) of the GLU 1 participants developed nephrotoxicity (serum creatinine level CTCAE v3.0 grade ≥ 1). 6 patients (6/16) developed CTCAE v3.0 grade 1 nephrotoxicity and half of those did not complete their treatment due to renal

impairment. One patient (1/16) developed grade 2 nephrotoxicity and one patient (1/16) developed grade 4 nephrotoxicity. Both of those patients did not complete study treatment due to their renal impairment. The patient who developed grade 2 nephrotoxicity had received rescue with glucarpidase/folinic acid. The patient who developed grade 4 nephrotoxicity received standard rescue with folinic acid.

Fourteen (14/27) GLU 1 MTX cycles were given with glucarpidase/folinic acid rescue. Amongst those, nine (9/14) cycles were not associated with nephrotoxocity, four (4/14) led to grade 1 nephrotoxicity and one (1/14) led to grade 2 nephrotoxicity. Thirteen (13/27) MTX cycles were given with folinic acid alone. Amongst those, eight (8/13) were not associated with nephrotoxocity, four (4/13) led to grade 1 nephrotoxicity and one (1/13) led to grade 4 nephrotoxicity.

d. MYELOSUPPRESSION

Anaemia CTCAE v3.0 grades 1, 2 and 3 complicated 21% (3/14), 43% (6/14) and 7% (1/14) of glu/FA respectively and 19% (4/13), 19% (4/13) and 23% (3/13) of FA cycles respectively. Anaemia grade 4 was not documented in either treatment cycles. The majority of treatment cycles were not associated with neutropenia, with only 14% (2/14) and 8% (1/13) of glu/FA and FA treatment cycles respectively leading to CTCAE v3.0 grade 1-2 neutropenia. More severe neutropenia (CTCAE v3.0 grade ≥ 3) was not documented. Similarly, the majority of treatment cycles were not associated with thrombocytopenia, with only 19% (4/14) and 31% (4/13) of glu/FA and FA treatment cycles respectively leading to CTCAE v3.0 grade 1-2 thrombocytopenia. More severe thrombocytopenia (CTCAE v3.0 grade ≥ 3) was not documented.

The difference in toxicity outcomes between cycles given with glucarpidase and cycles given without glucarpidase was examined. The severity of MTX related toxicity was graded from 0 to 4 (ordinal scale) as per the CTCTAE version 3.0. However, for most variables the majority of values were located at the lower end of the scale. To be able to easily allow for the data structure, toxicity outcomes were divided into two categories, a low and high toxicity and as such toxicity outcomes were considered as binary variables. The exact cut-off to create these groups varied depending on the individual adverse event and was decided bearing in mind that grading is not uniform across different adverse events in CTCAE version 3.0; i.e. grade 3 ALT is not necessarily equally severe as a grade 3 creatinine. (Table 27)

Table 27: MTX RELATED TOXICITY: LOW AND HIGH TOXICITY SCORE

Toxicity	Low toxicity score	High toxicity score
Mucositis clinical	Grade 0-1	Grades 2-4
Mucositis functional	Grades 0-1	Grades 2-4
Creatinine	Grade 0	Grades 1-4
ALT	Grades 0-3	Grade 4
Bilirubin	Grade 0	Grades 1-4
Phosphate	Grades 0-2	Grades 3-4
Hb	Grade 0-2	Grades 3-4
Neutrophils	Grade 0	Grades 1-4
Platelets	Grade 0	Grades 1-4

The paired exact test was used to compare the toxicity outcomes between when glu/FA cyles and FA cycles, although this method did not take into account the period in which the treatment occurred. This analysis was restricted to the 11 subjects with toxicity data on both cycles and the results are shown in Table 28. The figures reported are the number and percentage of patients with a high toxicity score in each arm. No

differences in the toxicity outcomes between glu/FA cyles and FA cycle were observed. However, it should be noted that the number of patients in the analysis was small, and so there was a low power to detect differences in these outcomes between arms.

Table 28: EFFECT OF GLUCARPIDASE ON THE TOXICITY OUTCOMES

Toxicity	High toxicity score	No Glucarpidase N (%)	Glucarpidase N (%)	P-value
Mucositis clinical	Grades 2-4	4 (36%)	3 (27%)	1.00
Mucositis functional	Grades 2-4	2 (18%)	3 (27%)	1.00
Creatinine	Grades 1-4	2 (18%)	4 (36%)	0.50
ALT	Grade 4	3 (27%)	3 (27%)	1.00
Bilirubin	Grades 1-4	3 (27%)	0 (0%)	0.25
Phosphate	Grades 3-4	5 (45%)	7 (64%)	0.50
Hb	Grades 3-4	2 (18%)	0 (0%)	0.50
Neutrophils	Grades 1-4	1 (9%)	1 (9%)	1.00
Platelets	Grades 1-4	3 (27%)	4 (36%)	1.00

We also examined the effects of MTX Cmax and AUC upon the various toxicity variables, such as mucositis (clinical), mucositis (functional), raised creatinine, raised ALT, hyperbilirubinaemia, hypophosphataemia, anaemia, neutropenia, and thrombocytopenia. There was no toxicity related to hypomagnesaemia and so it was not included in our analysis.

The analysis of MTX Cmax was performed for cycles given with and without glucarpidase combined. Multilevel logistic regression was used to examine the effect of MTX Cmax upon the toxicities. The analysis results are shown in Table 29. The first column indicates what constitutes a 'high' toxicity value. The next column gives the size of effect of MTX Cmax upon each outcome in the form of an odds ratio. These are presented as the change in the odds of a high toxicity value for a 100-unit increase in

MTX Cmax. An odds ratio above 1 would imply an increased likelihood of a high toxicity with a higher MTX Cmax value.

Table 29: EFFECT OF MTX Cmax UPON METHOTREXATE-RELATED TOXICITY IN ALL TREATMENT CYCLES COMBINED.

Toxicity	High toxicity score	Odds Ratio (95% CI)	P-value
Mucositis clinical	Grades 2-4	1.06 (0.84, 1.33)	0.65
Mucositis functional	Grades 2-4	0.95 (0.73, 1.24)	0.71
Creatinine	Grades 1-4	1.70 (0.73, 3.94)	0.22
ALT	Grade 4	1.33 (0.80, 2.21)	0.26
Bilirubin	Grades 1-4	1.36 (0.96, 1.93)	0.09
Phosphate	Grades 3-4	1.04 (0.54, 2.01)	0.91
Hb	Grades 3-4	0.70 (0.42, 1.16)	0.17
Neutrophils	Grades 1-4	0.93 (0.32, 2.71)	0.90
Platelets	Grades 1-4	1.40 (0.56, 3.47)	0.47

No strong evidence was found that MTX Cmax was associated with any of the toxicity outcome. There was very weak evidence that higher values of MTX Cmax were associated with an increased occurrence of higher bilirubin toxicity, but this result was not quite statistically significant.

The analysis of AUC was performed separately for glu/FA cycles and FA cycles; standard logistic regression was used for the analyses. The results are shown in Table 30, where the odds ratios represent the change in the odds of a high toxicity for a 1000-unit increase in AUC. No evidence that AUC was associated with any of the toxicity outcomes was observed.

Table 30: EFFECT OF AUC UPON METHOTREXATE-RELATED TOXICITY IN GLU/FA CYCLES AND FA CYCLES

Toxicity	High toxicity score	Odds Ratio (95% CI)	P-value
FA cycles			
Mucositis clinical	Grades 2-4	0.79 (0.50, 1.27)	0.34
Mucositis functional	Grades 2-4	0.80 (0.50, 1.28)	0.35
Creatinine	Grades 1-4	1.13 (0.77, 1.66)	0.55
ALT	Grade 4	1.16 (0.76, 1.79)	0.48
Bilirubin	Grades 1-4	(*)	-
Phosphate	Grades 3-4	1.14 (0.78, 1.66)	0.50
Hb	Grades 3-4	(*)	-
Neutrophils	Grades 1-4	1.00 (0.53, 1.88)	0.99
Platelets	Grades 1-4	1.02 (0.70, 1.49)	0.89
glu/FA cycles			
Mucositis clinical	Grades 2-4	1.22 (0.88, 1.70)	0.24
Mucositis functional	Grades 2-4	1.06 (0.76, 1.48)	0.74
Creatinine	Grades 1-4	1.53 (0.93, 2.52)	0.10
ALT	Grade 4	1.30 (0.89, 1.88)	0.18
Bilirubin	Grades 1-4	1.43 (0.92, 2.22)	0.11
Phosphate	Grades 3-4	1.00 (0.76, 1.33)	0.99
Hb	Grades 3-4	0.69 (0.39, 1.20)	0.19
Neutrophils	Grades 1-4	1.01 (0.68, 1.50)	0.96
Platelets	Grades 1-4	1.04 (0.76, 1.41)	0.82

(*) No patients with toxicity when glucarpidase was not given

The effects of age upon MTX-related toxicity were examined. Separate analyses were performed for glu/FA cycles and FA cycles. The toxicity outcomes were dealt with on a binary scale, and so Fisher's exact test was used. As shown in Table 31, age was not significantly associated with any of the toxicity outcomes in either of the two study arms.

Table 31: EFFECTS OF AGE UPON MTX-RELATED TOXICITY

Toxicity	High toxicity score	Age < 20, N (%)	Age ≥ 20, N (%)	P-value
FA cycles		(n=8)	(n=4)	
Mucositis clinical	Grades 2-4	1 (13%)	2 (50%)	0.24
Mucositis functional	Grades 2-4	2 (25%)	1 (25%)	1.00
Creatinine	Grades 1-4	1 (13%)	3 (75%)	0.07
ALT	Grade 4	3 (38%)	0 (0%)	0.49
Bilirubin	Grades 1-4	0 (%)	0 (0%)	(*)
Phosphate	Grades 3-4	4 (50%)	3 (75%)	0.58
Hb	Grades 3-4	0 (0%)	0 (0%)	(*)
Neutrophils	Grades 1-4	0 (0%)	1 (25%)	0.33
Platelets	Grades 1-4	1 (13%)	3 (75%)	0.07
glu/FA cycles		(n=8)	(n=6)	
Mucositis clinical	Grades 2-4	2 (25%)	4 (67%)	0.28
Mucositis functional	Grades 2-4	1 (13%)	2 (33%)	0.54
Creatinine	Grades 1-4	2 (25%)	3 (50%)	0.58
ALT	Grade 4	2 (25%)	2 (33%)	1.00
Bilirubin	Grades 1-4	1 (13%)	3 (50%)	0.25
Phosphate	Grades 3-4	3 (38%)	3 (50%)	1.00
Hb	Grades 3-4	1 (13%)	1 (17%)	1.00
Neutrophils	Grades 1-4	0 (0%)	2 (33%)	0.17
Platelets	Grades 1-4	1 (13%)	3 (50%)	0.25

(*) No patients with toxicity when glucarpidase was not given

Similar analysis was performed to examine the effects of gender upon MTX related toxicity. Again, separate analyses were performed for glu/FA cycles and FA cycles. The toxicity outcomes were dealt with on a binary scale, and so Fisher's exact test was used. As shown in Table 32, there were no significant differences in toxicity outcomes between genders either for glu/FA or FA cycles.

Table 32: EFFECTS OF GENDER UPON TOXICITY

Toxicity	High toxicity score	Male N (%)	Female N (%)	P-value
FA cycles		(n=10)	(n=2)	
Mucositis clinical	Grades 2-4	3 (30%)	0 (0%)	1.00
Mucositis functional	Grades 2-4	3 (30%)	0 (0%)	1.00
Creatinine	Grades 1-4	3 (30%)	1 (50%)	1.00
ALT	Grade 4	3 (30%)	0 (0%)	1.00
Bilirubin	Grades 1-4	0 (%)	0 (0%)	(*)
Phosphate	Grades 3-4	6 (60%)	1 (50%)	1.00
Hb	Grades 3-4	0 (0%)	0 (0%)	(*)
Neutrophils	Grades 1-4	1 (10%)	0 (0%)	1.00
Platelets	Grades 1-4	4 (40%)	0 (0%)	0.52
glu/FA cycles		(n=11)	(n=3)	
Mucositis clinical	Grades 2-4	4 (36%)	2 (67%)	0.54
Mucositis functional	Grades 2-4	3 (27%)	0 (0%)	1.00
Creatinine	Grades 1-4	4 (34%)	1 (33%)	1.00
ALT	Grade 4	2 (18%)	2 (67%)	0.18
Bilirubin	Grades 1-4	3 (27%)	1 (33%)	1.00
Phosphate	Grades 3-4	4 (36%)	2 (67%)	0.54
Hb	Grades 3-4	1 (9%)	1 (33%)	0.40
Neutrophils	Grades 1-4	2 (18%)	0 (0%)	1.00
Platelets	Grades 1-4	4 (36%)	0 (0%)	0.51

(*) No patients with toxicity when glucarpidase was not given

We also examined the GFR measurements on day 15 in glu/FA and FA cycles. There was no major difference in GFR measurements on day 15 among treatment cycles given with glucarpidase/folinic acid rescue and cycles given with folinic acid rescue alone (Table 33). For all treatment cycles, the median and average GFR on day 15 were 124 and 129.5 mls/min/1.73m^2 respectively. The median and average GFR on day 15 were 119.5 and 127.6 mls/min/1.73m^2 respectively for treatment cycles given with

glucarpidase and folinic acid and 124 and 131.8 respectively for treatment cycles given with folinic acid alone.

Table 33: COMPARISON OF GFR ON DAY 15 IN CYCLES WITH GLUCARPIDASE AND FOLINIC ACID RESCUE (glu/FA) AND CYCLES WITH FOLINIC ACID RESCUE ALONE (FA)

	Median (mls/min/1.73m^2)	**Average** (mls/min/1.73m^2)	**Range** (mls/min/1.73m^2)
All cycles	124	129.5	44-228
Glu/FA cycles	119.5	127.6	45-228
FA cycles	124	131.8	22-215

In order to evaluate the severity of mucositis further four different assessment tools were used; the World Health Organisation Toxicity Criteria for Oral Mucositis, the National Cancer Institute Common Terminology criteria for Adverse Events, version 3.0 (CTCAE v 3.0), the Oral Assessment Guide (OAG) and the Oral Mucositis Weekly Questionnaire (OMWQ). Grading of mucositis severity as per these assessment tools is shown on Table 34.

Table 34: MUCOSITIS GRADING ACCORDING TO WHO, OMWQ AND CTCAE V 3.0

ID	Cycle	GLU 1=YES 0=NO	WHO	OAG 1	OAG 2	OAG 3	OAG 4	OAG 5	OAG 6	OAG 7	OAG 8	OMW Q1	OMW Q2	OMW Q3a	OMW Q3b	OMW Q3c	OMW Q3d	OMW Q3e	OM WQ4	OM WQ5	OM WQ6	CTCTAE clinical	CTCTAE functional
1	1	1	0	1	1	1	1	1	1	1	1	8	1	0	0	0	0	0	1	0	0	0	0
1	2	0	3	1	1	1	1	1	3	1	1	1	3	3	3	3	2	0	9	1	1	3	2
2	1	0	2	1	1	3	1	1	1	1	1	7	0	0	0	0	0	0	0	1	2	2	0
2	1	1	2	1	1	3	1	1	1	1	1	7	0	0	0	0	0	0	0	1	2	2	0
3	1	1	2	1	1	3	1	1	1	1	1	5	3	2	0	1	0	0	5	0	0	2	0
3	2	0	1	1	1	1	1	1	1	1	1	7	1	0	0	0	0	0	1	0	0	1	0
4	1	1	0	1	1	1	1	1	1	1	1	1	4	3	2	1	3	1	7	0	0	0	3
6	1	0	4	1	1	1	1	1	1	1	1	7	4	1	4	4	3	1	9	0	0	3	3
6	2	1	0	1	1	1	1	1	1	1	1	9	1	0	1	1	1	0	1	0	0	0	0
7	1	1	0	1	1	1	1	1	1	1	1	9	2	2	1	2	0	1	5	0	0	0	0
7	2	0	0	1	1	1	1	1	1	1	1	9	1	0	0	0	0	0	1	0	0	0	0
8	1	1	0	1	1	1	1	1	1	1	1	6	4	3	3	4	3	2	9	2	5	0	0
8	2	0	0	1	1	1	1	1	1	1	1	4	2	0	0	0	0	0	0	1	1	0	0
9	1	0	0	1	1	1	1	1	1	1	1	8	2	3	3	3	1	0	8	0	0	0	0
9	2	1	0	1	1	1	1	1	1	1	1	9	0	0	0	0	0	0	0	0	0	0	0
10	1	0	3	1	2	1	1	2	3	1	1	6	3	3	3	3	2	3	8	1	1	3	2
10	2	1	0	1	1	1	1	1	1	1	1	8	3	3	3	2	1	3	8	0	0	0	0
11	1	0	0	1	1	1	1	1	1	1	1	8	1	0	0	0	0	0	1	2	3	0	0
11	2	1	0	1	1	1	1	1	1	1	1	8	0	0	0	0	0	0	0	0	0	0	0
12	1	1	2	1	1	3	1	1	1	1	1	6	1	0	0	0	0	0	3	3	7	2	2
13	1	1	0	1	1	1	1	1	1	1	1	7	0	0	0	0	0	0	0	0	0	2	0

ID	Cycle	GLU 1=YES 0=NO	WHO	OAG 1	OAG 2	OAG 3	OAG 4	OAG 5	OAG 6	OAG 7	OAG 8	OMW Q1	OMW Q2	OMW Q3a	OMW Q3b	OMW Q3c	OMW Q3d	OMW Q3e	OM WQ4	OM WQ5	OM WQ6	CTCTAE clinical	CTCTAE functional
14	1	0	0	1	1	1	1	1	1	1	1	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	0	0
15	1	1	2	1	1	3	1	1	2	1	1	9	0	0	0	0	0	0	0	0	0	0	0
15	2	0	2	1	1	3	1	1	1	1	1	7	3	3	2	3	2	1	7	0	0	2	1
16	1	0	2	1	2	3	1	1	3	1	1	9	3	2	0	2	1	0	6	0	0	1	2
16	2	1	0	1	1	1	1	1	1	1	1	9	3	1	0	1	1	0	6	0	0	0	0

We compared the severity of mucositis in cycles given with and without glucarpidase assessed by the WHO criteria, OMWQ, CTCAE version 3.0 and OAG. Amongst these tools there were several mucositis related variables, each of which was measured on an ordinal scale. Due to the nature of the outcomes, and the small amount of data, it is difficult to perform any analysis which accounts for the period in which each treatment arm is administered. Therefore, for the purposes of these analyses the period in which the treatment was given was omitted from the analyses.

The WHO, OMWQ and CTCAE variables had a range of scores, and thus the Wilcoxon matched-pairs test was used to compare between when glucarpidase was used and when it wasn't. The results of this analysis are summarised in Table 35. The figures presented are the median and range of grades in cycles given with and without glucarpidase, and the p-values indicating the significance of the results.

Significant difference in CTCAE mucositis-functional between glu/FA and FA cycles was found. Although the median values were 0 in both groups, there were higher values in cycles without glucarpidase rescue. There was also slight evidence of a difference between groups for the first question of OMWQ and CTCAE clinical, although these results were not quite statistically significant. No other differences between glu/FA and FA cycles were observed.

Table 35: SEVERITY OF MUCOSITIS IN CYCLES GIVEN WITH AND WITHOUT GLUCARPIDASE (ASSESSED BY WHO, OWMQ AND CTCAE VERSION 3.0)

Outcome	No Glucarpidase Median (range)	Glucarpidase Median (range)	P-value
WHO	2 (0, 4)	0 (0, 2)	0.13
OMWQ1	7 (1, 9)	9 (5, 9)	0.06
OMWQ2	2 (0, 4)	1 (0, 4)	0.34
OMWQ3a	1 (0, 3)	0 (0, 3)	0.50
OMWQ3b	0 (0, 4)	0 (0, 3)	0.34
OMWQ3c	2 (0, 4)	1 (0, 4)	0.34
OMWQ3d	1 (0, 3)	0 (0, 3)	0.17
OMWQ3e	0 (0, 3)	0 (0, 3)	0.92
OMWQ4	6 (0, 9)	1 (0, 9)	0.47
OMWQ5	0 (0, 2)	0 (0, 2)	0.29
OMWQ6	0 (0, 3)	0 (0, 5)	0.40
CTCAE clinical	1 (0, 3)	0 (0, 2)	0.07
CTCAE functional	0 (0, 3)	0 (0, 0)	0.03

For the analysis of the OAG we used a different approach since pretty much all eight assessments (swallow, lips and corners of mouth, tongue, saliva, mucus membrane, mucus, teeth, voice) were graded as 1 for the vast majority of patients, with few other values. Therefore, this variable was categorised as a grade of 1 or grades of 2-3, and the paired exact test was used for the analyses (Table 36). No differences between cycles given with glucarpidase and cycles given without glucarpidase were observed.

Table 36: SEVERITY OF MUCOSITIS IN CYCLES GIVEN WITH AND WITHOUT GLUCARPIDASE (ASSESSED BY OAG)

Outcome	No Glucarpidase N (%)*	Glucarpidase N (%)*	P-value
OAG1	0 (0%)	0 (0%)	-
OAG2	2 (18%)	0 (0%)	0.50
OAG3	3 (27%)	3 (27%)	1.00
OAG4	0 (0%)	0 (0%)	-
OAG5	1 (9%)	0 (0%)	1.00
OAG6	3 (27%)	1 (9%)	0.63
OAG7	0 (0%)	0 (0%)	-
OAG8	0 (0%)	0 (0%)	-

* Number and percentage of patients in each arm who had a grade of 2 or more.

5.5. PHARMACOKINETIC ANALYSIS OF MTX AND DAMPA

Following each of the 4 MTX doses (on days 1 & 8 of cycles 1 and 2), blood samples were collected for MTX and DAMPA HPLC analysis at the following time points: at 0 hours (prior to starting MTX), 4 hours (immediately prior to the end of the MTX infusion), 24 hours (prior to glucarpidase administration), 24:20 hours (15 minutes after glucarpidase administration), 48 hours and 72 hours after starting MTX and then daily until plasma MTX levels [MTX], measured by HPLC, were < 0.2 µmol/L. In addition, following each of the 4 MTX doses (on days 1 & 8 of cycles 1 and 2), blood samples were collected for plasma MTX analysis by immunoassay at the following time points: at 24 hours (prior to glucarpidase administration), 48 hours and 72 hours after starting MTX and then daily until [MTX] were < 0.2 µmol/L.

A total of 51 MTX courses were administered in the GLU1 clinical trial. Twenty eight of fifty one (55%) MTX courses were given with glucarpidase/folinic acid rescue and 23 of 51 (45%) MTX courses were given with folinic acid rescue alone.

[MTX] at different time points per patient and per methotrexate course are shown in Table 37. For MTX courses given with both rescue regimens (folinic acid and glucarpidase/folinic acid), patients required a median of 72 hours (range, 48 to 312) to achieve [MTX] of < 0.2 µmol/L. For MTX courses given with folinic acid alone, patients required a median of 72 hours (range, 72 to 144) to achieve [MTX] of < 0.2 µmol/L, whereas for MTX courses given with glucarpidase/folinic acid, patients required a median of 60 hours (range 48 to 312 hours) (Table 38 and Table 39).

Table 37: [MTX] AT DIFFERENT TIME POINTS PER PATIENT AND PER MTX COURSE

[MTX] in µM at different time points														
Courses given with glucarpidase highlighted	**0 h HPLC**	**4 h HPLC**	**24 h**		**24.20 h HPLC**	**48 h HPLC**	**72 h HPLC**	**96 h HPLC**	**120 h HPLC**	**144 h HPLC**	**168 h HPLC**	**192 h HPLC**	**216 h HPLC**	**240 h HPLC**
			HPLC	**Immuno -assay**										
GLU1-01														
Course 1	0		4.99	5.34	0.16	0.08	0.09							
Course 2	0.3	1303.84	7.96	7.02	0.6	0.06	0.11							
Course 3	0	1144.8	14.47	15.57	11.86	0.71	0.20							
Course 4	0	1439.6	12.5	13.01	1.84	0.67	0.23							
GLU1-02														
Course 1	0	1298.42	18.30	22.79	14.54	0.54	0.13							
Course 2	0	896.2	16.71	20.47	11.89	0.46	0.06							
Course 3	0	1276.4	15.67	32.29	0	0	0							
Course 4	0	985.6	21.16	23.74	0.09	0.01	0							
GLU1-03														
Course 1	0	1673.68	11.78	9.12	0	0.03	0							
Course 2	0.10	1228	54.37	63.46	0.415	0.02	0.12							
Course 3	0.04	1370	20.13	24.45	17.76	1.37	0.81	0.47	0.25	0.18				
GLU1-04														
Course 1	0.03	845.4	12.17	17.44	0.07	0.01	0.02							

Table 37: [MTX] AT DIFFERENT TIME POINTS PER PATIENT AND PER MTX COURSE

[MTX] in µM at different time points														
Courses given with glucarpidase highlighted	**0 h HPLC**	**4 h HPLC**	**24 h**		**24.20 h HPLC**	**48 h HPLC**	**72 h HPLC**	**96 h HPLC**	**120 h HPLC**	**144 h HPLC**	**168 h HPLC**	**192 h HPLC**	**216 h HPLC**	**240 h HPLC**
			HPLC	**Immuno -assay**										
Course 2	0.04	1179.4	54.32	70.16	0.79	0.14	0.45	0.4	0.34	0.26	0.28	0.16	0.1	
GLU1-05 (glucarpidase given as emergency treatment at 72 h post methotrexate, measurement pre and post glucarpidase available)														
Course 1	0	1400	30.11	33.22	13.94	1.39	0.58/0.03	0.03	0.09	0.15	0.11			
GLU1-06														
Course 1	0.02	922.46	10.85	12.55	7.16	0.27	0.07							
Course 2	0.06	932.44	3.79	3.55	2.77	0.22	0.10							
Course 3	0	983.4	13.3	12.76	0.21	0.02	0.04							
Course 4	0	1294.72	13.14	14.74	0.09	0.28	0.08							
GLU1-07														
Course 1	0.08	1176.4	95.66	95.92	0.852	0.06	0.17	0.08						
Course 2	0.12	1319.2	135.86	141.69	1	0.09	0.2	0.161						
Course 3	0.02	N.A.	152.62	149.78	135.24	8.61	0.98	0.39	0.24	0.15				
Course 4	0.318	1638	141.6	114.83	107.02	4.66	1.01	0.53	0.21					
GLU1-08														
Course 1	0.05	587.18	3.27	4.49	0.04	3.2?	0.04							
Course 2	0.08	524.42	7.69	9.06	0.10	0.09	0							

Table 37: [MTX] AT DIFFERENT TIME POINTS PER PATIENT AND PER MTX COURSE

[MTX] in µM at different time points														
Courses given with glucarpidase highlighted	**0 h HPLC**	**4 h HPLC**	**24 h**		**24.20 h HPLC**	**48 h HPLC**	**72 h HPLC**	**96 h HPLC**	**120 h HPLC**	**144 h HPLC**	**168 h HPLC**	**192 h HPLC**	**216 h HPLC**	**240 h HPLC**
			HPLC	**Immuno -assay**										
Course 3	0.15	723.76	5.54	5.57	3.51	0.25	0.19							
Course 4	0.05	535.46	4.48	5.16	4.65	0.2	0.05							
GLU1-09														
Course 1	0.03	1337.72	7.31	7.26	6.15	0.33	0.09							
Course 2	0	1355.58	8.33	7.6	6.24	0.34	0.13							
Course 3	0.09	1219	6.95	8.4	0.09	0.02	0.04							
Course 4	0.03	1370.9	10.01	9.44	0.16	0.05	0.05							
GLU1-10														
Course 1	0.02	1355.9	29.83	28.42	25.6	1.13	0.15							
Course 2	0.1	1181.6	23.10	22.77	13.96	1.0	0.2							
Course 3	0.02	1558	36.06	41.23	0.26	0.48	0.07							
Course 4	0.06	1577.44	43.68	55.76	0.7	1.23	0.276	0.192						
GLU1-11														
Course 1	0.09	1741.60	31.95	28.03	19.10	0.84	0.23							
Course 2	0.07	1452.60	12.22	11.05	8.662	0.58	0.16							
Course 3	0.10	1387.20	16.18	16.94	0.40	0.19	0.18							

Table 37: [MTX] AT DIFFERENT TIME POINTS PER PATIENT AND PER MTX COURSE

[MTX] in µM at different time points														
Courses given with glucarpidase highlighted	**0 h HPLC**	**4 h HPLC**	**24 h**		**24.20 h HPLC**	**48 h HPLC**	**72 h HPLC**	**96 h HPLC**	**120 h HPLC**	**144 h HPLC**	**168 h HPLC**	**192 h HPLC**	**216 h HPLC**	**240 h HPLC**
			HPLC	**Immuno -assay**										
Course 4	0.10	1370	18.9	20.44	0.53	0.23	0.14							
GLU1-12														
Course 1	Samples unsuitable for analysis as explained in Section 3.3.3.						0.19	0.27	0.2					
Course 2	0.19	1968.44	253.54	259.94	3.19	3.6	3.15	1.82	1.12	0.83	0.9	0.74	0.54	0.43
	264 h: 0.32; 288 h: 0.27; 312 h: 0.16													
GLU1-13														
Course 1	0	1698	16.35	17.47	0.43	0.61	0.63	N.A.	N.A.	0.18				
Course 2	0.17	2225.8	44.4	43.86	0.82	0.83	0.57	0.30	0.11					
GLU1-14														
Course 1	0.09	1901.72	25.62	30.04	16.96	1.04	0.34	0.09						
GLU1-15														
Course 1	0.17	709.92	10.76	12.78	0.41	0.5	0.32							
Course 2	0	811.84	15.18	16.89	1.022	0.425	0.16							
Course 3	0.07	954.68	23.01	26.12	15.34	1.07	0.41	0.13						
Course 4	0.08	1296.08	16.76	23.19	9.76	0.96	0.58	0.15						
GLU1-16														

Table 37: [MTX] AT DIFFERENT TIME POINTS PER PATIENT AND PER MTX COURSE

[MTX] in µM at different time points														
Courses given with glucarpidase highlighted	**0 h HPLC**	**4 h HPLC**	**24 h**		**24.20 h HPLC**	**48 h HPLC**	**72 h HPLC**	**96 h HPLC**	**120 h HPLC**	**144 h HPLC**	**168 h HPLC**	**192 h HPLC**	**216 h HPLC**	**240 h HPLC**
			HPLC	**Immuno -assay**										
Course 1	0	1155.8	14.96	18.38	10.78	0.80	0.209							
Course 2	0.04	1127.1	11.8	20	10.2	0.82	0.24							
Course 3	0.05	1321.2	18.9	26	0	0.44	0.16							
Course 4	0.07	967	14.4	16.97	0.7	0.48	0.09							

Table 38: HOURS REQUIRED TO ACHIEVE [MTX] OF < 0.2 µM FOR BOTH RESCUE REGIMENS, PER PATIENT AND PER COURSE

GLU1-01		**GLU1-09**	
Course 1	48 h	Course 1	72 h
Course 2	48 h	Course 2	72 h
Course 3	72 h	Course 3	48 h
Course 4	72 h	Course 4	48 h
GLU1-02		**GLU1-10**	
Course 1	72 h	Course 1	72 h
Course 2	72 h	Course 2	72 h
Course 3	48 h	Course 3	72 h
Course 4	48 h	Course 4	96 h
GLU1-03		**GLU1-11**	
Course 1	48 h	Course 1	72 h
Course 2	48 h	Course 2	72 h
Course 3	144 h	Course 3	48 h
GLU1-04		Course 4	72 h
Course 1	48 h	**GLU1-12**	
Course 2	192 h	Course 1	120 h
GLU1-05		Course 2	312 h
Course 1	96* h	**GLU1-13**	
GLU1-06		Course 1	144 h
Course 1	72 h	Course 2	120 h
Course 2	72 h	**GLU1-14**	
Course 3	48 h	Course 1	96 h
Course 4	72 h	**GLU1-15**	
GLU1-07		Course 1	72 h
Course 1	48 h	Course 2	72 h
Course 2	48 h	Course 3	96 h
Course 3	144 h	Course 4	96 h
Course 4	120 h	**GLU1-16**	
GLU1-08		Course 1	72 h
Course 1	72 h	Course 2	72 h
Course 2	48 h	Course 3	72 h
Course 3	72 h	Course 4	72 h
Course 4	72 h		

(courses given with glucarpidase are highlighted)
* glucarpidase given as emergency treatment 72 hours pot methotrexate

Table 39: HOURS REQUIRED TO ACHIEVE [MTX] OF < 0.2 µM FOR EACH RESCUE REGIMEN AND FOR BOTH REGIMENS

Hours to achieve [MTX] of 0.2 µM (from the start of MTX infusion)			
	Both rescue regimens	**folinic acid rescue regimen**	**Glucarpidase/folinic acid rescue regimen**
Mean	82 h	84.5 h	80 h
Median	72 h	72 h	60 h
Range	48-312 h	72-144 h	48-312 h

Median [MTX] measured by **HPLC** at 24 hours after starting the infusion was 15.9 µM (range, 3.27 to 253.54) for courses given with both rescue regimens (Table 20 and Table 23). For courses given with folinic acid alone, the median [MTX] measured by HPLC at 24 hours after starting the infusion was 16.71 µM (range, 3.79 to 152.62). For courses given with glucarpidase/folinic acid the median [MTX] measured by HPLC at 24 hours after starting the infusion was 15.67 µM (range, 3.27 to 253.54) (Table 40).

Median [MTX] measured by **immunoassay** at 24 hours after starting the infusion was 19.2 µM (range, 3.55 to 259.94) for courses given with both rescue regimens (Table 37 and Table 40). For courses given with folinic acid alone median [MTX] measured by immunoassay at 24 hours after starting the infusion was 20.47 µM (range, 3.55 to 149.78). For courses given with glucarpidase/folinic acid median [MTX] measured by immunoassay at 24 hours post starting the infusion was 17.44 µM (range, 4.49 to 259.94) (Table 40).

Median [MTX] measured by HPLC at 4 hours post starting the infusion was 1295.4 µM (range, 524.42 to 2225.8) for courses given with both rescue regimens (Table 37 and Table 40). For courses given with folinic acid alone median [MTX] measured by HPLC at 4 hours post starting the infusion was 1298.42 µM (range, 535.46 to 1901.72). For courses given with glucarpidase/folinic acid, median [MTX] measured

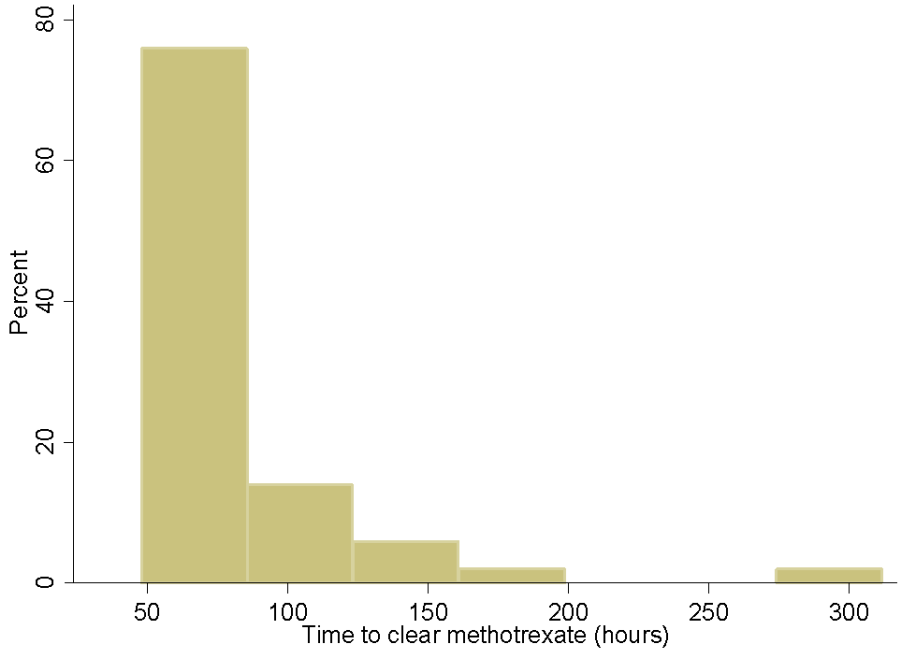
by HPLC at 4 hours post starting the infusion was 1294.72 µM (range, 524.42 – 2225.8) (Table 40).

Table 40: [MTX] AT 4 HOURS AND 24 HOURS POST STARTING THE INFUSION OF MTX

	[MTX] at 4 hours (HPLC, µM)			[MTX] at 24 hours (HPLC, µM)			[MTX] at 24 hours (immunoassay, µM)		
	Both rescue regimens	**Placebo & folinic acid**	**Glucarpidase & folinic acid**	**Both rescue regimens**	**Placebo & folinic acid**	**Glucarpidase & folinic acid**	**Both rescue regimens**	**Placebo & folinic acid**	**Glucarpidase & folinic acid**
Median	1295.40	1298.42	1294.72	15.92	16.71	15.67	19.19	20.47	17.44
Mean	1244.25	1242.42	1263.81	31.85	27.65	35.43	33.94	27.99	39.01
Minimum	524.42	535.46	524.42	3.27	3.79	3.27	3.55	3.55	4.49
Maximum	2225.8	1901.72	2225.8	253.54	152.62	253.54	259.94	149.78	259.94

In order to compare the effect of glucarpidase on the time to eliminate MTX, the distribution of our data was examined. Our measurements were found to have highly skewed distribution (Figure 9). Since it was not possible to normalize it by log transformation (or any other transformation) the outcome measurements were assumed to have a Poisson distribution.

Figure 9: GRAPHICAL ILLUSTRATION OF THE DISTRIBUTION OF DATA RELATED TO TIME TO ELIMINATE MTX



Additionally, as the study had a crossover design, it was necessary to take account of the repeat measurements for each subject in the analysis. This was done using multilevel statistical methods, assuming that individual measurements were nested within patients. To allow for both the data structure and distribution of values, multilevel Poisson regression was used for the analysis.

The multilevel Poisson regression analyses suggested that there was some evidence of a carryover of the effects of glucarpidase on time to clear MTX (p=0.007). The presence of such interaction suggests that the effects of glucarpidase were different in the two treatment cycles (i.e. different effects of glucarpidase when it was given in the first treatment cycle to when it was given in the second) and a carryover of the effects of glucarpidase to the second treatment cycle.

Two different approaches were used to examine the effect of glucarpidase on the time to eliminate MTX: a) data from both treatment cycles were analysed assuming that the period by treatment effect was not present, and b) only the results from the first treatment cycle were analysed as these were unaffected by carryover.

The time to clear MTX was found to be significantly less with the use of glucarpidase when data from both cycles was analysed (p <0.001, Table 41). There was no significant effect when the analysis was restricted to the first cycle only. However, given the significant period by treatment interaction, all these analyses should be viewed with some caution.

Table 41: EFFECT OF GLUCARPIDASE ON THE TIME TO ELIMINATE MTX

Outcome	Data used in analysis	Ratio* (95% CI)	P-value
Time to eliminate methotrexate	Both periods	0.70 (0.65, 0.74)	<0.001
	1st cycle only	1.23 (0.82, 1.87)	0.32

*The effect of glucarpidase on the time to eliminate MTX is reported in the form of ratios. This is given as the ratio of the outcome value when glucarpidase was given compared to when it wasn't. A ratio of above 1 suggests higher values of the outcome when glucarpidase was given, whilst a ratio below 1 would suggest lower values.

For MTX courses given with standard rescue, [MTX] measured by HPLC and immunoassay (IA) are shown on Table 42.

Table 42: MTX COURSES WITH STANDARD RESCUE: AGREEMENT BETWEEN HPLC AND IMMUNOASSAY (IA)

MTX courses with standard rescue: [MTX] in µM at different time points												
	24h		**48 h**		**72 h**		**96 h**		**120 h**		**144 h**	
	HPLC	**IA**	**HPLC**	**IA**	**HPLC**	**IA**	**HPLC**	**IA**	**HPLC**	**IA**	**HPLC**	**IA**
GLU1-01												
Course 3	14.47	15.57	0.71	0.94	0.20	0.27						
Course 4	12.5	13.01	0.67	0.64	0.23	0.19						
GLU1-02												
Course 1	18.30	22.79	0.54	0.65	0.13	0.16						
Course 2	16.71	20.47	0.46	0.53	0.06	0.11						
GLU1-03												
Course 3	20.13	24.45	1.37	1.92	0.81	1.18	0.47	0.44	0.25	0.24	0.18	0.14
GLU1-05												
Course 1	30.11	33.22	1.39	1.8	0.58	0.97	Post 72 hours, glucarpidase given as emergency treatment					
GLU1-06												
Course 1	10.85	12.55	0.27	0.35	0.07	0.09						
Course 2	3.79	3.55	0.22	0.22	0.1	0.19						
GLU1-07												
Course 3	152.62	149.78	8.61	8.66	0.98	1.31	0.39	0.43	0.24	0.28	0.15	0.13
Course 4	141.6	114.83	4.66	4.24	1.01	0.92	0.53	0.41	0.21	0.14		

MTX courses with standard rescue: [MTX] in µM at different time points												
	24h		48 h		72 h		96 h		120 h		144 h	
	HPLC	IA	HPLC	IA	HPLC	IA	HPLC	IA	HPLC	IA	HPLC	IA
GLU1-08												
Course 3	5.54	5.57	0.25	0.25	0.19	0.08						
Course 4	4.48	5.16	0.2	0.21	0.05	0.07						
GLU1-09												
Course 1	7.31	7.26	0.33	0.35	0.09	0.09						
Course 2	8.33	7.6	0.34	0.32	0.13	0.08						
GLU1-10												
Course 1	29.83	28.42	1.13	0.68	0.15	0.09						
Course 2	23.10	22.77	1	0.96	0.2	0.17						
GLU1-11												
Course 1	31.95	28.03	0.84	0.6	0.23	0.11						
Course 2	12.22	11.05	0.58	0.57	0.16	0.1						
GLU1-14												
Course 1	25.62	30.04	1.04	0.86	0.39	0.25	0.09	0.10				
GLU1-15												
Course 3	23.01	26.12	1.07	1.24	0.41	0.3	0.13	0.13				
Course 4	16.76	23.19	0.96	0.73	0.58	0.42	0.15	0.18				

MTX courses with standard rescue: [MTX] in µM at different time points												
	24h		48 h		72 h		96 h		120 h		144 h	
	HPLC	IA	HPLC	IA	HPLC	IA	HPLC	IA	HPLC	IA	HPLC	IA
GLU1-16												
Course 1	14.96	18.38	0.80	0.39	0.209	0.13						
Course 2	11.8	20	0.82	0.58	0.24	0.15						

The agreement between [MTX] measured by HPLC and immunoassay (IA) was examined. Since both methods measure the same quantity, it seemed appropriate to examine the agreement between them rather than the correlation. This was assessed using the intra-class correlation (ICC) coefficient which divides the total variability in the data in that between patients, and that between repeat measurements of the same patient. The ICC is the proportion of the total variability that is between patients. If the agreement is good, then the majority of the variability in the data should be between patients, with very little variability between methods for the same patient, and thus the ICC value should be close to 1. For this analysis, data from all time points were analysed in a single analysis. Additionally due to the skewed nature of outcome values, the analysis was performed on the log scale.

The analysis indicated an ICC value of 0.99, i.e. 99% of all variability was between different observations, and only 1% of variability in the data was due to differences between the chromatography and immunoassay methods for the same measurements. This extremely high value suggests very good agreement between the two methods.

For MTX courses given with glucarpidase/folinic acid rescue, plasma concentrations of MTX and DAMPA 15 minutes after glucarpidase as well as the reduction of plasma MTX concentrations 15 minutes after glucarpidase are shown in Table 18. Blood samples taken for HPLC analysis at T=0 hours, 4 hours, 24 hours, 24.20 hours and 48 hours from the GLU1-12 patient following his first course of MTX became jelly like with the addition of HCL. Therefore they were unsuitable for HPLC analysis. In the remaining twenty seven courses, plasma MTX concentrations decreased from a median of 15.67 µM (range, 3.27 to 253.4) prior to glucarpidase to a median of 0.4 µM (range, 0 to 3.195) 15 minutes after glucarpidase administration.

Glucarpidase resulted in a rapid 92.5 to100% reduction (median, 98.05%) in all patients.

The median DAMPA plasma concentration 15 minutes after glucarpidase was 4.92 µM (range, 1.7 to 75.70) as shown in Table 43. DAMPA plasma concentration 15 minutes after glucarpidase administration was 15-62% of methotrexate plasma concentration prior to glucarpidase administration (median 34%, mean 35.4%).

A small rebound increase in [MTX] was observed in 21% (6/28) of MTX courses given with glucarpidase/folinic acid rescue (Table 44). The median [MTX] prior to glucarpidase administration was 24.87 µM (range 10.76 to 253.54 µM) in those patients. The median [MTX] 15 minutes after glucarpidase administration was 0.335 µM (range 0 to 3.19 µM). The median peak rebound [MTX] was 0.49 µM (range 0.03 to 3.6 µM). Rebound occurred at a median of 24 hours after administration of glucarpidase.

Table 43: [MTX] AND [DAMPA] 15 MINUTES POST GLUCARPIDASE AND REDUCTION OF [MTX] 15 MIN POST GLUCARPIDASE

	Plasma concentration measured by HPLC (µM)			**Reduction of [MTX], 15 minutes post glucarpidase (%)**
	[MTX] at 24 h	**[MTX] at 24:20 h**	**[DAMPA] at 24:20 h (*)**	
GLU1-01				
Course 1	4.99	0.16	2.2 (44%)	96.8
Course 2	7.96	0.6	2.5 (31%)	92.5
GLU1-02				
Course 3	15.66	0	5.86 (37%)	100
Course 4	21.16	0.09	8.60 (41%)	99.6
GLU1-03				
Course 1	11.78	0	3.96 (34%)	100
Course 2	54.37	0.41	33.5 (62%)	98.5
GLU1-04				
Course 1	12.17	0.07	5.09 (42%)	99.4

	Plasma concentration measured by HPLC (µM)			Reduction of [MTX], 15 minutes post glucarpidase (%)
	[MTX] at 24 h	[MTX] at 24:20 h	[DAMPA] at 24:20 h (*)	
Course 2	54.32	0.79	31.92 (59%)	98.5
GLU1-06				
Course 3	13.3	0.208	1.96 (15%)	98.4
Course 4	13.14	0.1	2.58 (20%)	99.2
GLU1-07				
Course 1	95.66	0.85	40.54 (42%)	99.1
Course 2	135.86	1	45.18 (34%)	99.3
GLU1-08				
Course 1	3.95	0.04	1.81 (46%)	99
Course 2	7.69	0.1	1.7 (22%)	98.7
GLU1-09				
Course 3	6.95	0.09	2.21 (32%)	98.7
Course 4	10.01	0.16	2.95 (29%)	98.4
GLU1-10				
Course 3	36.06	0.26	14.08 (39%)	99.3
Course 4	43.68	0.7	16.12 (37%)	98.4
GLU1-11				
Course 3	16.18	0.40	6.4 (40%)	97.5
Course 4	18.9	0.53	5.6 (30%)	97.2
GLU1-12				
Course 1	Samples unsuitable for analysis		Not applicable	
Course 2	253.54	3.195	75.70 (30%)	98.7
GLU1-13				
Course 1	16.35	0.43	4.36 (27%)	97.4
Course 2	44.4	0.82	23.5 (53%)	98.1
GLU1-15				
Course 1	10.76	0.41	2.47 (23%)	96.2
Course 2	15.18	1.02	4.46 (29%)	93.3
GLU1-16				
Course 3	18.9	0	4.92 (26%)	100
Course 4	14.4	0.7	4.8 (33%)	95.1
Mean reduction of [MTX] 15 min post glucarpidase				98.05%
Median reduction of [MTX] 15 min post glucarpidase				98.5%
Range of reduction of [MTX] 15 min post glucarpidase				92.5%-100%

*[DAMPA] at 24.20 hours in comparison to [MTX] at 24 hours

Table 44: REBOUND INCREASE IN [MTX] AFTER GLUCARPIDASE ADMINISTRATION

Number of treatment courses given with glucarpidase/folinic acid rescue	28
Number of treatment courses given with glucarpidase/folinic acid rescue where rebound increase in [MTX] noted	8
[MTX] pre glucarpidase at 24 h	
Median	24.87 µM
Range	10.76 µM - 253.54 µM
[MTX] post glucapidase at 24.20 h	
Median	0.335 µM
Range	0 µM - 3.19 µM
Peak rebound* in [MTX]	
Median	0.49 µM
Range	0.03 µM - 3.6 µM

*Rebound occurred at a median of 24 hours after the administration of glucarpidase

[MTX] at different time points per patient and per MTX course as measured by HPLC are also presented in graphs (Figure 10.1 to Figure 10.16)

Figure 10: [MTX] AT DIFFERENT TIME POINTS PER PATIENT AND PER MTX COURSE AS MEASURED BY HPLC

Figure 10.1: PATIENT GLU1-01

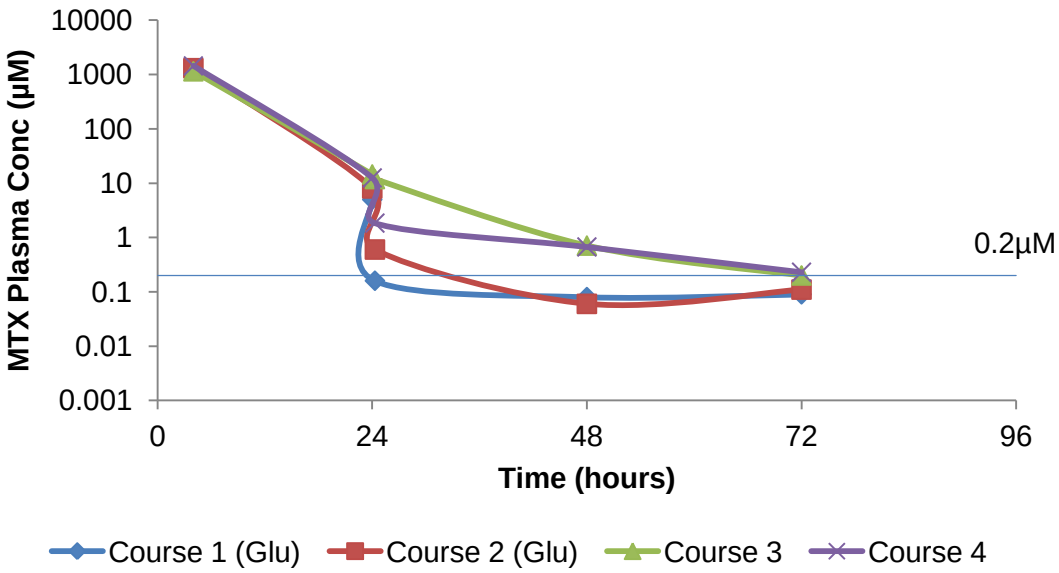


Figure 10.2: PATIENT GLU1-02

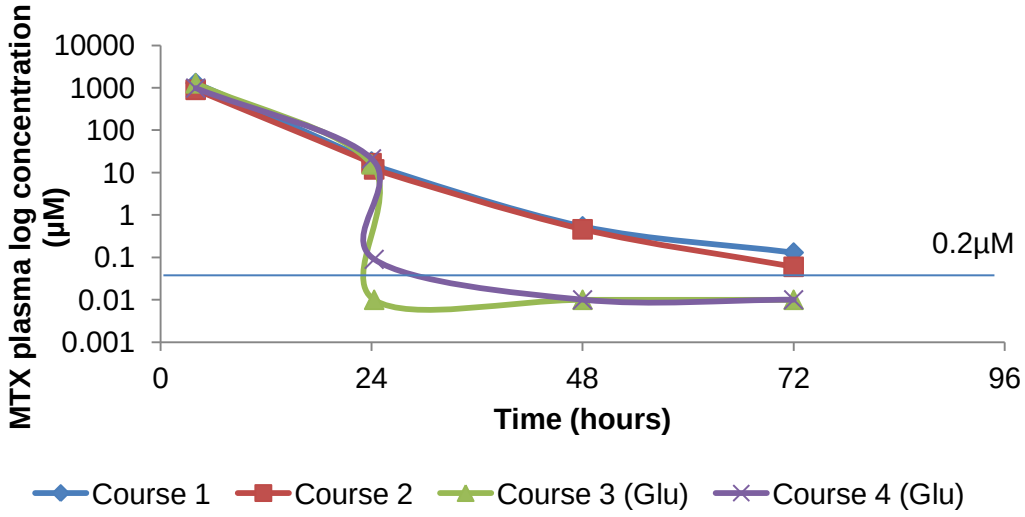


Figure 10.3: PATIENT GLU1-03

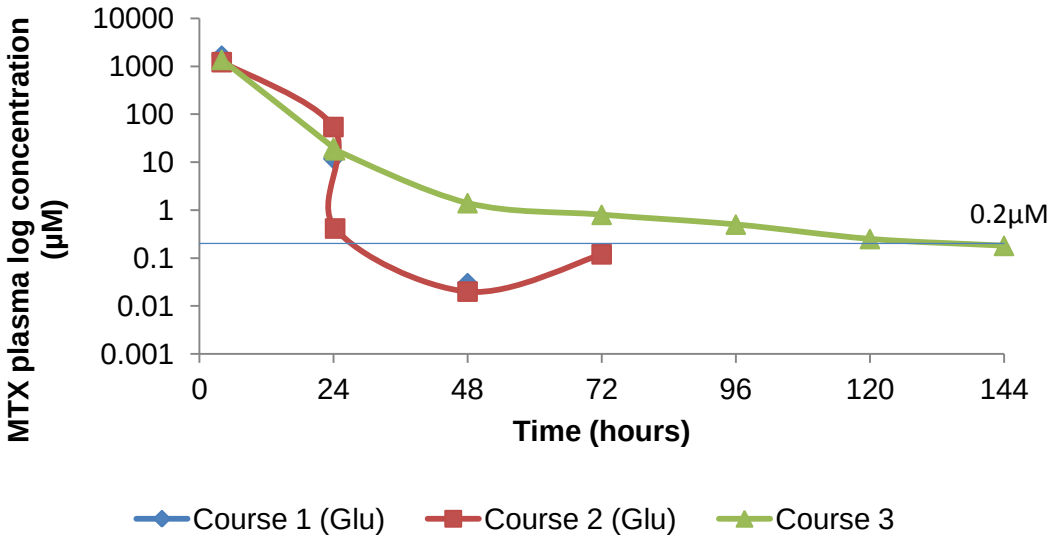


Figure 10.4: PATIENT GLU1-04

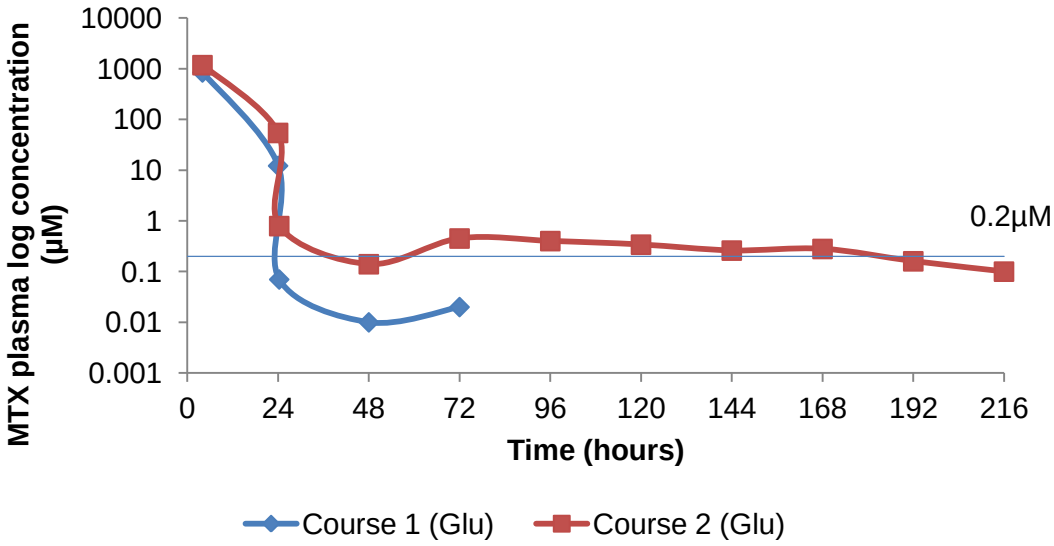


Figure 10.5: PATIENT GLU1-05

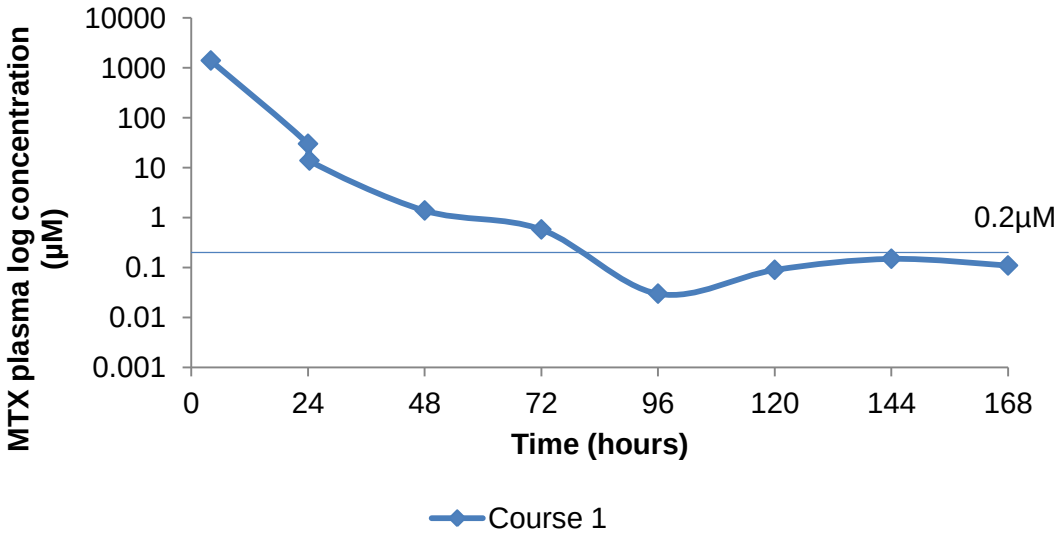


Figure 10.6: PATIENT GLU1-06

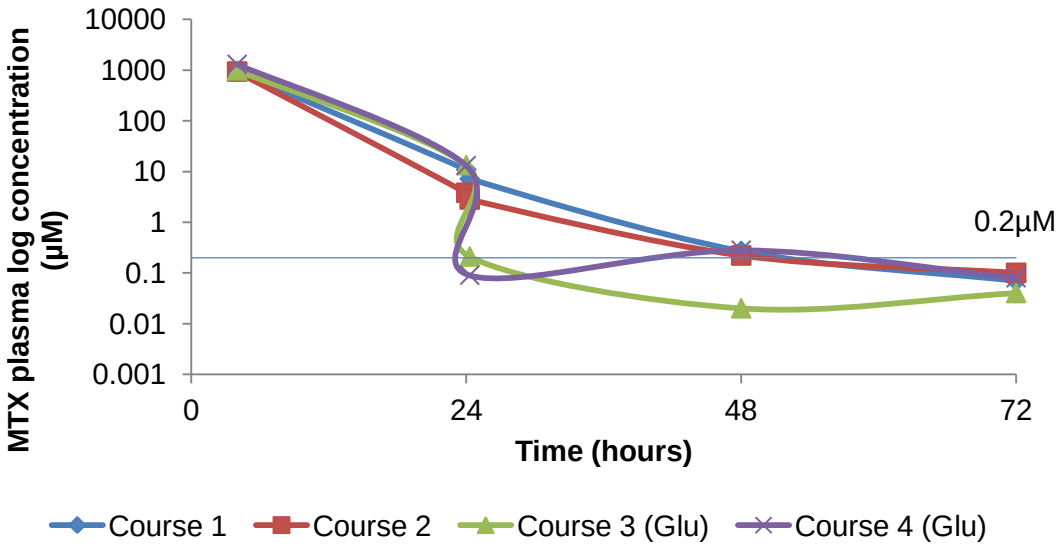


Figure 10.7: PATIENT GLU1-07

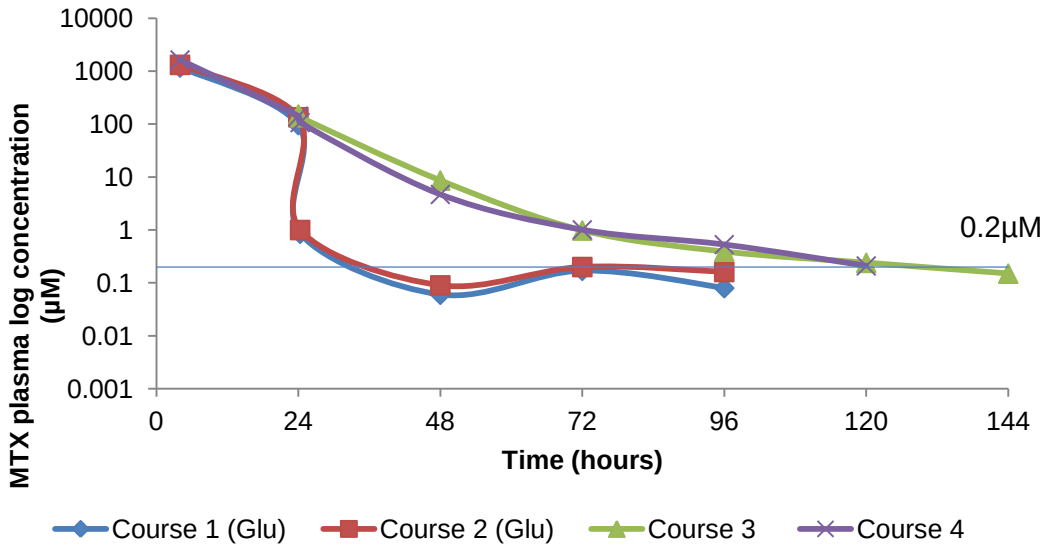


Figure 10.8: PATIENT GLU1-08

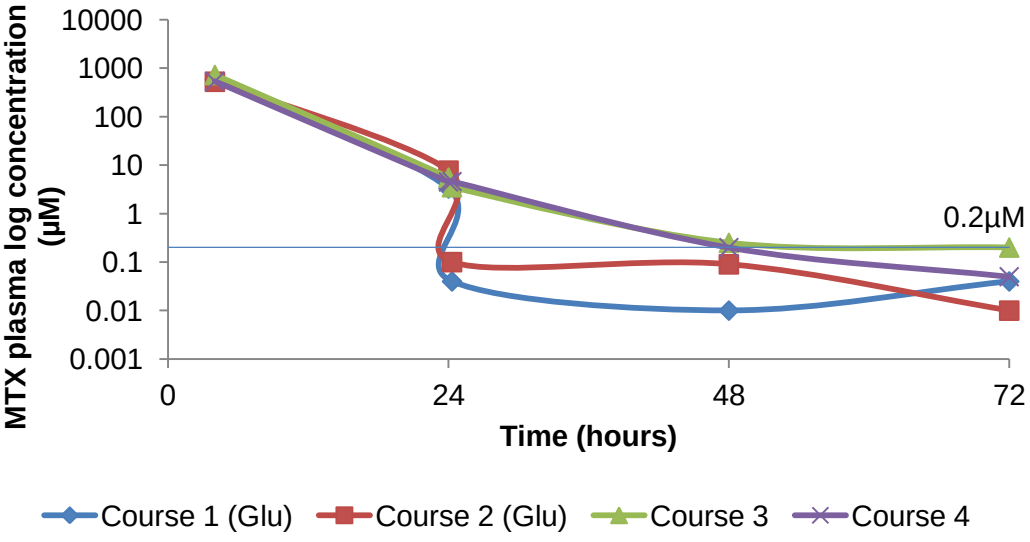


Figure 10.9: PATIENT GLU1-09

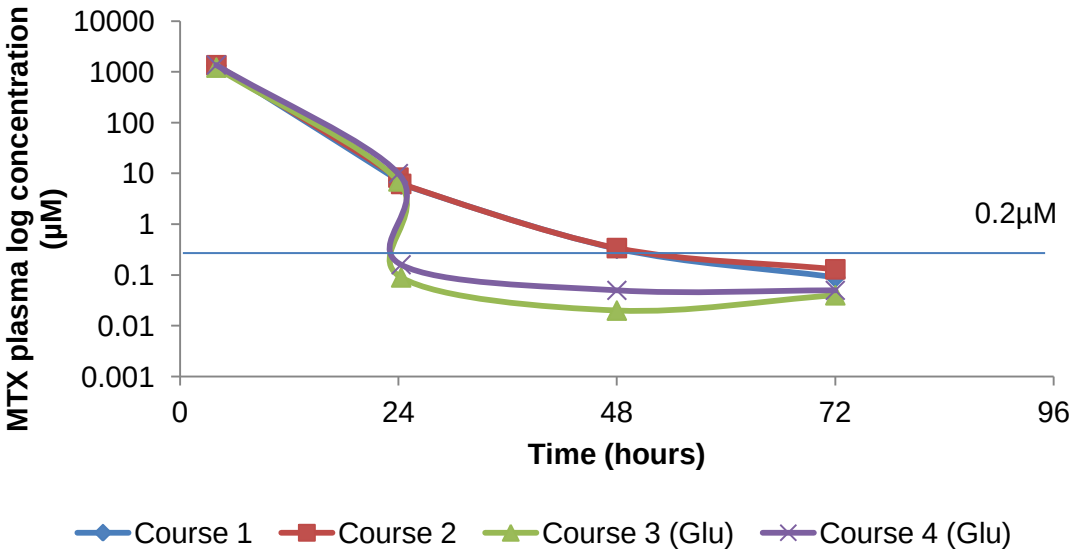


Figure 10.10: PATIENT GLU1-10

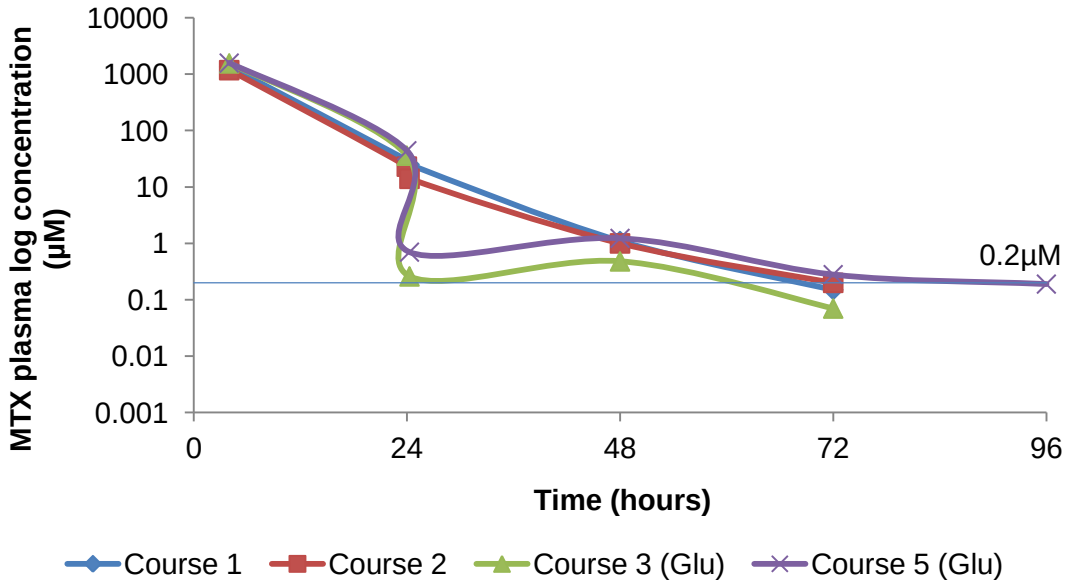


Figure 10.11: PATIENT GLU1-11

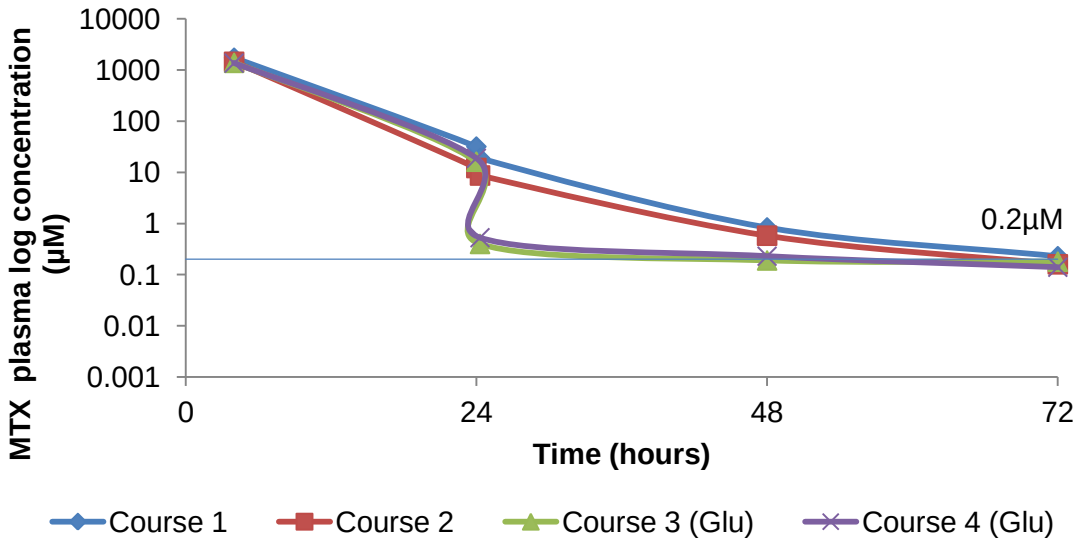


Figure 10.12: PATIENT GLU1-12

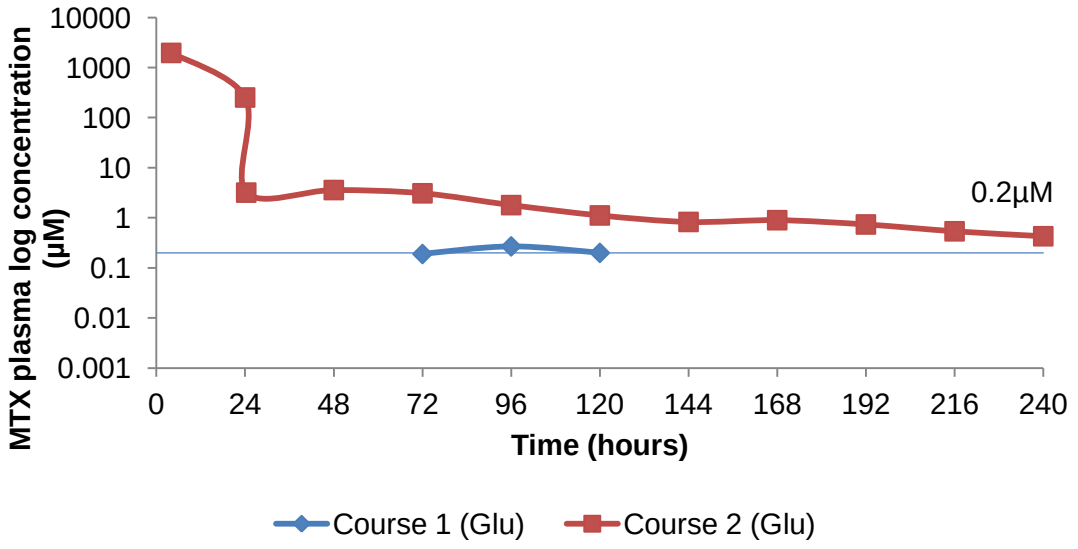


Figure 10.13: PATIENT GLU1-13

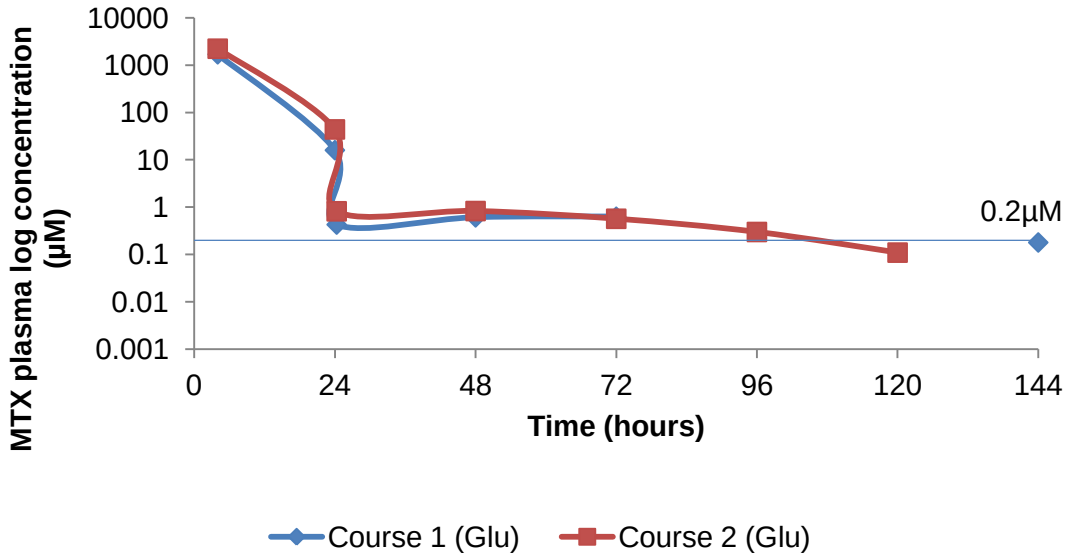


Figure 10.6: PATIENT GLU1-14

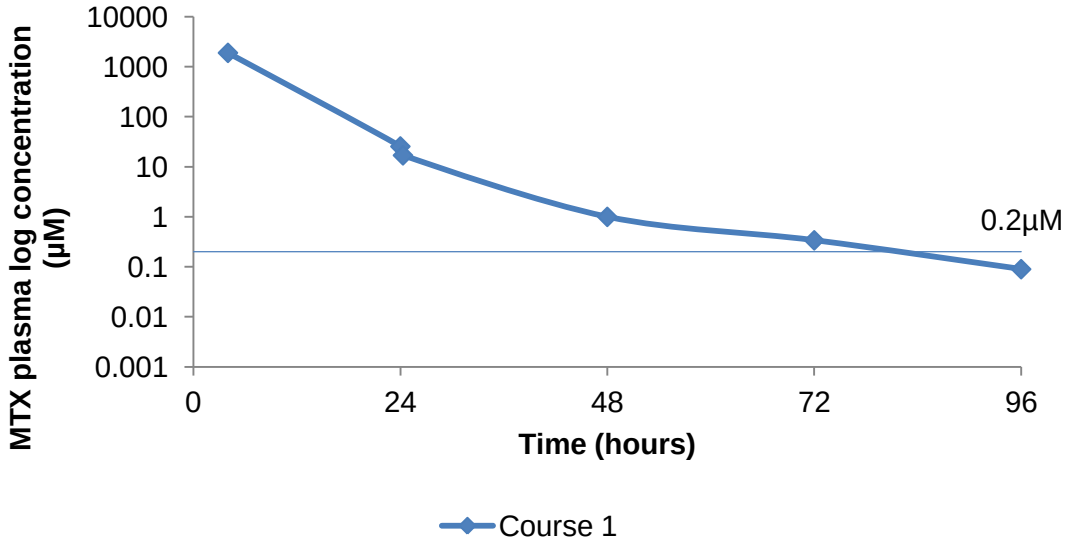


Figure 10.15: PATIENT GLU1-15

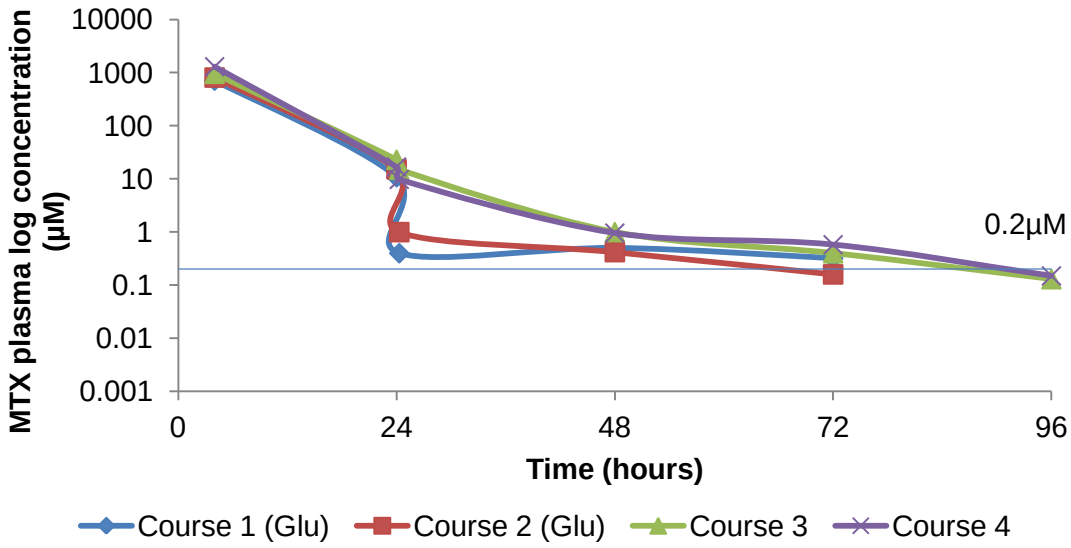
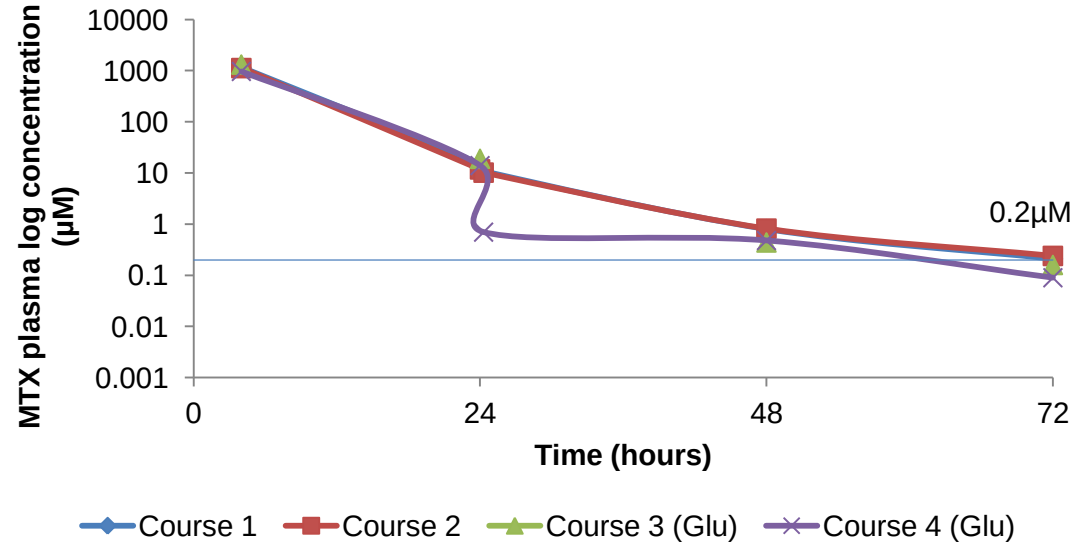


Figure 10.16: PATIENT GLU1-16



Drug exposure during MTX courses was expressed as $AUC_{0\text{-}T}$ (area under the curve from the start of MTX infusion to the time the last blood sample was collected) and calculated using the trapezoidal rule by the summation of integrals defined by the

drug concentrations and the respective time spans over which they had been obtained (Table 45). AUC_{0-24} (area under the curve from the start of MTX infusion to 24 hours) and AUC_{24-72} (from 24 hours to 72 hours after the start of the infusion) were also calculated for treatment courses given with and without glucarpidase (Table 46).

The median AUC_{0-T} in MTX courses given with both regimens (i.e. folinic acid alone and glucarpidase/folinic acid rescue) was 15101 µMh (range, 5874 to 27192 µMh). There was very little difference in the AUC observed in MTX courses given with folinic acid rescue and MTX courses given with glucarpidase/folinic acid rescue. The median AUC_{0-T} in MTX courses given with folinic acid rescue was 14653 µMh (range, 6462 to 23290 µMh) whereas the median AUC_{0-T} in MTX courses given with glucarpidase/folinic acid rescue was 15233 µMh (range, 5874 to 27192 µMh) (Table 29). This is because AUC_{0-24} represents the greatest proportion (98%) of the AUC_{0-T}, and reflects the exposure to MTX prior to glucarpidase administration.

Table 45: AUC AND PEAK [MTX] (Cmax) PER PATIENT AND COURSE OF MTX

GLU 1 patient	COURSE	AUC(0-T)	AUC(0-24)	AUC(24-72)	Cmax	GLUC 1: yes 0: no
01	1	12267.8467	12257.41666	4.992466666	1018	1
	2	15741.928	15714.81466	9.739999999	1303.84	1
	3	14046.647	13872.79	159.8198333	1144.8	0
	4	17431.071	17388.20333	40.47766666	1439.6	0
02	1	15901.8206	15709.2165	179.516875	1298.42	0
	2	11006.9851	10845.42417	142.627625	896.2	0
	3	15454.7773	15452.03614	0	1276.4	1
	4	11967.8043	11962.17644	1.224166667	985.6	1
03	1	20069.4279	20061.505	0.69375	1673.68	1
	2	15233.5173	15197.96583	6.827499999	1228	1
	3	16926.1923	2740.074	249.3433334	1370	0
04	1	10265.4515	10259.52471	1.294166666	845.4	1
	2	14755.2037	14686.24536	18.01366666	1179.4	1

GLU 1 patient	COURSE	AUC(0-T)	AUC(0-24)	AUC(24-72)	Cmax	GLUC 1: yes 0: no
05	1	N/A	N/A	N/A	1400	0
06	1	11270.0468	11170.28242	90.45375	922.46	0
	2	11259.5272	11219.55403	38.8245	932.44	0
	3	9483.83171	9468.65	3.195541666	983.4	1
	4	15667.9637	15657.12118	8.52625	1294.72	1
07	1	14678.5806	14596.5315	12.87716667	1176.4	1
	2	17252.0646	17177.1065	16.11825	1319.2	1
	3	N/A	N/A	N/A	N/A	0
	4	20997.3277	19441.336	1259.293334	1638	0
08	1	7090.48285	7080.879074	1.8693	587.18	1
	2	5874.24277	5837.986	3.101666667	524.42	1
	3	8347.15811	8285.060775	45.80154167	723.76	0
	4	6462.9393	6402.937775	57.33802501	535.46	0
09	1	16200.3296	16114.57943	79.13342501	1337.72	0
	2	16451.6036	16338.90407	81.32	1355.58	0
	3	14692.5626	14687.46375	1.982166667	1219	1
	4	16422.7749	16338.90407	81.32	1355.58	1
10	1	16910.7263	16557.87062	331.6176666	1355.9	0
	2	14653.0678	14400.5525	191.4026666	1181.6	0
	3	19071.2825	19043.6565	15.333	1558	1
	4	19406.9292	19353.05416	40.86299999	1577.44	1
11	1	21195.2204	20923.28834	240.4649167	1741.6	0
	2	17341.0917	17187.3505	113.6346667	1452.6	0
	3	16718.1677	16689.06833	11.225	1387	1
	4	15560.8803	15529.65417	12.60566667	1370	1
12	1	N/A	N/A	N/A	N/A	1
	2	26550.1785	26140.64678	161.3121666	1968.44	1
13	1	20613.2894	20525.21375	26.17375	1698	1
	2	27192.6615	27135.02166	34.37866667	2225.8	1
14	1	23290.8083	23060.9344	216.6788334	1901.72	0
15	1	8309.7118	8266.64	19.9214	709.92	1
	2	9273.61223	9204.696665	22.57765	811.84	1
	3	11198.1271	10953.1253	195.535	954.68	0
	4	14190.3097	14014.02	128.8066666	1296.08	0
16	1	13894.5843	13726.51	142.3086667	1155.8	0

GLU 1 patient	COURSE	AUC(0-T)	AUC(0-24)	AUC(24-72)	Cmax	GLUC 1: yes 0: no
	2	13446.4534	13263.64666	136.695	1127.1	0
	3	14968.7619	14926.75	11.9301075	1321.2	1
	4	11408.36	11380.115	20.06583334	967	1
Median		15101.1396	14807.1068	36.6016	1285.56	
Mean		14966.9653	14546.8022	97.2761	1235.9662	
Minimum		5874.2428	2740.074	0	524.42	
Maximum		27192.6615	27135.0217	1759.2933	2225.8	

Table 46: MEDIAN, MEAN, MINIMUM AND MAXIMUM AUC AND MTX Cmax FOR BOTH RESCUE REGIMENS AND EACH RESCUE REGIMEN (PLACEBO/FOLINIC ACID AND GLUCARPIDASE/FOLINIC ACID)

	AUC(0-T)			AUC(0-24)			AUC(24-72)			CMAX		
	Both rescue regimens	**Placebo & FA**	**Glu & FA**	**Both rescue regimens**	**Placebo & FA**	**Glu & FA**	**Both rescue regimens**	**Placebo & FA**	**Glu & FA**	**Both rescue regimens**	**Placebo & FA**	**Glu & FA**
Median	15101.1396	14653.0678	15233.5173	14807.1068	14014.02	15197.9658	36.6016	142.3086	11.9301	1285.56	1296.08	1276.4
Mean	14966.9653	14877.2398	15036.7517	14546.8022	13981.6981	14986.3275	97.2761	196.2426	20.3023	1235.9662	1226.7390	1243.1429
Minimum	5874.2428	6462.9393	5874.2427	2740.074	2740.074	5837.986	0	38.8245	0	524.42	535.46	524.42
Maximum	27192.6615	23290.8083	27192.6615	27135.0217	23060.9344	27135.0216	1759.2933	1259.2933	161.3122	2225.8	1901.72	2225.8

(AUC(0-T): area under curve from 0 hours to T=last blood sample collected, AUC(0-24): area under curve from 0 hours to 24 hours, AUC (24-72): area under curve from 24 hours to 72 hours)

To assess whether there was a difference in AUC_{0-24}, AUC_{24-72} and AUC_{0-T} between treatment courses given with and without glucarpidase, an ANOVA model that accounted for the crossover design with repeated measures was fitted. In addition to the treatment term (i.e. with or without glucarpidase), carryover, sequence and period effects were included. The model was fitted to each of the 3 outcomes AUC_{0-24}, AUC_{24-72} and AUC_{0-T}.

The use of glucardpidase was estimated to reduce the AUC_{0-T} value by an average of (-)1060.3 µMh (95%CI: -2671.1, 550.6), although this was not statistically significant (p=0.188). For AUC_{0-24} the treatment effect was not significant either (p=0.667), estimated as 471.4 µMh (95%CI: -1750.9, 2693.6). However, the effect of glucardpidase on AUC_{24-72} was highly significant (p=0.0008), reducing the AUC by an average of (-)206.1 µMh (95% CI: -317.8, -94.4). In all 3 models there was no evidence at all of any carryover, sequence or period effects.

The correlation between MTX C_{max} and AUC_{0-T} was studied. For all MTX courses (either with standard folinic acid or glucarpidase/folinic acid rescue) there was strong and highly significant correlation between MTX C_{max} and AUC_{0-T} and less strong correlation between MTX C_{max} and AUC_{24-72}, although the latter was not statistically significant.

In particular, for MTX courses with standard folinic acid rescue, there was strong and highly significant correlation between C_{max} and AUC_{0-T} ($r=0.994$; $p<0.0001$), adjusting for the clustered data (repeated measures). There was less strong correlation between C_{max} and AUC_{24-72} although this was not statistically significant ($r=0.412$; $p=0.158$).

Similarly, for MTX courses given with glucarpidase/folinic acid rescue, there was strong and highly significant correlation between C_{max} and AUC_{0-T} ($r=0.989$;

p<0.0001). There was less strong correlation between Cmax and AUC_{24-72} although this was not statistically significant (r=0.486; p=0.118).

The within-subject and inter-individual variation in MTX Cmax and AUC values was examined using the intra-class correlation (ICC) coefficient which divides the total variability in the data in that between patients, and that between repeat measurements of the same patient. The ICC was calculated from a multilevel linear regression model, with the MTX course number and the treatment arm accounted for. The variation was measured after taking account of any effects caused by course number and treatment with or without glucarpidase. The AUC values from 24-72 hours were found to have a highly skewed distribution, and thus the analysis of this outcome was performed on the log scale.

The results are shown in Table 47. There was a reasonably high ICC for AUC_{0-t}, suggesting that 73% of the total variation in AUC values can be put down to between subject variation, with the smaller amount down to variation between repeat measurements. However, this is still a reasonable amount of within-subject variation. The results for MTX Cmax, showed a similar amount of inter-individual variation to those for the AUC_{0-t} values. In particular 72% of the total variation in MTX Cmax values can be put down to between subject variation, with the smaller amount down to variation between repeat measurements. There was more inter-individual variation for the AUC_{0-24} and AUC_{24-72} measurements, where the ICC values were lower. For AUC_{0-24} the difference between repeat measurements of the same subjects was of a similar size to the differences between individuals. For AUC_{24-72} the within-individual variation was the major source of variation.

Table 47: WITHIN-SUBJECT AND INTER-INDIVIDUAL VARIATION IN MTX Cmax AND AUC VALUES

Outcome	Between-subject SD	Within-subject SD	ICC
AUC 0-t (µMh)	3837	2334	0.73
AUC 0-24 (µMh)	3411	3242	0.52
AUC 24-72 (µMh) (*)	0.41	0.84	0.20
MTX Cmax (µM)	305	191	0.72

(*) Variable analysed on the log scale

5.6. GLUCARPIDASE RELATED TOXICITY

In the previously described PD, NCI and Berlin Studies 52 glucarpidase-related adverse events (AEs) were reported in 26/318 patients (Section 1.4.3). Glucarpidase is a recombinant bacterial protein and it therefore has the potential to induce an immune response. Allergic reaction was the most common glucarpidase-related AE, occurring in 45 of patients treated. The most common non-allergic AE considered related to treatment with glucarpidase were paraesthesia (0.6% of patients), nausea (0.6% of patients) and hypotension (0.6% of patients). Two serious adverse events (SAEs) hypertension and arrhythmia that may have been related to glucarpidase administration were also reported.

In the GLU clinical trial, a total of 51 doses of HD-MTX were administered. Glucarpidase rescue was delivered following twenty seven of those doses (27/51, 53% of the doses). A further dose of glucarpidase was given to the GLU 1-05 patient as emergency treatment post his first dose of HD-MTX as he developed delayed MTX elimination due to impaired renal function. His treatment arm was unblinded as per study protocol; he was found to have been randomised to treatment arm B and therefore had not received glucarpidase as part of the study treatment. A total of 28 doses of glucarpidase were administered in 15 patients. The patient GLU 1-14,

randomised to treatment arm B, did not receive any glucarpidase as his treatment was discontinued early, due to knee effusion, following the first dose of HD-MTX.

AEs related to glucarpidase were recorded in the Glucarpidase Adverse Event form (part of the CRF) and followed-up until their resolution. AEs not related to glucarpidase were followed until the end of study, i.e. day 21 of cycle 2. No adverse events related to glucarpidase occurred to any of the GLU 1 participants.

5.7. ECONOMIC IMPACT OF USING GLUCARPIDASE VERSUS STANDARD RESCUE

In order to investigate the economic impact of using glucarpidase versus standard rescue, we reviewed the number of days required in hospital and total folinic acid dose per course. 28 MTX courses were given with glucarpidase and folinic acid (glu/FA) and 23 were given with folinic acid alone (FA).

Folinic acid requirements and length of hospital stay per patient and course are shown in Table 48.

The median and average folinic acid dose required in glu/FA courses was 189.5mg/m^2 and 478mg/m^2 respectively (range 117-5454 mg/m^2), whereas the median and average folinic acid dose required in FA courses was 192mg/m^2 and 336mg/m^2 respectively (range 116-1371 mg/m^2).

The median and average length of hospital stay per patient and course was 4 and 4.9 days respectively (range 4-13 days). The median and average length of patient's hospital stay per glu/FA course was 4 and 5.04 days respectively (range 4-13 days), whereas the median and average length of patient's hospital stay per FA course was 4 and 4.77 days respectively (range 4-10 days).

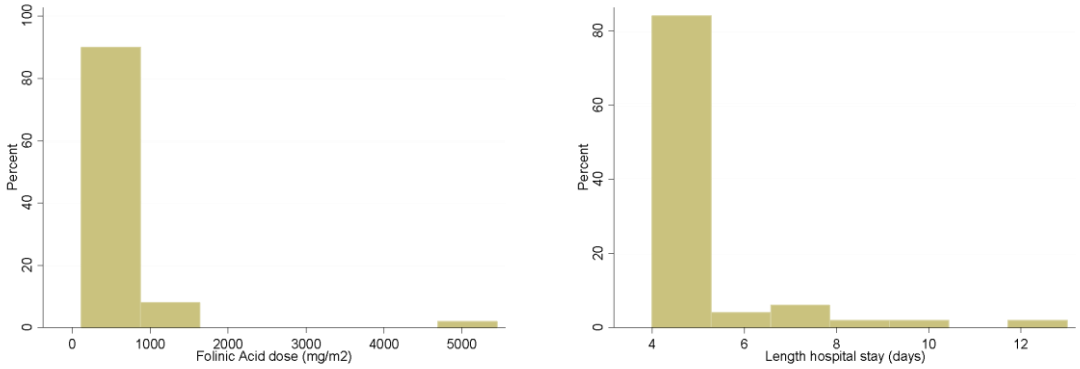
Table 48: FOLINIC ACID REQUIREMENTS PER PATIENT AND COURSE

GLU study patient	MTX courses (glu/FA courses highlighted)	FA dose per course	Days in hospital
01	Course 1	119 mg/m^2	4
	Course 2	119 mg/m^2	4
	Course 3	146 mg/m^2	4
	Course 4	116 mg/m^2	4
02	Course 1	199 mg/m^2	4
	Course 2	143 mg/m^2	4
	Course 3	250 mg/m^2	4
	Course 4	180 mg/m^2	4
03	Course 1	167 mg/m^2	4
	Course 2	466 mg/m^2	4
	Course 3	764 mg/m^2	7
04	Course 1	136 mg/m^2	4
	Course 2	1168 mg/m^2	10
05	Course 1	983 mg/m^2	10
06	Course 1	192 mg/m^2	4
	Course 2	199 mg/m^2	4
	Course 3	196 mg/m^2	4
	Course 4	194 mg/m^2	5
07	Course 1	722 mg/m^2	5
	Course 2	944 mg/m^2	5
	Course 3	1371 mg/m^2	7
	Course 4	967 mg/m^2	6
08	Course 1	131 mg/m^2	4
	Course 2	117 mg/m^2	4
	Course 3	191 mg/m^2	4
	Course 4	171 mg/m^2	4
09	Course 1	153 mg/m^2	4
	Course 2	153 mg/m^2	4
	Course 3	153 mg/m^2	4
	Course 4	134 mg/m^2	4
10	Course 1	209 mg/m^2	4
	Course 2	177 mg/m^2	4
	Course 3	243 mg/m^2	4
	Course 4	462 mg/m^2	5

GLU study patient	MTX courses (glu/FA courses highlighted)	FA dose per course	Days in hospital
11	Course 1	208 mg/m^2	4
	Course 2	158 mg/m^2	4
	Course 3	156 mg/m^2	4
	Course 4	153 mg/m^2	4
12	Course 1	326 mg/m^2	7
	Course 2	5454 mg/m^2	13
13	Course 1	225 mg/m^2	8
	Course 2	560 mg/m^2	6
14	Course 1	300 mg/m^2	5
15	Course 1	154 mg/m^2	4
	Course 2	248 mg/m^2	4
	Course 3	253 mg/m^2	5
	Course 4	320 mg/m^2	5
16	Course 1	129 mg/m^2	4
	Course 2	144 mg/m^2	4
	Course 3	165 mg/m^2	4
	Course 4	141 mg/m^2	4

We compared the effect of glucarpidase on the dose of folinic acid and length of hospital stay in cycles given with and without glucarpidase. Both outcomes measurements were found to have highly skewed distribution (Figure 11). Since it was not possible to normalize it by log transformation (or any other transformation) the outcome measurements were assumed to have a Poisson distribution.

Figure 11: GRAPHICAL ILLUSTRATION OF THE DISTRIBUTION OF DATA RELATED TO FOLINIC ACID DOSE AND LENGTH OF HOSPITAL STAY



Additionally, as the study had a crossover design, it was necessary to take account of the repeat measurements for each subject in the analysis. This was done using multilevel statistical methods, assuming that individual measurements were nested within patients. To allow for both the data structure and distribution of values, multilevel Poisson regression was used for the analysis.

The multilevel Poisson regression analyses suggested that there was some evidence of a carryover of the effects of glucarpidase on folinic acid dose ($p<0.001$), and to a less degree the length of hospital stay ($p=0.05$). The presence of such interaction suggests that the effects of glucarpidase were different in the two treatment cycles (i.e. different effects of glucarpidase when it was given in the first treatment cycle to when it was given in the second) and a carryover of the effects of glucarpidase to the second treatment cycle.

Two different approaches were used to examine the effect of glucarpidase on the dose of folinic acid and length of hospital stay: a) data from both treatment cycles were analysed assuming that the period by treatment effect was not present, and b)

only the results from the first treatment cycle were analysed as these were unaffected by carryover. Given the significant period by treatment interaction, the latter analysis should be viewed with some caution.

As shown in Table 49, there was no strong evidence of an effect of glucarpidase upon length of hospital stay using either analysis approach. Nevertheless, there was some slight evidence that glucarpidase increased the length of stay when only data from the first cycle was analysed, although this effect was not quite statistically significant.

There was a significant effect of glucarpidase upon the dose of folinic acid using both analysis approaches. Using the data from both treatment cycles, glucarpidase was found to reduce the folinic acid dose, on average by around 13%. However, if the analysis is restricted to the first treatment cycle, then glucarpidase is associated with 3 fold higher folinic acid dose.

This latter result highlighted that calculating the total folinic acid dose per course was not a good indicator of the economic impact of using glucarpidase versus standard rescue. GLU 1 participants had their dose of folinic acid adjusted based upon the pre-glucarpidase/placebo plasma MTX levels. The dose of folinic acid was maintained unchanged for 48 hours after glucarpidase/placebo. Hence, the total folinic acid dose did not reflect the effect of glucarpidase on MTX plasma levels at 48 hours and 72 hours.

Table 49: EFFECT OF GLUCARPIDASE ON FOLINIC ACID DOSE AND LENGTH OF HOSPITAL STAY.

Outcome	Data used in analysis	Ratio* (95% CI)	P-value
Length of hospital stay	Both periods	1.11 (0.86, 1.44)	0.42
	1st cycle only	1.37 (0.98, 1.94)	0.06

Folinic acid dose	Both periods	0.87 (0.84, 0.91)	<0.001
	1st cycle only	3.63 (1.68, 7.84)	0.001

*The effect of glucarpidase on the dose of folinic acid and length of hospital stay is reported in the form of ratios. This is given as the ratio of the outcome value when glucarpidase was given compared to when it wasn't. A ratio of above 1 suggests higher values of the outcome when glucarpidase was given, whilst a ratio below 1 would suggest lower values.

5.8. ANTI-GLUCARPIDASE ANTIBODY RESPONSE FOLLOWING GLUCARPIDASE ADMINISTRATION

Blood samples for anti-glucarpidase antibody assessment and enzyme neutralisation assay were collected as part of the GLU 1 study on days 1, 8 and 15 of cycle 1 and days 1, 8, 15 and 30 of cycle 2 and at 3 and 6 months after starting cycle 2 if the sample on day 30 of cycle 2 was positive.

Millipore BioPharma Services analysed all collected blood samples for anti-glucarpidase antibodies using Bridging ELISA. The interim analytical report follows in Appendix 13.

Covance Laboratories Ltd analysed all bloods samples with positive antiglucarpidase antibody response for enzyme neutralisation assay, using a UV spectrophotometric procedure. The draft report follows in Appendix 14.

The results of the anti-glucarpidase antibody response are shown on Table 50. 14 patients had more than one glucarpidase doses although no patients experienced allergic reaction following the second glucarpidase dose. Patient GLU1-05 required emergency treatment with glucarpidase (single dose) and had no blood samples assessed for anti-glucarpidase antibodies. Patient GLU1-14 only had one methotrexate course on the study and did not receive glucarpidase; therefore no blood samples were assessed for anti-glucarpidase antibodies for this patient.

Table 50: ANTIGLUCARPIDASE IgG RESPONSE PER GLU 1 PATIENT AND TREATMENT COURSE

Antiglucarpidase IgG response (positive/negative), samples taken post glucarpidase highlighted (processed by Millpore BioPharma Services, complete report in Appendix 13)																
Patient number	**01**	**02**	**03**	**04**	**05**	**06**	**07**	**08**	**09**	**10**	**11**	**12**	**13**	**14**	**15**	**16**
Received courses of methotrexate (received doses of glucarpidase)	**4 (2)**	**4 (2)**	**3(2)**	**2 (2)**	**1 (1)**	**4 (2)**	**4 (2)**	**4 (2)**	**4 (2)**	**4 (2)**	**4 (2)**	**2 (2)**	**2 (2)**	**1(1)**	**4 (2)**	**4 (2)**
Cycle 1																
Day 1	neg	neg	neg	neg	No samples processed	neg	neg	neg	neg	neg	neg	neg	neg	No sample processed	neg	neg
Day 8	neg	neg	neg	neg		neg	neg	neg	neg	neg	neg	neg	neg		neg	neg
Day 15	neg	neg	neg	neg		neg	neg	neg	neg	neg	neg	neg	-		neg	neg
Cycle 2																
Day 1	neg	neg	neg	-	Patient received glucarpidase as emergency treatment 72 hours post starting methotrexate	neg	pos	pos	-	neg	neg	-	-	No glucarpidase given	neg	neg
Day 8	neg	neg	-	-		neg	neg	neg	neg	neg	neg	-	-		neg	pos
Day 15	neg	neg	neg	neg on day 30 of cycle 1		neg	pos	neg	pos	neg	neg	neg on day 30 of cycle 1	neg on day 30 of cycle 1		pos	pos
Day 30	-	-	-	-		neg	pos day 23 of cycle 2	neg	neg	-	neg	-	-		pos on day 30 of cycle 2	-
Day 90	-	-	neg on day 120	-		neg	-	neg	neg	neg	neg	-	-		-	-

Antiglucarpidase IgG response (positive/negative), samples taken post glucarpidase highlighted
(processed by Millpore BioPharma Services, complete report in Appendix 13)

Patient number	**01**	**02**	**03**	**04**	**05**	**06**	**07**	**08**	**09**	**10**	**11**	**12**	**13**	**14**	**15**	**16**
Received courses of methotrexate (received doses of glucarpidase)	**4 (2)**	**4 (2)**	**3(2)**	**2 (2)**	**1 (1)**	**4 (2)**	**4 (2)**	**4 (2)**	**4 (2)**	**4 (2)**	**4 (2)**	**2 (2)**	**2 (2)**	**1(1)**	**4 (2)**	**4 (2)**
Day 180	neg on day 120	pos neutralising reactive	neg	-		-	neg	neg	-	-	-	-	-		-	-

Positive antibody response was found in 43% (6/14) of patients. Patient **GLU1-02** had positive response at a concentration of 2526.2 ng/mL on day 180 of cycle 2, having received 2 doses of glucarpidase in cycle 2 of trial treatment. Patient **GLU1-07** had positive response on days 1, 15 and 30 of cycle 2 having received glucarpidase in cycle 1. The measured antibody equivalent concentrations were 526.7 ng/mL, 172.4 ng/mL and 123.9 ng/mL on day 1, 15 and 23, respectively. His response was interestingly negative on day 8 of cycle 2 and also negative on the follow-up sample on day 180. Patient **GLU1-08** had positive response on day 1 of cycle 2 having received glucarpidase in cycle 1 of his treatment. His response was negative in the rest of blood samples on days 8, 15, 30, 90 and 180 of cycle 2. Patient **GLU1-09** had positive response on day 15 of cycle 2, having received glucarpidase in cycle 2 of his treatment. His response became negative on days 30 and 90 of cycle 2. Patient **GLU1-15** had positive response on days 15 and 30 of cycle 2, having received glucarpidase in cycle 1 of his treatment. There are no follow-up blood samples. Patient **GLU1-16** had positive response on days 8 and 15 of cycle 2, having received glucarpidase in cycle 2 of his treatment. Similarly no follow-up blood samples have been analysed. The positive samples from patients GLU1-08, GLU1-09, GLU1-15 and GLU1-16 gave low measured antibody equivalent concentrations that were below the limit of quantitation of the assay (<62.5 ng/mL). Patients GLU1-01, GLU1-03, GLU1-04, GLU1-06, GLU1-10, GLU1-11, GLU1-12 and GLU1-13 did not develop any anti-glucarpidase antibodies.

It is worth noting that no patients became antiglucarpidase antibody positive after a single dose of glucarpidase.

In total, 61 blood samples were taken after glucarpidase administration and analysed for antiglucarpidase IgG response. Of those, 16% (10/61) were found to be positive but

only 10% (1/10) of the samples with positive response were found to be neutralising reactive (Table 51). The activity of glucarpidase was inhibited in 17% (1/6) of patients with positive antibody response.

Table 51: NEUTRALISING ANTIGLUCARPIDASE ANTIBODIES

Neutralising antiglucarpidase antibodies
(processed by Covance Laboratories Ltd, draft report in Appendix 14)

Patient Number	Time Point	Antiglucarpidase IgG Response (positive/negative)	Neutralising Reactive (yes/no)
GLU1-02	Day 180 of cycle 2	positive	yes
GLU1-07	Day 1 of cycle 2	positive	no
	Day 15 of cycle 2	positive	no
	Day 23 of cycle 2	positive	no
GLU1-08	Day 1 of cycle 2	positive	no
GLU1-09	Day 15 of cycle 2	positive	no
GLU1-15	Day 15 of cycle 2	positive	no
	Day 30 of cycle 2	positive	no
GLU1-16	Day 8 of cycle 2	positive	no
	Day 15 of cycle 2	positive	no

5.9. ASSESSMENT OF QUALITY OF LIFE IN TRIAL PARTICIPANTS

For study participants aged 15 and under, Quality of Life was assessed using the "PedsQL 3.0 Cancer Module Acute Version questionnaire" (Appendix 10: Quality of Life questionnaires), whereas for study participants aged 16 and over at registration, quality of life was assessed using the European Organisation for Research and Treatment of Cancer (EORTC) QLQ-C30 version 3 (Aaronson et al. 1993; Fayers et al 2001) and the Functional Assessment of Cancer Therapy Scale-General (FACT-G) (Cella et al. 1993) questionnaires (Appendix 10: Quality of Life questionnaires).

Patient compliance with questionnaire completion was high and more than 94% of the scheduled questionnaires were returned (Table 52).

Table 52: PATIENT COMPLIANCE WITH COMPLETION OF QUALITY OF LIFE QUESTIONNAIRES

GLU 1 Patient's ID	QLQ-C30 or PedsQL (completed/requested questionnaires)	FACT-G return (completed/requested questionnaires)
1	6/6	6/6
2	6/6	6/6
3	5/5	5/5
4	3/3	3/3
5	1/1	1/1
6	6/6	N/A
7	5/6	5/6
8	5/6	5/6
9	6/6	N/A
10	6/6	6/6
11	6/6	6/6
12	3/3	3/3
13	2/3	2/3
14	1/1	1/1
15	5/6	5/6
16	6/6	6/6
Overall completion	72/76 **(95%)**	60/64 **(94%)**

The responses of the quality of life questionnaires were not formally analysed at this time point since the trial is still ongoing. Following discussions with national experts in this subject, it was felt that the Oral Mucositis Weekly Questionnaires should be instead reviewed since mucositis seemed to have been the main toxicity that could have

compromised the quality of life of trial participants. The results of the Oral Mucositis Weekly Questionnaires are described in Section 5.4.

5.10. GLU 1 INTERIM ANALYSIS: INDEPENDENT DATA MONITORING COMMITTEE (IDMC) REPORT

The IDMC reviewed the data of the trial after completion of trial treatment of the first 16 participants (Appendix 15: GLU 1 interim analysis, IDMC report).

Analysis of the primary outcome did not show a statistically significant benefit of glucarpidase with $P<0.005$. Treatment with glucarpidase and folinic acid was not found to be significantly worse than standard treatment using an one-sided test with $P<0.05$.

Recruitment rate was lower than expected. From June 2007 to May 2009 16 patients were recruited in over 22 months, while the expected accrual was 10-15 patients per year.

The drop-out rate was significantly higher than expected with only 11 out of 16 participants contributing to complete data set. The expected drop-out rate was 5% while the actual drop-out rate has been 31%. The trial sample size would need to be re-calculated assuming that the drop-out rate will remain around 30%.

There were no AEs related to the IMP.

Two SAEs were reported between the opening of the trial and the interim analysis, both unrelated to the IMP. The reported SAEs have not been considered to be a safety issue that might alter the current benefit-risk assessment.

SAE 1 was reported on 18 October 07 regarding the patient GLU1-05; Short description of the SAE 1: acute renal impairment after treatment with high-dose methoterexate;

reason for seriousness: prolongation of patient's hospitalization; causality assessment regarding the IMP: unrelated; causality assessment regarding protocol treatment apart from the IMP: related to MTX; resolution: 27 November 2007.

SAE 2 was reported on 27 March 08 regarding the patient GLU1-08; Short description of the SAE 2: right sided tension pneumothorax caused by patient's extensive pulmonary disease; reason for seriousness: life threatening required admission to Intensive Care Unit; causality assessment regarding protocol treatment apart from the IMP: unrelated; causality assessment regarding the IMP: unrelated; resolution: 09 April 2008

The expiry date on the Voraxaze trial stock (BN 2090601) was March 2009 (36 months post manufacture date). At the point of the GLU 1 interim analysis, there was new stability data to support extension of the IMP shelf-life to 48 months. A substantial amendment to the protocol was planned in order to increase the IMP shelf-life to 48 months (new expiry date: March 2010).

Another issue that was addressed at the interim analysis was the blinding of the study. In treatment cycles where patients received glucarpidase, both MTX and DAMPA were identified and measured by HPLC. In treatment cycles where patients received placebo, only MTX was identified and measured by HPLC. DAMPA was not present. Therefore it was obvious to the investigators from the HPLC findings when patients had received glucarpidase and when they had placebo; treatment was therefore not blind to the investigators.

The IDMC suggested that the design of the study needed to be changed from "double-blind randomised" to "unblind randomised". In order to minimize any bias both "Day 15"

assessments needed to be performed by the on duty registrars and not the investigators.

Also, the timings of the blood samples for anti-glucarpidase antibody assessment and enzyme neutralisation assay were reviewed at the interim analysis.

Blood samples for anti-glucarpidase antibody qualitative assessment and enzyme neutralisation assay were collected on days 0, 1, 8 and 15 of cycle 1 and days 1, 8, 15 and 30 of cycle 2. If anti-glucarpidase antibodies were present on day 30 of cycle 2, patients were followed up for a further blood test, at 3 and 6 months after starting cycle 2. However, there was a limited supply of one of the reagents used in the anti-glucarpidase antibody qualitative assay. In order to get the most use out of this reagent, 30 samples were analysed per assay run. This meant that sometimes it was not possible to have the results for the first time points (days 0, 1, 8 and 15 of cycle 1 and days 1, 8, 15 and 30 of cycle 2) and two further blood samples (at 3 and 6 months after starting cycle 2) were collected.

In view of that, the IDMC suggested that the trial protocol needed to be amended so that blood samples for anti-glucarpidase antibody qualitative assessment and enzyme neutralisation assay were collected on days 0, 1, 8 and 15 of cycle 1 and days 1, 8, 15 and 30 of cycle 2 and at 3 and 6 months after starting cycle 2.

Further to the GLU 1 interim analysis, a protocol amendment was planned to cover the following issues:

- Extension of IMP shelf life
- Amendment of the design of the study to unblind randomized

- Amendment of time points for collection of blood samples for anti-glucarpidase antibody assessment and enzyme neutralisation assay
- Administrative amendment: sponsor contact details for SAE submission
- Administrative amendment: updated arrangements for anti-glucarpidase antibody qualitative assessment and enzyme neutralisation analysis

6. DISCUSSION

6.1. MTX IN OSTEOSARCOMA TREATMENT

HD-MTX (12 g/m^2) with folinic acid rescue is one of the standard chemotherapy agents used in the management of patients with osteosarcoma and approximately two thirds of newly diagnosed patients with non-metastatic resectable primary osteosarcoma can achieve prolonged disease free survival with multi-agent chemotherapy regimens containing doxorubicin, cisplatin and HD-MTX (Meyers et al. 2008).

In early studies, MTX-related severe toxicity occurred in approximately 10% of patients and there was a 6% mortality rate (Ahmad et al. 1978). The introduction of pre-treatment hydration, alkalinisation of urine, routine monitoring of [MTX] and pharmacokinetically adjusted folinic acid rescue has decreased the incidence of severe and life threatening MTX-related toxicity to less than 1% (Jüergens et al. 1983; Allegra et al. 1990; Buchen et al. 2005).

However, despite currently used supportive measures, MTX related toxicity still occurs, resulting in increased morbidity, suboptimal chemotherapy doses, delays in subsequent chemotherapy administration and potentially reduced treatment efficacy.

It is evident in the literature that the fewer the MTX-related delays in osteosarcoma treatment, the better the outcome of those patients (Frei at al., 1980; French Tumour Study Group, 1988; Bacci et al., 2001; Delepine et al., 1996).

Nevertheless, there is currently no available literature on the incidence of delays in subsequent chemotherapy due to MTX toxicity in patients with osteosarcoma. Information is only available on the incidence of delayed MTX elimination in patients

with osteosarcoma which may then result in subsequent chemotherapy delays (Saeter et al., 1991; Winkler et al., 1988; Bacci et al., 2006; Zelcer et al., 2005).

6.2. DELAYS IN CHEMOTHERAPY ADMINISTRATION DUE TO MTX TOXICITY

In order to establish the incidence of chemotherapy delays due to MTX toxicity we reviewed the management of 56 osteosarcoma patients (aged 6 to 47 years, median age 20 years), treated with HD-MTX (12 g/m^2) at our institution between January 2004 and January 2005. MTX-induced toxicity resulted in delays in 47% of subsequent chemotherapy cycles in this cohort of patients. These findings were rather unexpected and were presented at the 38th Congress of International Society of Paediatric Oncology (SIOP) and the 12th annual meeting of the Connective Tissue Oncology Society (CTOS) (Appendix 16: SIOP 2006 poster and CTOS 2006 abstract). The feedback from the audience included surprise at this high incidence, though not supported by any similar data on MTX-induced treatment delays from their institutions. Valuable comments on the potential skew of our results due to the inclusion of patients aged ≥ 40 years led to a further audit where the incidence of chemotherapy delays due to MTX toxicity in different age groups (< 40 years and ≥ 40 years) in our institution was examined. However, no major difference in the delays in subsequent chemotherapy cycles between the two groups was seen. In the ≥ 40 years age group (median age 44.5 years) MTX-induced toxicity resulted in delays in 58.5% of subsequent chemotherapy cycles. In the < 40 years age group (median age 15 years) MTX-induced toxicity resulted in delays in 52.2% of subsequent chemotherapy cycles.

The main limitation in both audits was the subjectivity in clinical thresholds for initiating treatment. Although haematological and biochemical criteria for initiating treatment were objective (neutrophils ≥ 1x10^9/L and platelets ≥ 100x10^9/L; GFR ≥ 70 ml/min/1.73m^2;

bilirubin ≤ 1.5 x ULN, ALT ≤ 1.5 x ULN, albumin > LLN; urinary pH > 7.0 immediately prior to MTX and good urine output), assessment of patient's clinical status including mucositis was subjective and may have been influenced by the experience of the clinician. Less experienced clinicians may have been more reluctant to decide that a patient is fit to proceed with further chemotherapy whereas more experienced clinicians may have been more permissive.

It would have been particularly informative to be able to compare our findings against similar institutions in the UK and Europe. However, such information is not available up-to-date.

Both our audits showed that around half of subsequent chemotherapy cycles in the treatment of osteosarcoma were delayed due to MTX toxicity despite current supportive measures. Since fewer chemotherapy delays in osteosarcoma treatment are associated with better outcome, it was felt imperative to examine novel rescue regimens for MTX; less MTX toxicity would then lead to less chemotherapy delays and better treatment efficacy for patients with osteosarcoma.

6.3. GLU 1 CLINICAL TRIAL: COULD GLUCARPIDASE PROVIDE SOME ADVANTAGE IN THE DELIVERY OF HD-MTX?

Glucarpidase (Voraxaze™ or Carboxypeptidase G2) seemed to offer a promising opportunity for rescue from MTX toxicity. It has been used in the emergency setting to effectively treat patients with MTX-induced renal dysfunction (Widemann et al. 2006). A single intravenous dose of glucarpidase (50 units/kg) after MTX has resulted in the reduction of plasma MTX levels to the non-toxic range within minutes without causing toxicity. It has much higher affinity for MTX than for folinic acid so even high circulating

folinic acid levels are unlikely to interfere with extracellular MTX inactivation. Moreover, since glucarpidase is a high molecular weight protein and does not gain intracellular access, it is unlikely that it could counteract the anti-tumour effect of MTX. It was felt sensible to examine the use of glucarpidase in the routine setting after high-dose MTX.

In order to investigate the efficacy and safety of glucarpidase for routine use after high dose MTX in patients with bone sarcoma, a phase II clinical trial (GLU 1) was set up.

In view of the wide inter- and intra-patient variability in the pharmacokinetics and tolerance of MTX, a “randomised crossover” design was used. So as to minimize any cumulative dose effect which may interfere with MTX pharmacokinetics and associated toxicity, patients were randomised to receive either 2 courses of HD-MTX with standard rescue with folinic acid (cycle M) followed by 2 courses of HD-MTX with standard folinic acid rescue and glucarpidase (cycle GluM), or to receive cycle GluM first followed by cycle M. A crossover design was used to allow for smaller sample size, since patients acted as their own controls. The trial design was initially “double-blind randomised”. However, it became apparent that treatment with glucarpidase could not be blinded to the investigators since DAMPA, the catabolic product of glucarpidase action on MTX, was present in HPLC assays in all treatment cycles given with glucarpidase. Therefore, following the interim analysis, the trial design was changed from “double-blind randomised” to “unblind randomised”.

The trial sample size was calculated assuming that rescue with glucarpidase would result in “fitness on day 15” in at least 90% of treatment cycles. Based on our audit findings (Section 2.1), only 55% of the patients in the standard rescue group were expected to be treatable on day 15 of each cycle. Glucarpidase is currently used very rarely in the emergency setting and, therefore, is incredibly expensive. The assumption

that 90% of patients in the glucarpidase rescue group would be treatable on day 15 of each cycle was intentionally optimistic. It was felt that only a confidently advantageous result could be meaningful and persuasive and lead to practice change.

The primary objective of the trial was to examine whether glucarpidase rescue after HD-MTX reduces delay resulting from MTX toxicity to subsequent cycles of chemotherapy.

This endpoint was chosen as it is clinically relevant to patients with osteosarcoma treated with standard MAP chemotherapy (HD-**M**TX, **a**driamycin and cis**p**latin) who receive 2 courses/doses of MTX on consecutive weeks and are expected to be fit to receive further chemotherapy with adriamycin and cisplatin 15 days after the first MTX course/dose of each treatment cycle. The fitness criteria used in MAP regimen at that time point (general clinical condition permitting chemotherapy including no evidence of infection, no pyrexia, mucositis or diarrhoea; no serous effusions including ascites and pleural effusions; neutrophils ≥ $0.75x10^9$/L and platelets ≥ $100x10^9$/L; GFR ≥ 70 ml/min/1.73m^2; bilirubin ≤ 1.5 x ULN, ALT ≤ 1.5 x ULN, albumin > LLN; urinary pH >7.0 immediately prior to MTX and good urine output) are identical to the “day 15 fitness criteria” used for in the GLU 1 clinical trial.

Secondary trial objective included the examination of MTX and DAMPA pharmacokinetics prior and after glucarpidase administration. Plasma MTX and DAMPA concentrations were evaluated at regular time points. DAMPA is known to cross-react with MTX in most commercial immunological assays (Albertioni F et al. 1996; Allegra CJ. 1990; and Widemann BC et al. 1997) and MTX plasma concentrations determined by commercial laboratories are unreliable following treatment with glucarpidase. Plasma MTX concentrations in the GLU 1 study were measured by both immunoassay and high performance liquid chromatography (HPLC). Plasma DAMPA concentrations were

measured by HPLC. Since HPLC is not routinely available, I undertook the validation of a HPLC method for measurement of MTX and DAMPA in human plasma for trial patients and all HPLC assessments.

Another secondary trial objective was to establish whether routine glucarpidase rescue after HD-MTX reduces the incidence, severity and duration of MTX-related adverse effects. MTX-related toxicity was documented and graded using primarily CTCTAE v3.0.

Further secondary trial objectives included the evaluation of any adverse effects associated with the use of glucarpidase and the assessment of the anti-glucarpidase antibody response.

Finally, the economic impact of routine glucarpidase administration was evaluated by examining the length of hospital stay and the total dose of folinic acid use for each MTX course administered with and without glucarpidase.

Numerous time consuming factors such as confirmation of the sponsor of the trial, agreement between the sponsor and the pharmaceutical company providing the Investigational Medicinal Product (glucarpidase), review of the trial protocol by all parties (sponsor, statistician and pharmaceutical company), Regulatory Authorities and Ethical Committee approval, validation of HPLC assay for assessment of plasma MTX and DAMPA concentrations for trial patients, availability of glucarpidase, and an amendment to the protocol due to an update in the Summary of Product Characterictics of the IMP, delayed the opening of the GLU 1 trial for more than a year.

The first trial participant was enrolled in June 2007 and 16 patients were recruited until January 2009. An interim analysis was planned after completion of both courses in both

cycles of the first 14 trial participants (50% of sample size). However, this was revised to 16 patients as patients GLU1-05 and GLU1-14 did not contribute to the primary endpoint data. Patient GLU1-05 developed renal toxicity after one dose of MTX with standard rescue and trial treatment was discontinued. His treatment was unblinded as per trial protocol and he was offered emergency treatment with glucarpidase. Patient GLU1-14 developed postoperative knee effusion after one dose of MTX with standard rescue and his trial treatment was discontinued early. The interim analysis of the GLU 1 study was carried out in May 2009, following completion of both courses in both cycles of the first 16 trial participants.

In the GLU 1 interim analysis it was stated that the recruitment rate was lower than expected with 16 recruited patients in over 22 months (from June 2007 to May 2009), while the expected accrual was 10-15 patients per year. However, it is worth mentioning that since the IMP expired in March 2009, recruitment to the trial was not possible after January 2009. 16 patients were recruited in 19 months (between June 2007 and January 2009), which was not far from the expected accrual of 10-15 patients per year.

GLU 1 is still ongoing. Further amendments to the protocol in view of an extension in the shelf life of the IMP, the results of the interim analysis and the recruitment of a new co-investigator contributed to the delay in completing the trial. Here the GLU 1 results up to the interim analysis of the trial are described.

There were two major differences amongst the GLU 1 participants and patients with bone sarcoma treated on other clinical trials. Firstly, the GLU 1 participants were on average older (median age of 19 years; range, 13-47 years; IQR 17-30 years). The median age for patients treated on EURAMOS-1 was 14 years (IQR 11-17 years) (Whelan et al., 2012), whereas the median age of those treated on INT0133 study,

another large phase III osteosarcoma study, was 13 years (range 1-30 years) (Meyers et al., 2008).

Secondly, most of the GLU 1 participants (80%) had been exposed to chemotherapy prior to trial entry with nearly 20% of them (3/16) being treated for relapsed disease. This is because most newly diagnosed patients with osteosarcoma were recruited in an international phase III study (EURAMOS-1), which ran concurrently at our institution. Patients not eligible for the phase III study, such as patients > 40 years old or previously treated, were offered participation in the GLU 1 study. This resulted in treating relatively older patients in the GLU 1 trial.

6.4. ROUTINE USE OF GLUCARPIDASE AFTER HD-MTX: DOES IT REDUCE DELAYS IN OSTEOSARCOMA TREATMENT?

In order to examine whether glucarpidase rescue after HD-MTX reduces delay to subsequent cycles of chemotherapy due to MTX toxicity, the fitness of the GLU 1 participants to receive chemotherapy on day 15 of each cycle was assessed. “Day 15 fitness criteria” are described in section 2.

“Day 15 fitness criteria” were not met in 59% (16/27) of all given cycles. In other words, 59% of subsequent chemotherapy cycles would have been delayed if MTX was given as part of the MAP regimen. This outcome was similar to the findings of the previously described audits on the incidence of treatment delays in MAP chemotherapy due to MTX toxicity and the tolerance of HD-MTX (12 g/m^2) in patients aged ≥ 40 years. However, the GLU 1 findings were not the same when treatment cycles given with glucarpidase and treatment cycles given without glucarpidase were reviewed separately. Amongst cycles given with glucarpidase and folinic acid rescue, “day 15

fitness criteria" were met in 57% of cycles (8/14) and not met in 43% of cycles (6/14). Amongst cycles given with folinic acid rescue, "day 15 fitness criteria" were only met in 23% of cycles (3/13) and not met in 77% of cycles (10/13). The unexpectedly high incidence of delays in subsequent chemotherapy due to MTX toxicity in this study may be due to the fact that most trial participants had been pre-treated with chemotherapy prior to their recruitment and therefore likely less tolerant to MTX.

MTX-related toxicity in the GLU 1 study was not only associated with chemotherapy delays but also resulted in early discontinuation of treatment. The expected drop-out rate was 5% while the actual drop-out rate was 37.5%; only 62.5% (10/16) of the trial patients received all 4 courses of MTX.

The incidence of early discontinuation of treatment due to MTX toxicity in GLU 1 was higher than that observed at our UCH audit on chemotherapy delays due to MTX toxicity. 10% (5/56) of audited patients with median age of 20 years had their treatment with MTX stopped early due to MTX adverse events. However, the drop-out rate was significantly higher in the second UCH audit involving patients aged ≥ 40 years (median age 44.5 years); 75% (12/16) of those had their treatment with MTX discontinued early due to MTX toxicity. Half of those patients developed mucositis and the other half developed nephrotoxicity.

The incidence of early discontinuation of treatment due to MTX toxicity in GLU 1 was generally higher than that observed in the literature. A similar drop-out incidence was only reported by Rosen et al. (1984) who reviewed patients treated on the T10 protocol (neoadjuvant chemotherapy with 4 weekly courses of MTX at a dose 12 g/m^2 for children and 8 g/m^2 for adolescents). The majority of patients did not tolerate 4 weekly MTX treatments due to toxicity and approximately 30% had their treatment substituted

with bleomycin because of MTX toxicity. On the other hand, Goorin et al. (1987) observed a drop-out rate of 7% (3/46 patients) on the DFCI/TCH study III due to MTX-related CNS toxicity. Similarly, in a more recent review of 65 osteosarcoma patients (median age 18 years; range, 9 to 51 years) who received a total of 288 MTX courses at a dose 12 g/m^2 over 4 hours at the Norwegian Radium Hospital between 1994 and 2003, 7.7% of patients (5/65) were excluded from further MTX treatment due to MTX toxicity (Holmboe et al., 2012). In a further review of 97 patients with osteosarcoma (median age 16 years) who received 376 HD-MTX courses (8-12 g/m^2) on the SSG-II clinical trial between 1982 and 1989, 4% of patients (4/97) had their HD-MTX treatment terminated due to MTX toxicity (Saeter et al. 1991). In another review of 343 patients who receive pre-operative treatment with MTX (8-12 g/m^2) at the Instituti Orthopedici Rizzoli between 1983 and 2004, 2% of the patients (7/343) did not complete pre-operative treatment with MTX due to toxicity (Bacci et al. 2006).

A possible explanation for the high early drop-out rate in GLU 1 is the older age of the GLU 1 participants. The GLU 1 patients in whom study treatment was discontinued early due to MTX induced toxicity had a median age of 30 years. In addition, the majority of the trial participants had been exposed to chemotherapy prior to trial entry and were potentially less able to tolerate further treatment. It is unlikely that renal function at trial entry could have predicted early trial drop out since median GFR at trial entry was 108 ml/min/1.73 m^2 in patients completing all 4 MTX courses and 90 ml/min/1.73 m^2 in patients receiving less than 4 MTX courses.

6.5. ROUTINE USE OF GLUCARPIDASE AFTER HD-MTX: MTX AND DAMPA PHARMACOKINETICS

As part of the GLU 1 study, the pharmacokinetics of MTX and DAMPA after glucarpidase administration were studied and MTX plasma concentrations, MTX AUC and DAMPA plasma concentrations were compared in MTX cycles given with standard folinic acid rescue and MTX cycles given with folinic acid and glucarpidase.

Plasma MTX concentration decreased from a median of 15.67 µM prior to glucarpidase to a median of 0.4 µM 15 minutes after glucarpidase administration and glucarpidase resulted in a median reduction of serum MTX concentration of 98.05%.

Our findings are comparable to those described in the literature although in most reports glucarpidase was mainly used as emergency treatment in the setting of delayed MTX elimination and MTX related renal dysfunction. Widemann et al. (1995) reported a 98% decrease in MTX plasma concentration within 15 minutes after the first glucarpidase dose in a patient with osteosarcoma who experienced MTX-induced acute renal dysfunction. Similarly, Zoubek et al. (1995) reported a 99.7% decrease in MTX plasma concentration after 5 doses of glucarpidase (50 U/kg) in a patient with osteosarcoma who developed acute renal failure associated with delayed MTX elimination after HD-MTX. Likewise, Widemann et al. (1997) reviewed the use of glucarpidase (50 U/kg) in 20 patients with MTX related nephrotoxicity and reported a median reduction of 98.7% in plasma MTX concentrations within 15 minutes after the administration of glucarpidase. 6 of the patients received second and third doses of glucarpidase which did not result in a further decrease in plasma MTX concentrations. Also, Monty et al. (2000) described an 80% decrease in MTX plasma concentration in less than 15 minutes after the infusion of glucarpidase in a patient with MTX-related

severe renal failure. However, plasma MTX concentration was determined by fluorescence polarization immunoassay and not by HPLC. More recently, Buchen et al. (2005) reported 82 patients treated with HD-MTX (1-12 g/m^2) with delayed MTX excretion and renal impairment who received glucarpidase at a dose of 50 U/kg and noted that serum MTX concentration was reduced by 97% 15 minutes after glucarpidase administration.

The only study where glucarpidase was used routinely was reported by De Angelis et al. (1996). In this paper 4 patients with recurrent primary CNS lymphoma and normal renal function were treated with HD-MTX (3 g/m^2) and received routinely 2 doses of glucarpidase (50 U/kg). Patients had a 97.2% decrease of MTX plasma concentration within 5 minutes of glucarpidase administration. The second dose of glucarpidase did not further diminish the already low plasma MTX level.

In GLU 1 a small rebound increase in plasma MTX concentrations was observed in 21% (6/28) of MTX courses given with glucarpidase/folinic acid rescue (Section 5.5, Table 44). Due to its molecular size glucarpidase is restricted to the extracellular compartment and the intracellular MTX concentration is initially unaffected by its use. In time, the changed equilibrium between intracellular and extracellular MTX results in the efflux of intracellular MTX back into the serum, resulting in a rise of serum MTX levels some hours after glucarpidase administration (Buchen et al., 2005). A rebound increase in serum/plasma MTX concentration after treatment with glucarpidase has been described in the literature and our findings are in keeping with those described by Widemann et al. 1997 and Monty et al. (2000). On the contrary, Widemann et al (1995) and De Angelis et al. (1996) reported no rebound increase in plasma MTX concentrations.

Nevertheless, despite the observed rebound increase in plasma MTX concentrations in treatment cycles given with glucarpidase in GLU 1, the time to eliminate MTX was found to be significantly less with the use of glucarpidase and therefore the rebound increase in plasma MTX concentrations after glucarpidase did not have any clinical significance (Section 5.5, Table 37 and Table 41).

Following glucarpidase, MTX is rapidly metabolized to DAMPA and glutamate. In GLU 1, DAMPA plasma concentrations 15 minutes after glucarpidase administration were on average 35% of the pre glucarpidase MTX serum concentrations (Section 5.5, Table 43). In the literature, DAMPA plasma concentrations are generally equivalent to pre glucarpidase MTX plasma concentrations after systematic exposure to glucarpidase (De Angelis el al., 1996; Widemann et al., 1997; and Buchen et al., 2005).

The difference in DAMPA plasma concentrations in relation to pre glucarpidase MTX plasma concentrations between our study and previous publications (De Angelis et al., 1996, Widemann et al., 1997, and Buchen et al., 2005) may be due to the fact that, although blood samples for MTX and DAMPA plasma concentration analysis were collected 20 minutes after the start of glucarpidase administration, blood samples for MTX plasma concentration pre glucarpidase were not always collected immediately prior to glucarpidase administration. This was due to hospital logistics as all blood samples for MTX analysis by immunoassay had to be collected by 1 pm in order to be processed on the same day. In response to that, we aimed to start all MTX infusions at 12 o'clock noon. However, this was not always possible. Patients whose MTX infusion started later than 1 pm had to have their 24 hour blood samples for MTX analysis taken at the latest by 1 pm. Since glucarpidase was always given at 24 hours after the start of MTX infusion, the time between pre glucarpidase MTX plasma concentration

measurements and post glucarpidase DAMPA plasma concentration measurements was longer than 20 minutes for some patients. If all pre glucarpidase MTX plasma concentration measurements were taken immediately prior to glucarpidase administration, DAMPA plasma concentrations 15 minutes post glucarpidase administration might have been similar to pre glucarpidase MTX plasma concentrations.

6.6. MTX-RELATED TOXICITY WITH AND WITHOUT GLUCARPIDASE

One of the objectives of the GLU 1 study was to examine whether routine glucarpidase rescue after HD-MTX reduces the incidence, severity and duration of MTX-related adverse effects. The incidence and grading of mucositis, renal toxicity, liver toxicity, neutropenia and thrombocytopenia was compared in MTX cycles given with standard folinic acid rescue and MTX cycles given with folinic acid rescue and glucarpidase.

Overall, there was no statistical difference in MTX-related toxicity between glu/FA cycles and FA cycles. However, since only data from the interim analysis are available, the number of patients in this report was small, and so there was a low power to detect differences in cycles given with and without glucarpidase.

The most commonly observed adverse events were, as expected, elevated alanine transaminase, mucositis, hypophosphataemia, renal impairment and mild bone marrow suppression. None of the study participants developed neurotoxicity or skin related toxicity.

Hepatotoxicity (mainly elevated alanine transaminase) was the most commonly seen MTX related toxicity and all the GLU 1 treatment cycles were associated with acute transient ALT elevation. However, it was temporary and it contributed neither to lack of “fitness on day 15” nor to early discontinuation of trial treatment. ALT CTCTAE v3.0

grade ≥ 3 complicated 93% and 77% of glu/FA and FA treatment cycles respectively. Mild hyperbilirubinaemia (CTCTAE v3.0 grades 1 and 2) complicated 21% and 8% of glu/FA and FA treatment cycles respectively. We did not document any severe hyperbilirubinaemia (CTCTAE v3.0 grades 3 and 4). No statistically significant differences in hepatotoxicity between glu/FA cyles and FA cycle were observed. However, as mentioned already the number of patients in the analysis was small, and so there was a low power to detect differences. Our findings were similar to those reported in the literature for treatment cycles given with standard rescue (Saeter et al., 1991 and Holmboe et al., 2012) although Zelcer et al. (2008) observed lower incidence of ALT CTCTAE v3.0 grade ≥ 3.

Mucositis (clinical and functional) was the second most common toxicity observed; it complicated 44% (12/27) of treatment cycles and led to lack of “fitness on day 15” in 33% (8/27) of treatment cycles. The majority of these (6/27) were given with standard rescue and only 2 were given with glucarpidase. Mucositis did not contribute to early discontinuation of trial treatment.

Mucositis-clinical complicated 36% and 54% of glu/FA and FA treatment cycles respectively. More severe mucositis-clinical (CTCAE v3.0 clinical grades ≥ 3) complicated 7% and 23% of glu/FA and FA treatment cycles respectively. These differences were not statistically significant but may indicate a trend in favour of glucarpidase.

Interestingly, a significant difference in CTCAE mucositis-functional was found between the 2 rescue regimens with FA cycles being associated with higher CTCAE grades of mucositis-functional. Mucositis-functional complicated 15% and 38% of glu/FA and FA treatment cycles respectively. More severe mucositis-functional (CTCAE v3.0 grades ≥

3) complicated 8% FA treatment cycles whereas severe mucositis-functional was not documented in any glu/FA treatment cycles.

The incidence of MTX related mucositis in the literature was similar to our findings for treatment cycles given with standard rescue (FA cycles) (Sonis at al., 2004; Saeter et al., 1991; Holmboe et al., 2012).

Apart from the National Cancer Institute Common Terminology criteria for Adverse Events (CTCAE v 3.0), 3 other assessment tools were used to evaluate the severity of mucositis in the GLU 1 patients and ensure comprehensive assessment; the World Health Organisation Toxicity Criteria for Oral Mucositis, the Oral Assessment Guide (OAG) and the Oral Mucositis Weekly Questionnaire (OMWQ). The CTCAE v 3.0 and WHO criteria were used because they are the most commonly used criteria for mucositis evaluation in clinical studies (Sonis et al., 2004). The Oral Assessment Guide (Eilers et al., 1988) was chosen as it is popular amongst nursing staff. The Oral Mucositis Weekly questionnaire was adapted from the Oral Mucositis Daily Questionnaire (Bellm et al., 2002 and Stiff et al, 2006) and chosen because it provided a patient-reported measure of oral mucositis. However, it was retrospectively felt that the CTCAE v3.0 tool would have been comprehensive enough to capture the severity of mucositis in the GLU 1 study; it is sensitive, universally used and takes into account both objective (i.e. assessor) and subjective (i.e. patient) mucositis assessment.

Renal dysfunction was relatively common amongst the GLU 1 patients. Of the 16 trial patients, 8 developed nephrotoxicity (CTCAE v.3.0, grade ≥ 1) and 5 of those did not complete their treatment due to renal impairment. Routine administration of glucarpidase did not seem to make a difference in the incidence or severity of MTX-related nephrotoxicity. The incidence of grade ≥ 2 nephrotoxicity in GLU 1 was 12.5%,

seven times greater than the incidence documented by Widemann et al. (2004) and nine times greater than that reported by Saeter et al. (1991). The incidence of severe nephrotoxicity (serum creatinine grade 3 or 4) was 6.25%, 10 times greater than that identified by Widemann et al. (2006). Nevertheless, there were no deaths in the trial due to nephrotoxicity.

The high incidence of nephrotoxicity in GLU 1 could be explained as mentioned earlier by the older age of the participants and their previous exposure to chemotherapy. It is unlikely that renal function at trial entry could have predicted the development of nephrotoxicity since median GFR at trial entry was 108 ml/min/1.73 m^2 in patients who did not develop nephrotoxicity during their trial treatment and 96 ml/min/1.73 m^2 in patients who developed nephrotoxicity.

6.7. ROUTINE USE OF GLUCARPIDASE: ECONOMIC IMPACT

In order to investigate the economic impact of using glucarpidase versus standard rescue, we compared the number of days required in hospital for MTX cycles given with standard folinic acid rescue and MTX cycles given with folinic acid rescue and glucarpidase. The average length of patient's hospital stay per glu/FA and FA course was 5.04 days (range 4-13 days) and 4.77 days (range 4-10 days) respectively.

At present, it is a regulatory requirement that rescue with folinic acid continues for at least 48 hours after the administration of glucarpidase in order to compensate for any reduced exposure to folinic acid caused by its potential interaction with glucarpidase (Voraxaze™ Investigator's brochure, 2009). Interestingly, this requirement increased the hospital stay of the GLU 1 patients receiving rescue with glucarpidase by more than 1 day. If patients were discharged home as soon as MTX plasma concentration was <

0.2 µM, the average length of patient's hospital stay per glu/FA course would have been 3.88 days rather than 5.04 days.

We also compared the total folinic acid dose required in MTX cycles given with standard folinic acid rescue with MTX cycles given with folinic acid rescue and glucarpidase. Nevertheless, it became apparent that the total folinic acid dose per course was not a good indicator of the economic impact of using glucarpidase versus standard rescue. This was because the GLU 1 participants had their dose of folinic acid adjusted based upon the pre-glucarpidase/placebo plasma MTX levels and the dose of folinic acid was maintained unchanged for 48 hours after glucarpidase/placebo. Hence, the total folinic acid dose did not reflect the effect of glucarpidase on MTX plasma levels at 48 hours and 72 hours.

Currently available information on the interaction of folinic acid with glucarpidase is based on the LV interaction study (section 1.4.2.2.). When glucarpidase was administered in healthy subjects, their exposure to folinic acid was reduced by about 50% in the absence of methotrexate (Voraxaze™ Investigator's brochure, 2009). Further studies are needed to examine the interaction of glucarpidase with folinic acid **in the presence of methotrexate**. Since glucarpidase has > 10-fold higher affinity for MTX than for folinic acid, it might not be necessary to use such high doses of folinic acid in patients who are "rescued" with glucarpidase and/or it might be possible that patients are discharged home as soon as MTX plasma concentration was < 0.2 µM.

6.8. GLUCARPIDASE RELATED ADVERSE EVENTS AND ANTIBODY RESPONSE IN GLU 1

No adverse events related to glucarpidase were experienced by any of the GLU 1 patients. Our findings are similar to those published by De Angelis et al. (1996) and Mohty et al. (2000) who observed no glucarpidase-related adverse events. Nonetheless, Widemann et al. (1995, 1997, and 2006), Buchen et al. (2005) and Schwartz et al. (2004) observed easily reversible glucarpidase related toxicity in a small minority of patients treated.

Glucarpidase is a recombinant bacterial protein and it therefore has the potential to induce an immune response. This could produce an allergic reaction on subsequent administration of glucarpidase or reduce its efficacy by neutralizing the activity of glucarpidase.

In GLU 1 none of the 14 patients who had more than one glucarpidase dose experienced an allergic reaction. However, positive anti-glucarpidase IgG response was observed in 43% (6/14) of trial patients although the activity of glucarpidase was only inhibited in 17% (1/6) of patients with positive antibody response. Our results are in keeping with the literature (Voraxaze™ Investigator's Brochure, PR001-CLN-IB2007, released in February 2009).

Positive antiglucarpidase IgG response in patients GLU1-07, GLU1-08 and GLU1-09, became negative in follow-up samples. Patient GLU1-02, who had a positive antiglucarpidase IgG response on day 180 of cycle 2, had no further follow-up samples. This was in accordance with the trial protocol as "day 180 of cycle 2" is the last time point blood samples are collected for anti-glucarpidase antibody analysis. However, it is

unfortunate there is no follow-up data since this is the only patient where neutralising response was observed. Follow-up samples on patients GLU1-15 and GLU1-16, who developed positive antiglucarpidase IgG response, have not been analysed yet.

It is essential to know if repeated doses of glucarpidase could result in the development of anti-glucarpidase antibodies and subsequent inhibition of the activity of glucarpidase. However, it is reassuring that despite the presence of anti-glucarpidase antibodies in almost half of the trial patients, the activity of the enzyme was found to be inhibited only in 1 patient.

6.9. GLU 1 CLINICAL TRIAL: LIMITATIONS

There are some limitations with the GLU 1 clinical trial. Recruitment to GLU 1 was rather slow because most of the patients with bone sarcoma were eligible for and recruited to a phase III clinical trial which ran concurrently with GLU 1. This also explains why the GLU 1 participants were older compared to the average patient with bone sarcoma and why the majority of them had previous exposure to chemotherapy. GLU 1 included patients with relapsed disease who had chemotherapy-induced toxicity prior to entering the trial. Older age of trial participants and previous exposure to chemotherapy agents have likely contributed to the increased renal toxicity seen.

Other limitations included potential cumulative toxicity from other chemotherapy agents administered to study participants prior to their enrolment to GLU 1 (such as doxorubicin and cisplatin) and carryover of the effects of glucarpidase in the second GLU 1 treatment cycle for patients who received glucarpidase as part of their first treatment cycle. Also, a limited number of four MTX courses were studied instead of the

twelve MTX courses that are given to patients with osteosarcoma as part of the MAP regimen.

In addition, although most of the “fitness criteria on day 15”, such as haematological and biochemical parameters, are objective, assessment of patient’s clinical status including assessment of mucositis is subjective and possibly depends on the experience of the clinician. Furthermore, if a patient was due to start trial treatment on a Friday but found unfit, he/she would have been asked to attend for review the Monday of the following week, leading to an at least 3-day delay. However, if the same patient was due treatment in the beginning of the week but found unfit, his/her treatment would have perhaps been less delayed, i.e. 1- or 2-day delay.

Finally, the impact of glucarpidase on the anti-tumour effect of MTX was intentionally not included in the trial objectives. It was felt that this would have been beyond the remit of a phase II study. Nonetheless, there was no difference in peak plasma MTX concentration with and without glucarpidase as glucarpidase was given twenty hours after peak plasma MTX concentrations were achieved. Moreover, there was no statistical difference in the MTX AUC_{0-T} in courses given with and without glucarpidase. Peak plasma MTX concentration and MTX AUC_{0-T} could perhaps be used as surrogate endpoints for the anti-tumour effect of MTX in view of their relationship with the outcome of osteosarcoma treatment (Bacci et al. 1998; Bacci et al. 1996; Delepine et al. 1988; Graf et al. 1994; Crews et al. 2004; Aquerreta et al. 2004).

6.10. Conclusions - Implications for Future Research

HD-MTX has been an essential component in the management of osteosarcoma in the last forty years. There are no indications that this will change soon. However, although

well established supportive care is standard practice, HD-MTX is still associated with significant morbidity. Glucarpidase is the only available agent which offers a promising opportunity for rescue from MTX-related toxicity. A phase II study has been undertaken to investigate the efficacy and safety of glucarpidase for routine use after HD-MTX in patients with bone sarcoma. My responsibilities included the design of the GLU 1 trial and writing of the trial protocol, submission of all appropriate documentation and subsequent amendments to the trial protocol to the Regulatory Authorities and Ethics Committee, validation of a HPLC assay for the measurement of MTX and DAMPA plasma levels and assessment of MTX and DAMPA plasma levels for trial patients with HPLC, informed consent and enrolment of trial participants, involvement in the clinical management of trial participants, nursing and medical staff training on the trial, and data management and safety management of the trial. Here the findings up to the interim analysis of the study are presented.

So far it is apparent that glucarpidase can be safely administered as part of routine rescue after HD-MTX with reduction in MTX levels after a single dose. Furthermore, interim analysis of the study revealed a lower incidence of severe mucositis in patients receiving glucarpidase. If the trial, when completed, indicates that routine rescue with glucarpidase improves the delivery of HD-MTX in patients with bone sarcoma it would be necessary to be followed up by a larger phase III clinical trial where glucarpidase is administered after all the 12 MTX courses included in the MAP chemotherapy regimen. Further studies are necessary to examine the interaction of glucarpidase with folinic acid in the presence of MTX. Additional hurdles that need to be overcome before glucarpidase could be introduced routinely as standard rescue after HD-MTX should include the assessment of the inhibition of the enzyme with repeated administrations of

glucarpidase and the relationship of glucarpidase with the anti-tumour effectiveness of MTX.

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APPENDICES

APPENDIX 1. GLU 1 CLINICAL TRIAL PROTOCOL

University College London Hospitals NHS
NHS Foundation Trust

GLU 1

(PR001-CLN-pro009)

A RANDOMISED, CROSS-OVER, PHASE II STUDY, TO INVESTIGATE THE EFFICACY AND SAFETY OF GLUCARPIDASE FOR ROUTINE USE AFTER HIGH DOSE METHOTREXATE IN PATIENTS WITH BONE SARCOMA

CHIEF INVESTIGATOR: DR JEREMY WHELAN

CO-INVESTIGATOR: DR MARTHA PERISOGLOU

SPONSOR: UNIVERSITY COLLEGE LONDON

EudraCT number: 2006-003203-40
Sponsor's project ID number: BRD/06/085

Clinical trial protocol
Version 4.0, 26 August 2009

We gratefully acknowledge the financial support

from the Richard Scowcroft Foundation for this study

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E) GLOSSARY OF ABBREVIATIONS AND DEFINITION OF TERMS

AE	Adverse Event
ADL	Activities of Daily Living
ALT	Alanine aminotransferase
AR	Adverse Reaction
AUC	Area under the curve
BP	British Pharmacopoeia
CI	Chief Investigator
CPG1	Carboxypeptidase G1
CPG2	Carboxypeptidase G2
CRF	Case Record Form
CTCAE v.3.0	Common Terminology for Adverse Events, version 3
DAMPA	4-deoxy-4-amino-N10-methylpteroic acid
DFS	Disease Free Survival
DHFR	Dihydrofolate reductase
EFS	Event Free Survival
ELISA	Enzyme Linked Immunosorbent Assay
EURAMOS-1	European and American Osteosarcoma Study -1
FAR	Folinic acid rescue
GFR	Glomerular Filtration Rate
GI	Gastro-intestinal
GLP	Good Laboratory Practice
HD-MTX	High-dose methotrexate
HFR	Human Folate Receptor
HPLC	High Performance Liquid Chromatography
ICH GCP	International Conference on Harmonisation, Good Clinical Practice
IMP	Investigational medicinal product
ITMC	Independent Trial/Data Monitoring Committee
iv	Intravenous, intravenously
MAP	High-Dose **M**ethotrexate, Doxorubicin (**A**driamycin) and Cis**p**latin
MASCC	Multidisciplinary Association of Supportive Care in Cancer
MHRA	Medicines and Healthcare products Regulatory Agency
MTX	Methotrexate
MTX-PG	Methotrexate-polyglutamates
NCI	National Cancer Institute
OAG	Oral Assessment Guide

OMDQ	Oral Mucositis Daily Questionnaire
OMWQ	Oral Mucositis Weekly Questionnaire
OS	Osteosarcoma
REC	Research Ethics Committee
RFC	Reduced Folate Carrier
RTOG	Radiation Therapy Oncology Group
SAR	Serious adverse reaction
SAE	Serious adverse event
SUSAR	Suspected Unexpected Serious Adverse Reaction
TS	Thymidylate synthetase
TSC	Trial Steering Committee
UCH	University College Hospital
UCL	University College London
UCLH	University College London Hospitals
WCC	White Cell Count
WHO	World Health Organisation
WMA	World Medical Association

F) STUDY SYNOPSIS

TITLE OF THE STUDY	Randomised, cross-over, phase II study, to investigate the efficacy and safety of glucarpidase for routine use after high-dose methotrexate in patients with bone sarcoma. [This study will be conducted in compliance with the principles of ICH GCP, the Declaration of Helsinki, the Medicines for Human Use (Clinical Trials) regulations 2004 and all other applicable regulatory requirements]
STUDY DESIGN	Randomised, cross-over, phase II study
STUDY OBJECTIVES	**Primary Objective:** To investigate whether glucarpidase rescue after high-dose methotrexate reduces delay to subsequent cycle of chemotherapy due to methotrexate toxicity **Secondary Objectives:** 1.To investigate whether glucarpidase rescue after high-dose methotrexate reduces the incidence, severity and duration of methotrexate-associated adverse effects 2.To study the pharmacokinetics of methotrexate and 4-deoxy-4-amino-N10-methylpteroic acid (DAMPA) after glucarpidase administration 3.To evaluate any adverse effects associated with the use of glucarpidase 4.To investigate the economic impact of using glucarpidase plus standard rescue versus standard rescue alone 5.To investigate the effect of glucarpidase on the quality of life of patients treated with high-dose methotrexate 6.To assess the anti-glucarpidase antibody response
STUDY POPULATION	a) **Inclusion Criteria** 1. Signed informed consent from patient or parent /guardian 2. Diagnosis of high grade osteosarcoma, localised or metastatic

	or high grade osteosarcoma as a second maligancy **or** spindle cell sarcoma of bone **or** relapsed high grade osteosarcoma 3. Age: 5-50 years at registration 4. Ability to comply with study and follow up procedures (WHO performance scale 0-2) 5. No concomitant anti-cancer or investigational drugs during the study and complete resolution of toxicity related to previous treatment 6. Life expectancy of at least 3 months 7. Haematopoietic function: - Absolute neutrophil count ≥1 x10^9/L - Platelets ≥100 x10^9/L 8. Hepatic function: - Bilirubin: ≤ 1.5 x ULN, ALT ≤ 1.5 x ULN, Albumin > LLN 9. Renal function: GFR (radioisotope) ≥ 70 ml/min/1.73m^2
	b) <u>Exclusion Criteria</u> 1. Previous treatment with glucarpidase 2. Pregnant or breast feeding women (patients with reproductive potential must use contraception) 3. Concomitant treatment with agents which interact with methotrexate metabolism or excretion (appendix 8) 4. Serous effusions, including ascites and pleural effusions
TREATMENT AND DOSAGE	Patients will be randomised to **<u>arm A</u>** or **<u>B</u>**. Each arm consists of two cycles. Each cycle consists of two courses of methotrexate. In <u>arm A</u> patients will receive cycle M first followed by cycle GluM, whereas in <u>arm B</u>, patients will receive cycle GluM first followed by cycle M. <u>In arm A:</u> **Cycle M** starts with course **M_1** on day 1 followed by course **M_2** planned for day 8. **Cycle GluM** starts with course **$GluM_1$** on day 1 followed by **$GluM_2$** planned for day 8. Cycle GluM will not start for a minimum of 14 days from the beginning of course M_2 or until bone marrow, renal and hepatic functions have completely recovered and the patient is clinically ready to receive further chemotherapy (see section 7.6 for minimum requirements). A minimum of 14 days from M_2 to the beginning of the second cycle seems appropriate to allow complete recovery. <u>In arm B:</u> **Cycle GluM** starts with course **$GluM_1$** on day 1 followed by **$GluM_2$** planned for day 8. **Cycle M** starts with

	course $\mathbf{M_1}$ on day 1 followed by course $\mathbf{M_2}$ planned for day 8. Cycle M will not start for a minimum of 14 days from the beginning of course $GluM_2$, or until bone marrow, renal and hepatic function have completely recovered and the patient is clinically ready to receive further chemotherapy (see section 7.6 for minimum requirements). **Definition of courses:** **Course M:** Methotrexate (12 g/m^2/4 h x 1, iv) with standard folinic acid rescue **Course GluM:** Methotrexate (12 g/m^2/4 h x 1, iv) with folinic acid and glucarpidase rescue (50 units/kg x 1, iv)
ASSESSMENT OF EFFICACY	Primary endpoint: Assessment of fitness to receive chemotherapy on day 15 of each cycle (see section 8 for "Day 15" fitness criteria). Secondary endpoints: 1.Incidence of methotrexate-associated toxicity (CTCAE v3.0 grade ≥2) 2.Serum methotrexate and DAMPA concentrations 3.Anti-glucarpidase IgG antibody response and neutralisation of glucarpidase activity 4.Number of days required in hospital per cycle and total dose of folinic acid required per cycle Quality of life assessment (QLQ-C30 version 3, FACT-G and PedsQL 3.0 cancer module acute version)
ASSESSMENT OF SAFETY	Glucarpidase- and high-dose methotrexate-related Adverse Event and Serious Adverse Event reporting
PHARMACOKINETIC ASSESSMENT	- MTX pharmacokinetic assessment by immunoassay: For both cycles: On day 1 and 8: at 24 hours after starting MTX at 48 hours after starting MTX at 72 hours after starting MTX and then daily until MTX plasma levels <0.2 µmol/L - MTX and DAMPA pharmacokinetic assessment by High Performance Liquid Chromatography (HPLC): For both cycles:

	On day 1 and 8: at 0 hours, prior to starting MTX at 4 hours, at completion of MTX at 24 hours after starting MTX (prior to glucarpidase administration) at 24:20 hours after starting MTX (15 min after glucarpidase administration) at 48 hours after starting MTX at 72 hours after starting MTX and then daily until MTX plasma levels <0.2 µmol/L
DURATION OF STUDY	The planned duration of the study for each participant is 6 weeks. However, patients will be followed up on day 30 and at 3 and 6 months after starting cycle 2, to investigate anti-glucarpidase antibody response.. The planned duration of the entire study is 4 years.
NUMBER OF PATIENTS	With anticipated proportions of responses to standard rescue and glucarpidase+standard rescue of 55% and 90% respectively, the study will require 38 patients to give 80% power at a significance level of 5%, allowing for up to 30% dropout during the study.
STUDY PERIOD	First patient: July 2007 Anticipated accrual: 10 patients per year
STATISTICAL ANALYSIS	Primary outcome: An estimate of the difference in proportions of patients ready to receive chemotherapy on day 15 of each chemotherapy cycle comparing standard & placebo and standard & glucarpidase rescue will be provided. A 95% confidence interval will be calculated for this estimate and a McNemar's test carried out. A significance level of 0.048 will be used. The influence of period effects will be investigated. All the analyses will be carried out by intention to treat.
	Secondary outcomes: The three secondary outcomes to be analysed will be incidence of grade ≥2 (CTCAE v3.0) mucositis, neutropaenia and renal toxicity. The statistical methods used will be as for the primary outcome. Analysis of other outcomes will be descriptive using graphical or summary measures, as appropriate.

	Interim analysis: An interim analysis will be performed after data for all courses of treatment for 50% of the planned number of patients have been obtained. The ITMC will make a recommendation about study continuation at that time. In particular, termination of the study will be considered if: (i) Analysis of the primary outcome shows a statistically significant benefit of glucarpidase with $p < 0.005$ (ii) Treatment with glucarpidase and folinic acid is significantly worse than standard treatment by a one-sided test of the primary outcome with $p < 0.05$. (iii) Glucarpidase associated AEs, SAEs and SUSARs outweigh its benefit (iv) Recruitment rate appears inadequate to achieve the required sample size for the study. If the study continues to the full planned size, the final analysis will use an adjusted p value of 0.048 to preserve the overall probability of type 1 (false positive) error. Interim analysis results: Interim analysis was carried out following completion of both courses in both cycles of the first 16 study participants. In examining efficacy based on the primary outcome, the O'Brien-Fleming method for judging significance of results from a McNemar's test[71] was used. A significance level of 0.005 was used for this interim analysis. To assess whether glucarpidase may be delaying further chemotherapy, the McNemar's test with a one sided significance level of 5% was used. i) Analysis of the primary outcome did not show a statistically significant benefit of glucarpidase with $P<0.005$ ii) Treatment with glucarpidase and folinic acid was not found to be significantly worse than standard treatment using an one-sided test with $P<0.05$ iii) Glucarpidase associated SAEs and SUSARs did not outweigh its benefit. There were no AEs related to the IMP. Two SAEs were reported during this period, both unrelated to the IMP.

	iv) Recruitment rate did not appear inadequate to achieve the required sample size for the study. However, the drop-out rate has been significantly higher than expected with only 11 out of 16 participants contributing to complete data set. The expected drop-out rate was 5% while the actual drop-out rate has been 31%. The trial sample size has been re-calculated assuming that the drop-out rate will remain around 30%.

G) SCHEDULE OF ASSESSMENTS AND PROCEDURES

INVESTIGATIONS / PROCEDURES	CYCLE 1						CYCLE 2							
DAY OF CYCLE	-7 to 0	1	2	8	9	15	1	2	8	9	15	30	90	180
DAY OF STUDY	-7 to 0	1	2	8	9	15	22	23	29	30	36	51	111	201
Eligibility & Consent	X													
Medical history	X													
Physical examination, performance status,		X		X		X	X		X		X			
Methotrexate administration		X		X			X		X					
Glucarpidase / Placebo administration			X		X			X		X				
Haematology	X			Xa		X	X		Xa		X			
Biochemistry / LFTs	Xb			Xb		X	Xb		Xb		X			
Glomerular Filtration Rate	Xc			Xd		Xd	Xd		Xd		Xd			
Concomitant medication	X			X		X	X		X		X			
[MTX] & [DAMPA] by HPLC analysis		Xe		Xe			Xe		Xe					
[MTX] estimation by immunoassay		Xf		Xf			Xf		Xf					
Anti-glucarpidase antibodies and enzyme neutralisation assay	X			X		X	X		X		X	X	X	X
Glucarpidase adverse events			Xg		Xg			Xg		Xg				
MTX toxicity assessment		X		X		X	X		X		X			
Quality of Life questionnaires		X		X		X	X		X		X			

a: and at least every 3rd day until haematological recovery
b: and at least daily until completion of rescue
c: GFR (radio-isotopic assay)
d: estimated GFR (see Appendix 7)
e: at 0, 4, 24, 24:20*, 48, 72 hours after starting methotrexate and then daily until methotrexate plasma levels <0.2 µmol/L (*for glucarpidase cycles only)
f: at 24, 48, 72 hours after starting methotrexate and then daily until methotrexate plasma levels <0.2 µmol/L
g: daily until discharge from hospital

1. RATIONALE AND BACKGROUND

1.1 RATIONALE OF THE STUDY

High-dose methotrexate (HD-MTX) at a dose of 12 g/m^2, in combination with vigorous hydration and urinary alkalinisation along with a pharmacokinetically guided folinic acid "rescue" (FAR) schedule, is an essential component of osteosarcoma treatment. Folinic acid replenishes the intracellular source of reduced active folates. Although FAR may decrease the degree of methotrexate (MTX) toxicity, patients will remain at risk as long as elevated MTX levels persist in the circulation. Moreover, if the extracellular MTX concentration is very high, FAR may prove inadequate.

Despite current supportive measures, MTX-induced toxicity (myelosuppresion, mucositis, hepatic and renal toxicity) still occurs and results in increased morbidity, patient discomfort, increased costs and potentially reduced treatment efficacy, due to suboptimal chemotherapy doses and/or delays in chemotherapy administration. A review of 56 osteosarcoma patients treated in our institution between 2003 and 2006 revealed that MTX-induced toxicity resulted in delays in 47% of chemotherapy cycles.

Several studies have shown that the fewer chemotherapy delays in osteosarcoma treatment, the better the outcome. Frei at al.[1] reported that chemotherapy response in osteosarcoma improves by increasing MTX dose and worsens by increasing the time between MTX administrations. The French Tumour Study Group[2] revealed that delay in MTX course administration is a negative prognostic factor in osteosarcoma. Moreover, Bacci et al[3] showed that avoiding reductions in MTX doses and /or delays in chemotherapy is crucial in osteosarcoma outcome. A review of 30 studies by Delepine et al.[4] demonstrated that the total planned dose and dose intensity of MTX (total MTX dose during treatment divided by total number of weeks), correlates significantly with disease free survival and seems to be a major factor in predicting the outcome of patients with localised high grade osteosarcoma. These reports indicate that improving rescue from MTX toxicity is a worthwhile goal.

Glucarpidase (Voraxaze™, formerly known as Carboxypeptidase G2) is an enzyme that cleaves the terminal glutamate from folate and folate analogues such as MTX. In the case of MTX, its action results in the production of the inactive metabolite DAMPA (4-deoxy-4-amino-N10-methylpteroic acid). It is currently used effectively to treat patients with MTX-induced renal dysfunction, in order to avoid potentially fatal MTX-related toxicity. A single intravenous dose of 50 units/kg of glucarpidase after MTX results in the reduction of plasma MTX levels to the non-toxic range within minutes without causing toxicity. Glucarpidase has much higher affinity for MTX than folinic acid so even high circulating folinic acid levels are unlikely to interfere with extracellular MTX inactivation. Moreover, glucarpidase is a high molecular weight protein and does not gain intracellular access. Therefore it would not

counteract the anti-tumour effect of MTX trapped intracellularly in the form of polyglutamate. Glucarpidase offers a promising opportunity for rescue from MTX toxicity.

In this study we will investigate the role of glucarpidase in the routine rescue after HD-MTX in patients with bone sarcoma. Glucarpidase, if found to be effective and safe in maintaining the treatment intensity and reducing the incidence and severity of MTX-induced toxicity, could optimise treatment, improve patients' well-being, and reduce the use of health resources.

1.2 STANDARD TREATMENT OF OSTEOSARCOMA

1.2.1 NEWLY DIAGNOSED OSTEOSARCOMA

In the UK, standard treatment for newly diagnosed osteosarcoma includes 10 weeks of neoadjuvant chemotherapy with Doxorubicin (**A**driamycin), Cis**p**latin and High-Dose **M**ethotrexate (**MAP**), followed by surgery, followed by 18 weeks of adjuvant chemotherapy with the same agents (table 1.1)

Table 1.1: Schedule of standard osteosarcoma management

CYCLE	WEEK	TREATMENT
	1	DOXORUBICIN + CISPLATIN
	2	
1	3	
	4	HIGH-DOSE METHOTREXATE
	5	HIGH-DOSE METHOTREXATE
	6	DOXORUBICIN + CISPLATIN
	7	
2	8	
	9	HIGH-DOSE METHOTREXATE
	10	HIGH-DOSE METHOTREXATE
	11	SURGERY
	12	DOXORUBICIN + CISPLATIN
	13	
3	14	
	15	HIGH-DOSE METHOTREXATE
	16	HIGH-DOSE METHOTREXATE
	17	DOXORUBICIN + CISPLATIN
	18	
4	19	
	20	HIGH-DOSE METHOTREXATE
	21	HIGH-DOSE METHOTREXATE
	22	DOXORUBICIN
	23	
5	24	HIGH-DOSE METHOTREXATE
	25	HIGH-DOSE METHOTREXATE
	26	DOXORUBICIN
	27	
6	28	HIGH-DOSE METHOTREXATE
	29	HIGH-DOSE METHOTREXATE

Currently, an intergroup trial, the European and American Osteosarcoma Study (EURAMOS-1), is being undertaken by several European and North American study groups [the North American Children's Oncology Group (COG), the German-Austrian-Swiss Cooperative Osteosarcoma Study Group (COSS), the European Osteosarcoma Intergroup (EOI) and the Scandinavian Sarcoma Group (SSG)]. EURAMOS-1 is a phase III, open label, randomised controlled clinical trial of parallel groups with the intention to optimise therapy for patients with osteosarcoma.

The aim of EURAMOS-1 is to investigate whether it is feasible to improve outcome for both good and poor responders to induction chemotherapy through the addition of extra agents into the post-operative treatment schedule. All patients will receive 10 weeks of chemotherapy with MAP, followed by surgery to remove the primary tumour. Poor responders (≥10% viable tumour) will be randomised between MAP for 18 weeks and MAPIE (methotrexate, doxorubicin, cisplatin, ifosfamide and etoposide) regime for 29 weeks. Good responders (<10% viable tumour) will be randomised between MAP for 18 weeks and MAPinf, consisting of MAP for 18 weeks followed by maintenance therapy with interferon-α, which is continued for up to 2 years from diagnosis.

1.2.2 RELAPSED OSTEOSARCOMA

Surgery is the cornerstone of successful relapse therapy. However, chemotherapy should be offered to most patients who experience a relapse within the first three years after diagnosis and those with multiple metastases. The choice of drugs should be made on an individual basis. Patients, who have been previously treated with MAP, could potentially receive ifosfamide and etoposide. Nevertheless, there are patients with relapsed disease who have not received methotrexate at initial diagnosis, such as patients on the previous randomised MRC BO06 study[5], which compared standard and intensified doxorubicin and cisplatin regimens. In those patients high-dose methotrexate could be offered. In patients who relapsed, despite having received all five active chemotherapy agents (doxorubicin, cisplatin, methotrexate, ifosfamide and etoposide), but responded to methotrexate in the past, the use of further methotrexate could be considered.

1.3 METHOTREXATE - ROLE OF HD-MTX IN TREATMENT OF OSTEOSARCOMA

1.3.1 Description and use of methotrexate

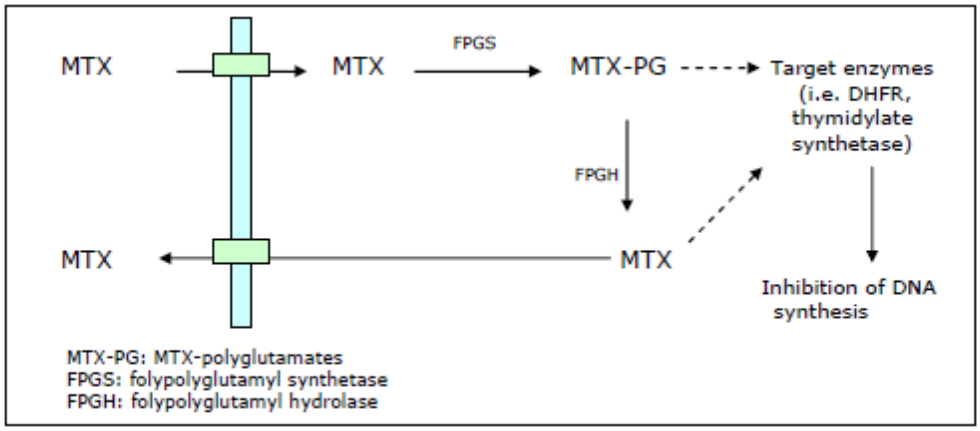
Methotrexate (MTX, Amethopterin, 4-NH_2-4-deoxy-N^{10}-methyl-pteroyglutamic acid) is an analogue of folic acid. The molecular structure of MTX differs from folic acid only in that it has a 4-amino group in place of the hydroxyl group on the pteridine ring, and a methyl group at the N^{10} position. It was first used in the treatment of childhood acute lymphoblastic leukaemia in 1948. Since then it has been used in the treatment of various malignancies

including osteosarcoma, non-Hodgkin's lymphoma, Hodgkin's disease, and breast cancer[6-8]. It is one of the few agents that can be given intrathecally and used for central nervous system involvement in leukaemia, lymphoma and solid tumours. Due to its immunosuppressive effects, it is used in rheumatoid arthritis, psoriasis and the prevention of graft-versus-host disease after bone marrow transplantation.

1.3.2 Mechanism of action of methotrexate (*figure 1.1*)

MTX inhibits dihydrofolate reductase (DHFR), the enzyme responsible for converting folic acid to reduced folate cofactors. Reduced folates are necessary for the transfer of 1-carbon units in a variety of biochemical reactions, such as the biosynthesis of thymidylic acid, the nucleotide specific to DNA, and the biosynthesis of inosinic acid, the precursor of purines necessary for both DNA and RNA synthesis[6].

Figure 1.1: Mechanism of action of methotrexate (Broken lines indicate enzyme inhibition)



1.3.3 Half life and transport of methotrexate

After intravenous administration, methotrexate disappearance from plasma is triphasic[9-10]. The initial half-life is 0.75 ± 0.11 h[9]. The second half-life has been reported as 2.06 ± 0.61[10], 3.49 ± 0.55[9] or 2.0-3.4 h[11]. The terminal half-life is 10.4 ± 1.8 h[10] and begins as the plasma antifolate concentration approaches 10^{-7}M, approximately 30-48 hrs after high-dose therapy. The first half-life is probably that of distribution and the second half-life that of renal clearance. The prolonged terminal phase probably represents a combined effect of release from deep compartments, enterohepatic circulation and renal tubular reabsorption, and is responsible for the major portion of gastrointestinal and bone marrow toxicity[6].

In human plasma, 50-70% of methotrexate is bound to protein, principally albumin. Alterations in plasma protein binding affect the amount of free extracellular methotrexate, which in turn influences the influx of methotrexate into cells and its rate of clearance by the kidneys[6].

The transport of MTX across the cell membrane is mediated by a saturable, energy dependent process which appears to be identical to that used for naturally occurring reduced folates[12-14]. Uptake is competitively inhibited by N^5-methyl-FH_4, the predominant circulating folate in mammals, as well as folinic acid (N^5-formyl-FH_4). The process involves two transport systems. The primary process is by the **reduced-folate carrier (RFC)**, which has high affinity for both reduced folates and methotrexate. The second transport system is the **human folate receptor (HFR)**, which has higher affinity for folic acid and reduced folates than MTX. At high extracellular concentrations (MTX serum levels >100µmol/L), MTX also enters cells by **passive diffusion**[15]. This appears to be the principal means of drug accumulation by MTX resistant cells that are deficient in carrier-mediated transport.

Once inside the cell, MTX undergoes polymerisation of the glutamic acid chain, similar to endogenous folates, to form methotrexate polyglutamates (MTX-PG). While both MTX and MTX-PG competitively inhibit DHFR, MTX-PG has enhanced binding to and inhibition of the enzyme and serves to enhance the retention and potency of MTX against target enzymes. The process of polymerisation happens more readily in tumour cells than in benign cells, which particularly explains selectivity of MTX for tumour cells. Formation of the MTX-PG is dependent upon intracellular MTX concentration and the duration of exposure.

1.3.4 Metabolism and excretion of methotrexate

Several metabolites have been found in human urine and plasma, particularly in patients receiving the highest doses of MTX[6]. The metabolites account for <10% of the total dose administered if MTX is given intravenously at 30mg/m^2. If given orally at the same dose, as much as 35% of the absorbed dose may be excreted as metabolites. The higher amount of metabolites after oral administration than after intravenous injection is consistent with the hypothesis that MTX metabolism in man occurs primarily in the gastrointestinal tract or enterhepatic circuit.

Under conditions of normal renal function MTX clearance from plasma is 110 cc/min/m^2, 103 cc/min/m^2 of which is due to renal clearance[16]. Approximately 41% of an intravenously administered dose is excreted unchanged in the urine within 6 h after administration, 90% within 24 h, and 95% within 30h[11, 17-18]. At very low plasma concentrations, MTX appears to be reabsorbed by the kidney[9]. At higher concentrations, the renal clearance of MTX is relatively constant[9] and exceeds that of inulin clearance[16], suggesting that MTX is not only

filtered but also actively secreted by renal tubular cells. One to 2% of an intravenously administered dose is excreted in the stool as the parent combound and metabolites[6].

1.3.5 Methotrexate resistance

MTX resistance may develop through a variety of mechanisms, including impaired transport of drug into the cell via the RFC[19], alterations in the affinity of DHFR for MTX[20-21] an increase in DHFR due to gene amplification or increased transcription[22-23], and diminished intracellular retention secondary to decreased polyglutamation[24].

In patients with osteosarcoma, conventional MTX dose therapy is ineffective and consequently high-dose MTX is used. Several retrospective studies have suggested that a threshold peak MTX level needs to be achieved to obtain good histological response to chemotherapy. Wei Guo et al.[25] have demonstrated that 65% of high grade OS samples at the time of initial biopsy were found to have decreased RFC expression, which suggests that impaired transport of MTX may be an important mechanism of intrinsic resistance in OS. This may partly explain why conventional dose of MTX is ineffective in the treatment of OS, as high doses may be needed to allow transport through alternatives means, such as passive diffusion. In the same study, although increased expression of DHFR was rare in the biopsy material, it was frequent in the recurrent pulmonary metastases and excision samples. Therefore it is possible that increased DHFR expression represents acquired MTX resistance, either through an acquired alteration in tumour cells or through selection of a previously resistant clone. Furthermore, Meyer et al. reported that xenografts of osteogenic sarcoma cells form predominantly short chain MTX polyglutamates, suggesting either relatively low FPGS activity, relatively high FPGH activity or a combination of these features[26].

Theoretically, all known mechanisms of MTX resistance could be overcome by administration of high doses of the drug. At high extracellular concentrations, passive diffusion of the drug may overcome resistance due to impaired membrane transport and the high intracellular levels achieved could overcome resistance due to the presence of increased DHFR levels or altered enzyme affinity. In addition, the availability of large amounts of free intracellular MTX might promote conversion to polyglutamate derivatives of the drug[6].

1.3.6 The role of HD-MTX in osteosarcoma

Before the use of chemotherapy, 80-90% of patients with non-metastatic osteogenic osteosarcoma died despite early radical surgery. With the use of multidrug chemotherapy, approximately two thirds of patients with non-metastatic resectable primary tumours can be cured[27]. The improved outcome has been attributed, in part, to the use of high-dose

methotrexate with folinic acid rescue as described by Jaffe et al. and emphasized by Rosen et al.[28-33]

The first effective drugs to be introduced into the treatment of osteosarcoma were doxorubicin and HD-MTX (high-dose MTX) .[29,34] Rosen et al.[32] combined these two drugs together with cyclophosphamide; bleomycin, cyclophosphamide and dactinomycin; and cisplatin. This innovative approach of aggressive multidrug chemotherapy provoked controversy and the role of HD-MTX was questioned in particular. Subsequently data from the study by Rosen et al.[32] were analyzed by Meyers et al.[35], who convincingly showed a histologically proven 19% response rate of single-drug HD-MTX in 54 patients.

Since then, Delepine et al.[36] in a review of 30 studies revealed that the total planned dose and dose intensity of HD-MTX correlates significantly with disease free survival in patients with localised high grade osteosarcoma. However, there is still controversy regarding the optimal MTX plasma levels and/or the duration of exposure that must be achieved for optimum efficacy. Some studies suggest that MTX plasma levels of ≥700 μM[37-38] or ≥1000 μM [39-40] at the end of a 4-6 h infusion improves histologic response and event free survival. These levels are achievable at the end of a 4 h intravenous MTX infusion of 12 g/m^2. [41] However, Crews et al.[41] reported that mean peak MTX levels >1500 μM is associated with lower event free survival, possibly because higher MTX levels are associated with more toxicity and therefore decreased dose intensity, or because increased folinic acid dosing in patients with very high MTX exposures may have compromised the antitumour effect of MTX. Others have observed significant differences in the disease free survival between patients whose mean AUC (area under the curve) was below or above 4000 μMh and recommended that the MTX dose should be increased such as to obtain an AUC >4000 μMh[42].

1.3.7 HD-MTX-induced toxicity

HD-MTX, 12 g/m^2, is administered with a combination of vigorous patient hydration and urinary alkalinisation along with a pharmacokinetically guided FAR schedule.

Despite currently used supportive measures, MTX-induced toxicity (myelosuppression, hepatotoxicity, nephrotoxicity, mucositis, and less commonly dermatitis and encephalopathy) still occurs, resulting in increased morbidity, suboptimal chemotherapy doses, delays in subsequent chemotherapy administration and possibly poorer outcome. There is wide inter- and intra-patient variability in relation to MTX tolerance[37-38, 43-45], the primary determinant of which appears to be variation in the pharmacokinetics of the drug.

Correlation of the toxic reactions with the drug's pharmacokinetics discloses certain time- and concentration-dependent relationships which appear to determine which target tissue is

at risk of toxicity. For bone marrow and gastrointestinal epithelium, the plasma concentration- and time-threshold appear to be 2 x 10^{-8} M and about 42 hours respectively[46-47]. The severity of toxicity is positively associated with the duration of MTX exposure beyond the time-threshold, and relatively less dependent on the magnitude of methotrexate elevation above the extracellular concentration-threshold[48].

In a recent internal review [49] of the medical records of 56 osteosarcoma patients treated with MAP regime at UCH, data from 175 chemotherapy cycles were collected in order to determine the incidence of delays in chemotherapy due to MTX toxicity. Fifty two percent of cycles (92/175) were delayed due to MTX toxicity, by a median of 7 days (range 1-28). Causes of delay included mucositis in 50.5% of cycles, bone marrow suppression in 28.5%, infection in 12%, nephrotoxicity in 8% and elevated liver enzymes in 1% of the cycles. Of 350 planned MTX courses, 19 (5.5%) were omitted due to previous MTX toxicity. Treatment with MTX was discontinued in 10% (6/56) of patients, due to MTX toxicity. Two FAR regimens were used. In regimen A, FA (folinic acid) dose was first adjusted according to 48 hour plasma MTX levels. In regimen B, which replaced regimen A, FA was adjusted based on 24 hour MTX levels. Regimen A was used in 98 cycles of which 57% (56/98) were delayed (median 7 days, range 1-28). Regimen B was used in 77 cycles of which 47% (36/77) were delayed (median 7 days, range 3-27).

1.4 ROLE OF FOLINIC ACID RESCUE AFTER HD-MTX[50]

Folinic acid is a racemic mixture of the stereoisomers of N^5-formyl-FH_4. The D- and L-isomers differ significantly in their cellular and clinical pharmacology, with only the L-isomer having the capacity to rescue cells from MTX toxicity. Following oral or parenteral administration, folinic acid is readily converted to N^5-methyl-FH_4, the primary circulating folate in humans.

The rationale for the use of folinic acid rescue is that provision of reduced folate to normal cells should circumvent the metabolic block produced by MTX and allow resumption of purine and pyrimidine synthesis. However, the selective rescue of normal tissues and not tumour cells has not been adequately explained, except perhaps when tumour cell resistance to MTX is caused by loss of the membrane transport system for reduced folates, thus excluding folinic acid from tumour cells[51].

Folinic acid competes with MTX for entry into the cell because it is actively transported by the same cell transport system as MTX. This observation forms the basis for one of the hypotheses of selectivity, because tumour cells with a defect in the folate transport system would not be rescued since insufficient folate would enter the cell. This is in contrast to normal cells with intact folate transport, which could be more easily rescued.

Experimental observations have shown that folinic acid is able to competitively displace MTX from DHFR, allowing its reactivation[52]. However, in the presence of MTX polyglutamates, such competitive displacement does not occur, and DHFR inhibition is sustained. The observation that most tumour cells synthesize much greater quantities of MTX polyglutamates than normal cells is central to understanding the selectivity of LV rescue. In **normal cells**, such as bone marrow precursors, which have few MTX polyglutamates, folinic acid administration promotes dissociation of MTX from DHFR, with consequent reactivation of the enzyme. Cellular levels of MTX polyglutamates then decrease rapidly, enabling reactivation of purine and thymidylate biosynthesis as reduced folate pools are restored. In **tumour cells** however, accumulation of MTX polyglutamates prevents competitive displacement from DHFR by folinic acid and DHFR inhibition is sustained. Thus cellular levels of MTX polyglutamates remain high and directly inhibit purine biosynthesis[51].

An important issue to consider is that the concentration of folinic acid needed to rescue normal cells from MTX is dependent upon the concentration of MTX present. The concentration of folinic acid must be high enough to compete effectively for transport into the cells of normal tissue. Importantly, with high MTX concentrations, even ten-fold higher folinic acid concentrations (concentrations that theoretically are achieved with supra-pharmacologic dosing of folinic acid) are unable to rescue normal haematopoietic cells[53].

1.5 ROLE OF GLUCARPIDASE (VORAXAZE™) RESCUE AFTER HD-MTX

Glucarpidase (Voraxaze™) (formerly Carboxypeptidase G2) is an enzyme originally isolated from *Pseudomonas sp* strain RV-308, cloned and now produced in *Escherichia coli*. It has a sub-unit molecular mass of 41,440 Da and dimeric molecular weight of approximately 83,000 Da[54].

Voraxaze™ is presented as a sterile, white lyophilized powder intended for single-use intravenous administration after reconstitution with 1.0 mL of sterile normal saline solution. Each vial of Voraxaze contains 1000 units of glucarpidase. One unit corresponds to the enzyme activity that cleaves 1 µmol/L MTX/min at 37°C. The product also contains approximately 10 mg of lactose as an inactive ingredient buffered to pH 7.0 to 8.0[55].

It is currently used effectively to treat patients with MTX-induced renal dysfunction, in order to avoid potentially fatal MTX-related toxicity. A single intravenous dose of 50 units/kg of glucarpidase after MTX results in the reduction of plasma MTX levels to the non-toxic range within minutes without causing toxicity.

1.5.1 Mechanism of action of glucarpidase

Glucarpidase hydrolyses the carboxyl terminal glutamate residue from folic acid and its analogues (e.g. MTX) [56]. The enzyme follows Michaelis-Menten kinetics with K_m values of 4

µmol/L for folate, 8 µmol/L for MTX, 34 µmol/L for 5-methyl-FH4, and 120 µmol/L for 5-formyl-FH4 (folinic acid). Glucarpidase has >10-fold lower affinity for folinic acid than for MTX, which is of significance when considering the potential combined use of glucarpidase and folinic acid for HD-MTX rescue[56]. Glucarpidase cleaves the MTX molecule into inactive metabolites, 4-deoxy-4-amino-N^{10}-methylpteroic acid (DAMPA) and glutamate, which are metabolised by the liver, and thus provides an alternative route of MTX elimination. This is particularly important in patients who develop renal dysfunction due to MTX nephrotoxicity[57-60] and would therefore not be able to renally excrete MTX. Such patients would be vulnerable to significant MTX toxicity due to sustained serum MTX concentrations.

1.5.2 Clinical studies with glucarpidase

The safety and effectiveness of glucarpidase on systemic MTX concentrations and MTX toxicities has been assessed in three clinical studies, the Berlin Study, PR001-CLN-001[61]; the NCI Study, PR001-CLN-002[62-63]; and the PD Study, PR001-CLN-006 (table 1.2). A study to determine the pharmacokinetics of glucarpidase in eight healthy normal subjects and four subjects with severe renal impairment was also performed (PK Study: PR001-CLN-005). In addition, an interaction study between glucarpidase and leucovorin (LV Interaction Study: PR001-CLN-010) was performed since it is known that leucovorin is also a substrate of glucarpidase.

Table 1.2: Clinical studies undertaken with glucarpidase for which efficacy data has been reported

Study reference (descriptor)	Years	Lot No.	Study title	No. & location of sites	No. of patients treated (ES)
PR001-CLN-006 (PD Study)	2004 – 2005	Lot 2090302	National Cancer Institute - Special Exception Protocol for the Use of Carboxypeptidase-G2 for MTX Toxicity	55 sites in USA, Australia and Canada	68[a] (23)
PR001-CLN-002 (NCI Study)	1993 – 2004	CAMR Lot 004	National Cancer Institute - Special Exception Protocol for the Use of Carboxypeptidase-G2 ± Thymidine for MTX Toxicity and Renal Failure	149 sites in USA, Australia, Canada, Hungary, Israel, Germany, Austria, France & Italy	227[b] (70)
PR001-CLN-001 (Berlin Study)	2000 – 2003	CAMR Lot 004	A study of Recombinant Carboxypeptidase G2 for the Management of Patients with Delayed Methotrexate (MTX) Clearance or Intrathecal MTX Overdose	28 sites in Germany	42[c] (23)

Note: All studies were open label design compassionate use
ES = Efficacy Subset (patients who had both a pre- and post-glucarpidase MTX concentrations measured by HPLC and had a pre-glucarpidase MTX concentration ≥1 µmol/L)
[a] Safety data for Voraxaze are available for 64 of the 68 patients who were treated
[b] Safety data for Voraxaze are available for 212 of the 227 patients who were treated
[c] Safety data for Voraxaze are available for all 42 patients who were treated

1.5.2.1. PK Study

The PK study was an open-label, single site, pharmacokinetic study of Voraxaze administered intravenously at a dose of 50 units/kg to eight subjects with normal renal function and four subjects with severe renal impairment. Two assay methods were used to quantify serum glucarpidase concentration, one measured glucarpidase enzyme activity and the other total glucarpidase. The pharmacokinetic data based on total glucarpidase indicated about 7% lower mean C_{max} in subjects with impaired renal function relative to those with normal renal function. However, the total glucarpidase exposure, as determined by $AUC_{0-\infty}$, was marginally higher, by about 5%, in subjects with impaired renal function relative to those with normal renal function. The median time to maximum serum concentration (T_{max}) was short for subjects with normal renal function, indicating a rapid equilibration of glucarpidase after completion of the short infusion. Based on total glucarpidase data, no significant differences were noted in mean $t_{½}$ between subjects with normal and impaired renal function. Large variability was noted especially for AUC_{0-t} and $AUC_{0-\infty}$, in the renally impaired subject group, with one subject having a higher exposure relative to the other three. Overall, the results of the PK Study showed little effect of renal impairment on the serum pharmacokinetics of glucarpidase.

1.5.2.2. LV Interaction Study

This study was a double-blind, placebo controlled, randomised, two-period crossover pharmacokinetic study. The primary objective was to assess the effect of glucarpidase on the pharmacokinetics of the active L-stereoisomer of leucovorin (6)L/S-LV following repeated doses of LV. The secondary objecyives of the sudy were to assess the effect of glucarpidase on the pharmacokinetics of 5-methyl-tetrafydrofolate, (6)L/S-LV-THF, the active metabolite of (6)L/S-LV. The study demonstrated that glucarpidase reduces the systemic availability of (6)L/S-LV, by up to 50%, and its active metabolite (6)L/S-LV-THF, but does not completely eliminate them. The reduced availability of (6)L/S-LV and its active metabolite could potentially lead to a reduction in the efficacy of LV.

1.5.2.3. PD, NCI and Berlin Studies

The above three studies were all compassionate use, multiple site, single arm, open label studies to provide access to the drug and assess the safety of 50 units/kg Voraxaze given intravenously to patients with delayed elimination of MTX due to renal impairment. The majority of patients (65%) were enrolled in the NCI Study. The primary efficacy endpoint for all three studies was the proportion of patients who achieved a clinically important reduction (CIR) in plasma MTX concentration as measured by HPLC. A CIR is defined as a plasma or serum MTX concentration that has decreased to ≤1 µmol/L in all post glucarpidase samples, indicating a sustained reduction of MTX. The primary efficacy

evaluation was performed on patients (the Efficacy Subset, ES) with valid primary efficacy data which included a pre-glucarpidase MTX concentration ≥1 µmol/L determined by HPLC and at least one MTX concentration by HPLC after the last glucarpidase dose. A comparison across the three studies regarding demographics, diagnosis, renal function, MTX level at entry and glucarpidase doses, is described in Table 1.3. Comparison of the Primary Efficacy Subsets across the studies is described in Table 1.4.

In all three studies the majority of patients treated with glucarpidase achieved CIR in MTX and thus met the primary endpoint. The reductions in MTX were consistently attained, rapid and generally sustained. The proportion of patients achieving CIR was 57% (13/23) for the PD Study, 57% (40/70) for the NCI Study and 83% (19/23) for Berlin Study. The primary analyses of CIR in all three studies were repeated for subgroups of patients based on pre-glucarpidase plasma/serum MTX concentrations, a diagnosis of osteosarcoma, gender and age (Table 1.5). In summary, a CIR was seen in 62% (72/116) of patients in the ES across studies and >50% of patients in the ES of each study had a CIR. The incidence of CIR was lower for the subgroups with higher pre-glucarpidase MTX concentrations, lower for patients with osteosarcoma, lower for female patients and higher for adult patients.

Secondary efficacy endpoints for the three clinical studies included the measurement of the reduction in plasma/serum MTX concentrations by HPLC immediately after the administration of glucarpidase (immediate reduction) and in all samples taken post glucarpidase (sustained reduction). Immediately (10-50 minutes, median 15 minutes) after glucarpidase administration, plasma/serum MTX concentrations for patients had fallen by >95% in the PD and NCI Studies and by >97.8% in the Berlin Study. There was a gradual increase in MTX concentration followimg the immediate post-glucarpidase reduction, presumably because some MTX redistributes out of the cells. When expressed as a ratio of per-glucarpidase value, the median sustained reduction in MTX was 98.7%, 97.8% and 96.5% for the PD, NCI and Berlin Studies respectively.

Table 1.3: Comparisons across studies (demographics, prior MTX treatment history and MTX level at entry, and glucarpidase doses) - PD, NCI, Berlin Studies

Demographic	PD Study		NCI Study		Berlin Study	
	No. (%[a]) of patients		No. (%[a]) of patients		No. (%[a]) of patients	
	Pediatric	Adult	Pediatric	Adult	Pediatric	Adult
No. patients	68		227		42	
Age - No. of patients with data	67/68		216/227		42/42	
Age by pediatric or adult (≥18) [≥65]	31 (46%)	36 (54%) [7(10%)]	115 (53%)	101 (46%) [31(14%)]	1 (2%)	41 (98%) [7(17%)]
Age range (median)	2-84 (20)		0-82 (17)		10-78 (52)	
Gender – No. of patients with data	N=68/68		N=197/227		N=0/42	
Male	42 (62%)		127 (58%)		-	
Female	26 (38%)		70 (32%)		-	
Cancer diagnosis[b] No. patients	68/68 (67 with age)		188/216		42/42	
Osteosarcoma (102)	17/25 (69%)	7/25 (28%)	55/77 (71%)	22/77 (29%)	-	-
Leukaemia (61)	9/13 (69%)	4/13 (31%)	24/36 (67%)	12/36 (33%)	1/12 (1%)	1/121 (92%)
Lymphoma (122)	3/24 (13%)	21/24 (88%)	22/70 (31%)	48/70 (69%)	-	28/28 (100%)
Other cancers (12)	2/6 (33%)	4/6 (67%)	-	4/4 (100%)	-	2/2 (100%)
Non Cancer (1)			-	1/1 (100%)	-	-
Unknown Total (28)	-	-	14 (45%)	14 (45%)	-	-
Patients with renal impairment (serum creatinine >2.2 mg/dL)[c]	15/28 (54%)	30/34 (88%)	58/105 (55%)	70/86 (81%)	0/1 (0%)	22/40 (55%)
MTX						
Dose range in g/m^2 (median)	1.0-20 (6.7) (68 patients)		0.4-19 (5.5) (217 patients)		0.9-12 (3) (42 patients)	
Pre-glucarpidase concentration in µmol/L (median)	3.47 - 708 (40.2) ES (23 patients)		1.1-849.10 (34.7) ES (70 patients)		1.1-166 (5.8) ES (23 patients)	
Glucarpidase dose range in Units/kg (median)	29.2-55 (50)		10.9-63.7 (49.8)		9.8-58 (50)	

Source: Voraxaze Investigator's Brochure edition number PR001-CLN-IB007

[a] Percentages have been rounded to the nearest whole number.

[b] One patient in the NCI Study did not have cancer and one patient in the PD Study had a diagnosis of osteosarcoma but age is not available.

[c] Patients with impairment equivalent to serum creatinine >1.5 x ULN post-MTX but pre-glucarpidase.

Table 1.4: Comparisons of the Primary Efficacy Subsets across studies (demographics, prior MTX treatment history and MTX level at entry, and glucarpidase doses) - PD, NCI, Berlin Studies

Demographic	PD Study		NCI Study		Berlin Study	
	No. (%[a]) of patients		No. (%[a]) of patients		No. (%[a]) of patients	
	Pediatric	Adult	Pediatric	Adult	Pediatric	Adult
No. patients	23/68		70/219		23/42	
Age - No. of patients with data	23/23		68/70		NA (data not collected)	
Age by pediatric or adult (≥18)	13 (57%)	10 (43%)	39 (57%)	29 (43%)	NA	NA
Gender – No. of patients with data	N=23/23		N=61/70		N=0/23	
Male	14 (61%)		35 (50%)		-	
Female	9 (39%)		26 (37%)		-	
Cancer diagnosis[b] No. patients	23/23		57/70		23/23	
Osteosarcoma (102)	11 (48%)		35 (61)		-	
Leukaemia (61)	3 (13%)		7 (12%)		8 (35%)	
Lymphoma (122)	8 (35%)		12 (21%)		13 (56%)	
Other cancers (12)	1 (4%)		3 (5%)		2 (9%)	
Non Cancer (1)	-		-		-	
Unknown Total (28)	-		13		-	
MTX						
Dose range in g/m^2 (median)	1.4-20 (8)		0.4-19 (8) (69 patients)		1.1-4.6 (2.9)	
Pre-glucarpidase concentration in µmol/L (median)	3.47 - 708 (40.2)		1.1-849.10 (34.7)		1.1-166 (5.8)	
Time between MTX & 1st dose glucarpidase (median)	1-6 (2) days		1-9 (3) days		1-4 (2) days	
Glucarpidase dose range in Units/kg (median)	39-52 (50)		15-55 (50)		11-58 (50)	

Source: Voraxaze Investigator's Brochure edition number PR001-CLN-IB007

[a] Patients with serum or plasma MTX concentration by HPLC prior to Voraxaze dosing, and in at least one sample after the last dose of Voraxaze and who had MTX ≥ 1 µmol/L prior to Voraxaze.

Table 1.5: Exploratory sub-group analyses of CIR based on pre-Voraxaze plasma/serum MTX concentration, cancer diagnosis and gender – PD, NCI and Berlin Studies

	Proportion of patients with a clinically important reduction (CIR) in plasma MTX [percentage] (95% CI)[a]		
Pre-Voraxaze plasma/serum MTX concentration	**PD Study**	**NCI Study**	**Berlin Study**
<1 µmol/L	3/3 [100%] (44% to 100%)	4/4 [100%] (51% to 100%)	3/3 [100%] (44% to 100%)
≥1 µmol/L and <10 µmol/L	4/5 [80%] (38% to 96%)	20/22 [91%] (72% to 97%)	13/14 [93%] (69% to 99%)
≥10 µmol/L and <100 µmol/L	7/11 [64%] (35% to 85%)	13/27 [48%] (31% to 66%)	6/8 [75%] (41% to 93%)
≥100 µmol/L	2/7 [29%] (8% to 64%)	7/21 [33%] (17% to 55%)	0/1 [0%] (0% to 79%)
Cancer diagnosis of osteosarcoma?			
Yes	3/11 [27%] (10% to 57%)	17/35 [49%] (33% to 64%)	ND
No	10/12 [83%] (55% to 95%)	14/22 [64%] (43% to 80%)	ND
Gender			
Male	11/14 [79%] (52% to 92%)	23/35 [66%] (49% to 79%)	NK
Female	2/9 [22%] (6% to 55%)	12/26 [46%] (29% to 65%)	NK

Source: Voraxaze Investigator's Brochure edition number PR001-CLN-IB007
ND = Not done (no patient in the Berlin Study had osteosarcoma); NK = Not known (gender was not recoded in the Berlin Study)
[a] Using the Newcombe & Altman statistical method

As of February 2009, a total of 337 patients treated with intravenous glucarpidase have been reported. In all patients with evaluable MTX concentration data pre- and post-administration of glucarpidase, plasma MTX concentrations decreased >95% by the time the first post-glucarpidase sample was taken, usually about 15 minutes after glucarpidase administration.

For patients at risk of MTX toxicity due to delayed MTX elimination or inadvertent overdose, glucarpidase rescue has been shown to be a safe and effective addition to FAR. Glucarpidase allows patients to then be managed with standard doses of folinic acid and, if administered early, greatly diminishes the risk of serious and/or life-threatening MTX toxicity. Moreover, patients avoid the risks associated with extracorporeal methods of MTX removal such as dialysis.

Glucarpidase rapidly converts MTX to the inactive metabolite DAMPA. *In vitro* experiments have shown that DAMPA has no cytotoxic potential and does not enhance MTX cytotoxicity[60]. DAMPA is known to be eliminated renally and by metabolism. Data from the

PD, NCI and Berlin Studies confirm that DAMPA is effectively eliminated in patients even though almost all had renal insufficiency, with a median half-life of about 11 hours. A theoretical concern regarding the use of glucarpidase is that the rapid formation of DAMPA, which is approximately 10-fold less soluble than MTX, could lead to further renal toxicity by precipitation in the renal tubules[64]. However, there is no indication from the clinical studies that DAMPA has caused renal toxicity.

Glucarpidase has >10-fold lower affinity for folinic acid than for MTX. However, folinic acid is a substrate for glucarpidase and thus may compete with MTX for glucarpidase binding sites. Hence, glucarpidase has the potential to reduce the efficacy of folinic acid. When glucarpidase was administered in healthy subjects, their exposure to folinic acid was reduced by about 50%, **in the absence of MTX**; exposure to folinic acid was reduced for about 26 hours. Therefore, in order to minimize any potential interaction, it is recommended that folinic acid should not be administered in the 2-4 hours prior to or in the 2-4 hours following glucarpidase. In order to compensate for any reduced exposure to folinic acid caused by its potential interaction with glucarpidase, it is advised that the dose of folinic acid should be based upon the pre-glucarpidase MTX plasma levels and maintained at this dose for at least 48 hours after the dosing with glucarpidase.

1.5.3 Adverse effects

Glucarpidase is a pure enzyme (>98% purity) with a specific activity to hydrolyse the N-carboxy terminal of folate-related molecules. It is administered specifically to hydrolyse MTX. It is not expected to have any adverse effects related to its pharmacological action when given as a single dose for the treatment of a single MTX course.

In the previously described PD, NCI and Berlin Studies 52 glucarpidase-related adverse events (AEs) were reported in 26/318 patients (Table 1.6); only two of the glucarpidase-related AEs were reported as serious. Allergic reaction was the most common glucarpidase-related AE, occurring in 4% of patients. Allergic reactions were temporally related and included symptoms of flushing, paraesthesia, and feelinghot/burning sensation. These reactions occurred primarily on the same day as dosing and all were transient. No anaphylactic reactions have been reported in any of the studies. The most common non-allergic AE considered related to treatment with glucarpidase was paraesthesia (0.6% of patients). Two serious adverse events (SAEs), hypertension and arrhythmia, that may have been related to glucarpidase administration, occurred on the NCI and PD Studies, respectively. No glucarpidase-related AEs were reported in the PK Study. One AE associated with glucarpidase was reported in the LV Interaction Study.

Table 1.6: All AEs considered related to glucarpidase- PD, NCI and Berlin Studies (N=318, Although 329 patients were treated in these studies and included in the study reports, data from follow-up assessments after glucarpidase dosing is only available for 318)[55]

Body System/Adverse Event	No. of patients with AE (N= 318*)	Percentage of patients
ALL	**26**	**8.2%**
Blood and lymphatic system disorders	**1**	**0.3**
Pancytopenia	1	0.3
Cardiac disorders	**2**	**0.6**
Arrhythmia	1	0.3
Tachycardia	1	0.3
Gastrointestinal disorders	**4**	**1.3**
Abdominal pain	1	0.3
Diarrhoea	1	0.3
Nausea	2	0.6
Oral discomfort	1	0.3
vomiting	1	0.3
General disorders and administration site conditions	**4**	**1.3**
Feeling hot	3	0.9
Pyrexia	1	0.3
Immune system disorders	**1**	**0.3**
Hypersensitivity	1	0.3
Investigations	**2**	**0.6**
Aspartate aminotransferase increased	1	0.3
Blood creatinine increased	1	0.3
Blood urea increased	1	0.3
Metabolism and nutrition disorders	**1**	**0.3**
Hypokalaemia	1	0.3
Hyponatraemia	1	0.3
Nervous system disorders	**11**	**3.5**
Burning sensation	3	0.9
Headache	3	0.9
Paraesthesia	7	2.2
Tremor	2	0.6
Renal and urinary disorders	**1**	**0.3**
Oliguria	1	0.3
Respiratory, thoracic and mediastinal disorders	**2**	**0.6**
Dyspnoea	1	0.3
Throat irritation	1	0.3
Throat tightness	1	0.3
Skin and subcutaneous tissue disorders	**2**	**0.6**
Dermatitis allergic	1	0.3
Hyperhidrosis	1	0.3
Pruritus	1	0.3
Vascular disorders	**11**	**3.5**
Flushing	7	2.2
Hot flush	1	0.3
Hypertension	1	0.3
Hypotension	2	0.6

Glucarpidase is a recombinant bacterial protein and it therefore has the potential to induce an immune response. This could produce an allergic reaction on subsequent administration of glucarpidase or reduce its efficacy by neutralizing the activity of glucarpidase.

Across the PD, NCI, Berlin and PK Studies 43% (26/61) of subjects developed anti-glucarpidase antibodies after administration of glucarpidase. Of the positive samples, 19% (5/27) subjects inhibited the activity of glucarpidase. The reduction in enzyme activity in the five subjects who had samples which caused a reduction were 35%, 49% and 84% for the PD Study, 23% for the Berlin Study and 44% for the PK Study. Only one of the positive samples in the PK Study inhibited the enzyme activity of glucarpidase by ≥20% (44%).

Since glucarpidase eliminates methotrexate in the blood, there is a risk that it could make methotrexate less effective. To mininise this risk glucarpidase is given at 24 hours after the beginning of methotrexate infusion. It is standard practice to start rescue from methotrexate at 24 hours after the beginning of methotrexate infusion.

Patients with allergic reactions can be symptomatically managed. Patients with Grade 1 allergic reactions can be pre-medicated (antihistamines, steroids, and/or acetaminophen) prior to receiving a subsequent administration of glucarpidase. All patients with Grade ≥2 allergic reaction or hypersensitivity will be removed from the study (Grading for these reactions is according to CTCAE V.3.).

2. STUDY OBJECTIVES

2.1 Primary Objective

1. To investigate whether glucarpidase rescue after high-dose methotrexate reduces delay to subsequent cycle of chemotherapy due to methotrexate toxicity

2.2 Secondary Objectives

1. To investigate whether glucarpidase rescue after high-dose methotrexate reduces the incidence, severity and duration of methotrexate associated adverse effects
2. To study the pharmacokinetics of methotrexate and 4-deoxy-4-amino-N10-methylpteroic acid (DAMPA) after glucarpidase administration
3. To evaluate any adverse effects associated with the use of glucarpidase
4. To investigate the economic impact of using glucarpidase versus standard rescue
5. To investigate the effect of glucarpidase on the quality of life of patients treated with high-dose methotrexate
6. To assess the anti-glucarpidase antibody response

3. STUDY ENDPOINTS

3.1 Primary Endpoint

1. Assessment of fitness to receive chemotherapy on day 15 of each cycle (see section 8 for fitness criteria)

3.2 Secondary Endpoints

1. Incidence and grading of mucositis, renal toxicity, liver toxicity, neutropaenia, thrombocytopaenia and infections
2. Plasma methotrexate and DAMPA concentrations
3. Incidence of glucarpidase related adverse effects
4. Number of days required in hospital per cycle and total dose of folinic acid required per cycle
5. Assessment of quality of life
6. Serum anti-glucarpidase IgG levels following glucarpidase administration

4. STUDY DESIGN

4.1 Description of study

This is a prospective, randomised, cross-over, phase II study, to investigate the efficacy and safety of glucarpidase for routine use after high dose methotrexate in patients with bone sarcoma.

This study will be conducted in compliance with the principles of ICH GCP, the Declaration of Helsinki (Appendix 1), the Medicines for Human Use (Clinical Trials) Regulations 2004 and all other applicable regulatory requirements.

Patients participating in the study will receive four consecutive methotrexate courses, of which two will be given with standard folinic acid rescue and two with a combination of folinic acid and glucarpidase rescue.

4.2 Definition of treatment courses

Course M: Methotrexate (12 g/m^2 x 1, intravenously) with standard folinic acid rescue and placebo (0.9% Normal Saline injection BP).

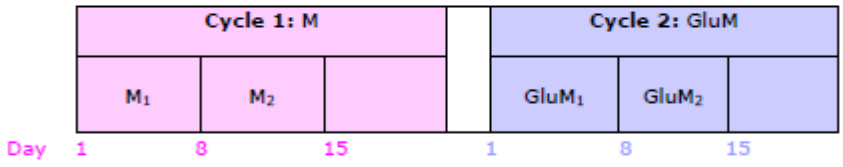
Course GluM: Methotrexate (12 g/m^2 x 1, intravenously) with folinic acid and glucarpidase rescue (50 units/kg x 1, intravenously).

Each cycle of treatment comprises of two courses of methotrexate (see section 4.3).

4.3 Scheduled treatment dates

Patients will be randomised to **arm A** or **B** in a 1:1 randomisation. Each arm consists of two cycles. In **arm A** patients will receive cycle M first followed by cycle GluM, whereas in **arm B**, they will receive cycle GluM first followed by cycle M.

Arm A:

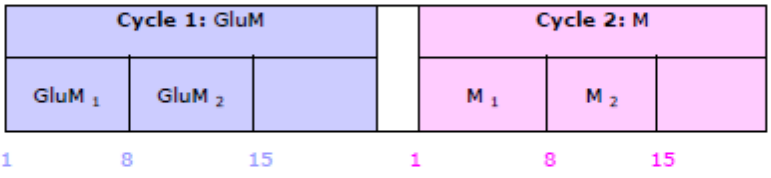


Cycle M starts with course **M_1** on day 1 followed by course **M_2** planned for day 8.

Cycle GluM starts with course **$GluM_1$** on day 1 followed by **$GluM_2$** planned for day 8. Cycle GluM will not start for a minimum of 14 days from the beginning of course M_2, or until bone

marrow, renal and hepatic functions have completely recovered and the patient is clinically ready to receive further chemotherapy (see section 7.5 for minimum requirements). A minimum of 14 days from M_2 to the beginning of the second cycle seems appropriate to allow complete recovery.

Arm B:



Cycle GluM starts with course **$GluM_1$** on day 1 followed by **$GluM_2$** planned for day 8.

Cycle M starts with course **M_1** on day 1 followed by course **M_2** planned for day 8. Cycle M will not start for a minimum of 14 days from the beginning of course $GluM_2$, or until bone marrow, renal and hepatic function have completely recovered and the patient is clinically ready to receive further chemotherapy (See section 7.6 for minimum requirements).

4.4 Study centres

The study will be undertaken at the University College Hospital (UCH), London, UK and Harley Street at UCH, London, UK.

4.5 Registration and randomisation

Eligible patients will be registered following fully informed consent. There is no time limit between confirmation of diagnosis and registration. A registration form for each patient will be completed by the investigators who will assign a unique study number to each participant. The registration form will be emailed and faxed to UCH Pharmacy Department within 24 hours from registration (UCH Pharmacy Department contact details in Appendix 12.1). UCH Pharmacy will assign the next available treatment slot according to the randomisation list provided by the UCLH Medical Statistics Department for this study and fax the randomisation details to Dr Jeremy Whelan on 0207 380 9055. The randomisation list will be computer generated and blocked to ensure approximately equal number of patients are allocated to the two sequences. For participants treated at Harley Street at UCH, UCH Pharmacy will fax the randomisation details to 020 7691 5867, for the attention of Tracey Patrick, Haematology Team Leader, HCA International Limited.

In order to minimize any cumulative dose effect which may interfere with methotrexate pharmacokinetics and associated toxicity, half of the patients will follow arm A and half, arm B. The cross-over design allows smaller sample size, since patients act as their controls.

4.6 Expected number of patients

Based on previous experience, nearly 55% of the patients in the standard rescue group are expected to be treatable on day 15 of each cycle[49]. The sample size calculation is based on a McNemar's test with an assumption that the responses to glucarpidase and folinic acid rescue are independent[65]. With anticipated proportions of responses to standard rescue & placebo and glucarpidase+folinic acid of 55% and 90% respectively, the study will require 38 patients to give 80% power at a significance level of 5% and to allow for up to 30% drop-out during the study.The O'Brien and Flemming boundary method will be used for testing significance of effect estimates.

4.7 Study duration

The planned duration of the study is 6 weeks for each participant, ie 21 days after starting cycle 2. However, patients will be followed up on day 30 after starting their second chemotherapy cycle, to investigate anti-glucarpidase antibody response. If anti-glucarpidase antibodies are present on that day, patients will be followed up for a further blood test, at 3 and 6 months after starting cycle 2.

The planned duration of the entire study is 4 years.

4.8 Feasibility of recruitment

The London Sarcoma Service at UCH is the largest clinical practice treating bone sarcomas in the UK and one of the largest in Europe, treating 30-40 patients with osteosarcoma every year. Anticipated accrual for this study is 10 patients per year.

5. SELECTION OF STUDY PATIENTS

5.1 Inclusion Criteria

Patients must fulfil the following criteria for registration on study:

- Written informed consent from patient or parent/guardian
- Diagnosis of high grade osteosarcoma, localised or metastatic
  or high grade osteosarcoma as a second maligancy
  or spindle cell sarcoma of bone
  or relapsed high grade osteosarcoma
- Age: 5-50 years at registration

- Ability to comply with study and follow up procedures (WHO performance scale 0-2)
- No concomitant anti-cancer or investigational drugs during the study and complete resolution of toxicity related to previous treatment
- Life expectancy of at least 3 months
- Haematopoietic function: Absolute neutrophil count ≥1 $x10^9$/L, Platelets ≥100 $x10^9$/L
- Hepatic function: Bilirubin ≤1.5 x ULN, ALT ≤1.5 x ULN, Albumin >LLN
- Renal function: Glomerular Filtration Rate (radioisotope) ≥ 70 ml/min/1.73m^2

5.2 Exclusion Criteria

- Previous treatment with glucarpidase
- Pregnant or breast feeding women (patients with reproductive potential of either gender must use contraception*)
- Concomitant treatment with agents which interact with methotrexate metabolism or excretion (see Appendix 8)
- Serous effusions, including ascites and pleural effusions

* Adequate means of contraception:

(i) Combined oral contraceptive pill
(ii) Long acting progesterone only methods (such as the 3-monthly depo injection or the implant)
(iii) Intrauterine contraceptive device or intrauterine device
(iv) Contraceptive patch
(v) Sterilisation

6. STUDY ASSESSMENTS

Clinical and laboratory assessments will take place as follows:

- Urine pregnancy test (pre-menopausal women only): Day -7 to 0
- Written informed consent: Day -7 to 0
- Medical History: Day -7 to 0
- Concomitant Medications: Day -7 to 0, 8, 15 of cycle 1 & day 1, 8, 15 of cycle 2
- Physical examination: Day 1, 8, 15 of cycle 1 & day 1, 8, 15 of cycle 2
- Performance status: Day 1, 8, 15 of cycle 1 & day 1, 8, 15 of cycle 2
- Weight, Body Surface: Day 1, 8 and 15 of cycle 1 & day 1, 8, 15 of cycle 2
- Haematology (haemoglobin, white cell count, absolute neutrophil count, platelets):
  Cycle 1: Day 0 or 1, day 8 and at least every 3rd day until haematological recovery, day 15
  Cycle 2: Day 1, day 8 and at least every 3rd day until haematological recovery, day 15
- Serum creatinine, urea, Na+, K+, Ca++, glucose, phosphate, magnesium:

Cycle 1: Day 0 or 1 and at least daily until completion of rescue, day 8 and at least daily until completion of rescue, day 15

Cycle 2: Day 1 and at least daily until completion of rescue, day 8 and at least daily until completion of rescue, day 15

- Glomerular Filtration Rate

  a) By radionuclide measurement/EDTA clearance: Day -7 to 0 of cycle 1

  b) By estimation (see Appendix 8): Day 8, 15 of cycle 1 & day 1, 8, 15 of cycle 2

- Hepatic Function (ALT, bilirubin, total protein, albumin):

  Cycle 1: Day 0 or 1 and daily until completion of rescue, day 8 and daily until completion of rescue, day 15

  Cycle 2: Day 1 and daily until completion of rescue, day 8 and daily until completion of rescue, day 15

- Methotrexate pharmacokinetic assessment by immunoassay:

  For both cycles:

  On day 1 and 8: at 24 hours after starting methotrexate
  at 48 hours after starting methotrexate
  at 72 hours after starting methotrexate
  and then daily until MTX plasma levels < 0.2 µmol/L

- Methotrexate and DAMPA pharmacokinetic assessment by High Performance Liquid Chromatography:

  For both cycles:

  On day 1 and 8: at 0 hours, prior to starting the methotrexate
  at 4 hours, at completion of methotrexate
  at 24 hours after starting methotrexate
  at 24:20 hours after starting methotrexate (for glucarpidase cycles only, 15 minutes after completion of glucarpidase administration)
  at 48 hours after starting methotrexate
  at 72 hours after starting methotrexate
  and then daily until MTX plasma levels < 0.2 µmol/L

- Anti-glucarpidase IgG levels and enzyme neutralisation assay:

  Cycle 1: Day 0, 8 and 15

  Cycle 2: Day 1, 8, 15, 30 and 3 and 6 months after starting cycle 2.

- Glucarpidase Adverse Events: Day 2 of cycle 1 and daily until discharge, day 9 of cycle 1 and daily until discharge, day 2 of cycle 2 and daily until discharge, day 9 of cycle 2 and daily until discharge
- Mucositis Assessment (Appendix 3): Day 1, 8, 15 of cycle 1 & day 1, 8, 15 of cycle 2
- Quality of Life Assessment (Appendix 4): Day 1, 8, 15 of cycle 1 & day 1, 8, 15 of cycle 2
- Total number of days in hospital for each cycle
- Total dose of folinic acid used for each cycle

7. STUDY MEDICATION AND TREATMENT PLAN

7.1 Agents used

The following agents will be used in this study:

- Methotrexate
- Folinic Acid
- Glucarpidase (Voraxaze™)

7.2 Drug availability and accountability

7.2.1 Methotrexate and folinic acid:

Methotrexate and folinic acid will be obtained from UCH Pharmacy or Harley Street at UCH Pharmacy. The supplier's recommendations regarding storage, stability, dilution, incompatibilities, and measures of caution should be followed (see appropriate package inserts and Appendix 6 for further information).

7.2.2 Glucarpidase (Voraxaze™)

Glucarpidase (Voraxaze™) is the Investigational Medicinal Product in this study.

Labelled vials of Voraxaze™, containing sterile, lyophilised glucarpidase (1000 units/vial), will be supplied. The drug substance is manufactured by Eurogentec S.A. Rue du Bois Saint Jean, 14 Liege Science Park B-4102 Seraing (Liege), Belgium. The sterile filling and freeze-drying is performed by Cangene Corporation, 104 Chancellor Matheson Road, Winnipeg, Manitoba, Canada R3T 5Y3. The unlabelled drug product is then QP released by Protherics UK Limited, Blaenwaun, Ffostrasol, Llandysul, Ceredigion, Wales, UK, SA44 5JT. The final labelled drug product is then supplied by Biotec Distribution Wales Ltd, 17 St Theodore's Way, Brynmenyn Industrial Estate, Bridgend, CF32 9TZ, UK. An approved copy of the label will be submitted to the Regulatory Authorities. Voraxaze™ will be supplied together with batch numbers and a certificate of release authorised by Protherics.

UCH Pharmacy and Harley Street at UCH Pharmacy will be responsible for appropriate storage of Voraxaze™ vials according to supplier's recommendations, to ensure stability and intergrity of glucarpidase. UCH Pharmacy and Harley Street at UCH Pharmacy will also be responsible for maintaining a careful record of receipt, use and disposition of unused supplies of Voraxaze™. The sponsor, ITMC and Protherics will be permitted, at intervals and upon request during the study, to check supplies storage, assembly procedures and records (provided that this does not unblind the study). Samples of the batch of Voraxaze™ used in the study will be retained by Protherics for 2 years after completion of the study.

Following completion of the study and sponsor review of accountability, all unused supplies will be destroyed and certificates of distruction provided to the sponsor and Protherics.

7.3 Prescription of methotrexate, folinic acid and glucarpidase

Methotrexate and glucarpidase will be prescribed by qualified medical staff, on ChemoCare®. Folinic acid will be prescribed by qualified medical staff, on ChemoCare® and standard UCH and Harley Street at UCH drug charts. All prescriptions will be kept within the participants' notes.

7.4 Mandatory assessments prior to each chemotherapy course

- Height, weight and surface area
- Clinical examination and concomitant medication
- Full blood count and differential white cell count
- Blood chemistry (creatinine, urea, sodium, potassium, magnesium, phosphate, albumin, ALT, bilirubin)
- Measurement of GFR:
  a) by radio-isotopic method prior to the first course of cycle 1 for all patients
  b) by estimation (see suggested formulae in Appendix 7) prior to all other courses
- Mucositis assessment (see Appendix 3)
- Quality of life assessment (see Appendix 4)
- Performance status (see Appendix 2)
- Blood sample for anti-glucarpidase antibodies and enzyme neutralisation assay

7.5 Minimum requirements prior to each chemotherapy course

7.5.1 Minimum requirements prior to course M_1 and $GluM_1$ in both arms

1. General clinical condition permitting chemotherapy including no evidence of infection, no pyrexia, mucositis or diarrhoea
2. No serous effusions including ascites and pleural effusions
3. Neutrophils $\geq 1x10^9$/L and platelets $\geq 100x10^9$/L
4. GFR ≥ 70 ml/min/1.73m^2
5. Bilirubin ≤ 1.5 x ULN, ALT ≤ 1.5 x ULN, Albumin > LLN
6. Urinary pH >7.0 immediately prior to MTX and good urine output

7.5.2 Minimum requirements prior to course M_2 and $GluM_2$ in both arms

1. General clinical condition permitting chemotherapy including resolving mucositis ≤ grade 1 but no evidence of infection, no pyrexia
2. No serous effusions including ascites and pleural effusions

3. Neutrophils ≥0.25x10^9/L and platelets ≥50x10^9/L
4. Bilirubin ≤1.5 x ULN
5. Transaminases: may be any value in the absence of other causes of liver dysfunction
6. GFR ≥70 ml/min/1.73m^2
7. Urinary pH >7.0 immediately prior to MTX and good urine output

7.6 Schedule of therapy

7.6.1 $GluM_1$ or $GluM_2$ courses

The administration of $GluM_1$ or $GluM_2$ courses is as follows:

- At -6 hours: Give 800 ml/m^2 4% glucose + 0.18% sodium chloride intravenously over 6 hours, with potassium chloride 20 mmol/L and sodium bicarbonate 50 mmol/L. Continue prehydration for at least 6 hours and until urinary pH >7.
- At 0 hours: Take blood for MTX levels immediately prior to starting MTX (**for HPLC analysis**). Urinary pH >7 must be confirmed before starting MTX. It may be necessary to give extra sodium bicarbonate at a dose of 50-100 mmol, intravenously or orally, to maintain alkaline urine (pH 7-8). Provided that urinary pH >7, give MTX 12 g/m^2 in 1000 ml 5% glucose over 4 hours along with iv hydration 4% glucose + 0.18% sodium chloride with potassium chloride 20 mmol/L and sodium bicarbonate 50 mmol/L, maintaining a total rate of 125 ml/m^2/h. At the end of MTX infusion take blood for MTX levels (**for HPLC analysis**).
- At +4 hours: Continue with post hydration, maintaining a combined oral/intravenous (4% glucose + 0.18% sodium chloride with potassium chloride 20 mmol/L and sodium bicarbonate 50 mmol/L) fluid intake at 3 L/m^2/day. Continue post hydration until plasma MTX levels are <0.2 µmol/L and for a minimum of 72 hours from the beginning of methotrexate infusion.
- At +24 hours: Take blood for MTX levels (=pre-glucarpidase plasma MTX levels), **for immunoassay and HPLC analysis**. Give glucarpidase as a slow intravenous injection over 5 min. Patients should be observed for 1 hour after completion of glucarpidase/placebo infusion for hypersensitivity reactions.
- At +24:20 hours: Take blood for MTX and DAMPA levels **for HPLC analysis**.
- At +26:00 hours: Start folinic acid rescue, 15 mg/m^2 orally, every 6 hours. Apart from the first dose of folinic acid which is standard (15 mg/m^2 orally), all other folinic acid doses should be adjusted according to plasma MTX levels. For the first 48 hours after glucarpidase/placebo, folinic acid dose should be adjusted based upon the pre-glucarpidase/placebo plasma MTX levels. After that, the folinic acid dose should be adjusted according to MTX plasma levels measured by HPLC. In patients who do not tolerate folinic

acid orally (due to vomiting, nausea etc), the same dose should be given intravenously.

- At +48 hours: Take blood for MTX and DAMPA levels **for immunoassay and HPLC analysis**. Apart from the first dose of folinic acid which is standard (15 mg/m^2 orally), all other folinic acid doses should be adjusted according to plasma MTX levels. For the first 48 hours after glucarpidase, folinic acid dose should be adjusted based upon the pre-glucarpidase plasma MTX levels. After that, the folinic acid dose should be adjusted according to MTX plasma levels measured by HPLC. In patients who do not tolerate folinic acid orally (due to vomiting, nausea etc), the same dose should be given intravenously.

- At +72 hours: Take blood for MTX and DAMPA levels **for immunoassay and HPLC analysis**. Adjust folinic acid dose according to plasma MTX levels measured by HPLC.

- Measure plasma MTX and DAMPA levels every 24 hours (**immunoassay and HPLC analysis)** until <0.2 µmol/L and for a minimum of 72 hours from the start of MTX infusion. Continue FAR, hydration and urinary alkalinisation until plasma MTX levels are <0.2 µmol/L, for a minimum of 72 hours from the start of MTX infusion. In order to compensate for any reduced potential exposure to folinic acid caused by its potential interaction with glucarpidase, the dose of folinic acid should be based upon the pre-glucarpidase/placebo MTX plasma levels and maintained for at least 48 hours after the dosing with glucarpidase. After that adjust folinic acid dose according to plasma MTX levels measured by HPLC. Ensure urinary output >1 ml/kg/h and urinary pH >7 at all times.

7.6.2 M_1 or M_2 courses

The administration of M_1 or M_2 courses is as follows:

- At -6 hours: Give 800 ml/m^2 4% glucose + 0.18% sodium chloride intravenously over 6 hours, with potassium chloride 20 mmol/L and sodium bicarbonate 50 mmol/L. Continue prehydration for at least 6 hours and until urinary pH >7.
- At 0 hours: Take blood for MTX levels immediately prior to starting MTX (**for HPLC analysis**). Urinary pH >7 must be confirmed before starting MTX. It may be necessary to give extra sodium bicarbonate at a dose of 50-100 mmol, intravenously or orally, to maintain alkaline urine (pH 7-8). Provided that urinary pH >7, give MTX 12 g/m^2 in 1000 ml 5% glucose over 4 hours along with iv hydration 4% glucose + 0.18% sodium chloride with potassium chloride 20 mmol/L and sodium bicarbonate 50 mmol/L, maintaining a total

rate of 125 ml/m^2/h. At the end of MTX infusion take blood for MTX levels (**for HPLC analysis**).

- At +4 hours: Continue with post hydration, maintaining a combined oral/intravenous (4% glucose + 0.18% sodium chloride with potassium chloride 20 mmol/L and sodium bicarbonate 50 mmol/L) fluid intake at 3 L/m^2/day. Continue post hydration until plasma MTX levels are <0.2 µmol/L and for a minimum of 72 hours from the beginning of methotrexate infusion.
- At +24 hours: Take blood for MTX levels **for immunoassay and HPLC analysis**. Start folinic acid rescue, 15 mg/m^2 orally, every 6 hours. Apart from the first dose of folinic acid which is standard (15 mg/m^2 orally), all other folinic acid doses should be adjusted according to plasma MTX levels measured by immunoassay. In patients who do not tolerate folinic acid orally (due to vomiting, nausea etc), the same dose should be given intravenously.
- At +48 hours: Take blood for MTX and DAMPA levels **for immunoassay and HPLC analysis**. Apart from the first dose of folinic acid which is standard (15 mg/m^2 orally), all other folinic acid doses should be adjusted according to plasma MTX levels measured by immunoassay. In patients who do not tolerate folinic acid orally (due to vomiting, nausea etc), the same dose should be given intravenously.
- At +72 hours: Take blood for MTX and DAMPA levels **for immunoassay and HPLC analysis**. Adjust folinic acid dose according to plasma MTX levels measured by immunoassay.
- Measure plasma MTX and DAMPA levels every 24 hours (**immunoassay and HPLC analysis)** until <0.2 µmol/L and for a minimum of 72 hours from the start of MTX infusion. Continue FAR, hydration and urinary alkalinisation until plasma MTX levels are <0.2 µmol/L, for a minimum of 72 hours from the start of MTX infusion. Ensure urinary output >1 ml/kg/h and urinary pH >7 at all times.

7.7 Hydration details

Hydration fluids pre HD-MTX:

Intravenous prehydration with 800 ml/m^2 4% glucose + 0.18% sodium chloride over 6 hours, with potassium chloride 20 mmol/L and sodium bicarbonate 50 mmol/L. Continue prehydration for at least 6 hours and until urinary pH >7. It may be necessary to give extra sodium bicarbonate at a dose of 50-100 mmol, intravenously or orally, to maintain alkaline urine (pH 7-8).

Hydration fluids during HD-MTX infusion:

Intravenous hydration with 4% glucose + 0.18% sodium chloride with potassium chloride 20 mmol/L and sodium bicarbonate 50 mmol/L at 3 L/m^2/day (125 ml/m^2/h). It may be necessary to give extra sodium bicarbonate at a dose of 50-100 mmol, intravenously or orally, to maintain alkaline urine (pH 7-8).

Hydration fluids post HD-MTX:

Continue with post hydration, maintaining combined oral/intravenous (4% glucose + 0.18% sodium chloride with potassium chloride 20 mmol/L and sodium bicarbonate 50 mmol/L) fluid intake at 3 L/m^2/day. Continue post hydration until serum MTX levels are <0.2 µmol/L and for a minimum of 72 hours from the beginning of methotrexate infusion. Ensure urinary output >1 ml/kg/h and urinary pH >7 at all times. It may be necessary to give extra sodium bicarbonate at a dose of 50-100 mmol, intravenously or orally, to maintain alkaline urine (pH 7-8).

7.8 Glucarpidase dose and administration

The Pharmacy Department of the appropriate centre (UCH Pharmacy or Harley Street at UCH Pharmacy) will be informed the day prior to any elective admissions of GLU 1 participants for HD-MTX. Each vial of Voraxaze™ will be reconstituted in with 1 ml sodium chloride 0.9%. The vials will be stored in the fridge (at 2° to 8°C) and once reconstituted should be kept in room temperature and used within 8 hours. Glucarpidase syringes will be labelled as showed below:

UCH label:

STORE AT ROOM TEMPERATURE (UNDER 25°C) **GLUCARPIDASE FOR GLU-1 STUDY** Label Audit Ref_____ EudraCT Number: 2006-003203-40 Chief Investigator: Dr Jeremy Whelan Patient_____ Patient's Trial Number_____ Prepared on: _____ at___:___hr B.No:_____ Do not use after: ____:____hr on _____	**Aseptic Dispensing Unit** **UCL Hospitals Foundation** **NHS Trust, London**

Harley Street Clinic at UCH label:

STORE AT ROOM TEMPERATURE (UNDER 25°C) **GLUCARPIDASE FOR GLU-1 STUDY** Label Audit Ref_____ EudraCT Number: 2006-003203-40 Chief Investigator: Dr Jeremy Whelan Patient_____ Patient's Trial Number_____ Prepared on: _____ at___:___hr B.No:_____ Do not use after: ____:____hr on _____	**Aseptic Dispensing Unit** **Harley Street Clinic at UCH**

Glucarpidase will be administered by qualified nursing staff, at a dose of 50 units/kg, by a slow intravenous injection over 5 minutes, 24 hours after the start of MTX infusion. Date and time of administration should be clearly signed and documented in the patient's ChemoCare® chart. The ChemoCare® chart will then be filed in the patient's notes.

Patients will be monitored for allergic reaction (rash, wheezing, pyrexia) and have their vital signs montored (heart rate, blood pressure and temperature) prior to glucarpidase and every 15 min minutes until 1 hour after glucarpidase.

If a patient has a grade 1 (CTCAE v3.0) anaphylactic reaction after glucarpidase, for any subsequent dose they will be given hydrocortisone (≤5 years: 50mg, ≥6 years: 100mg), by slow intravenous injection, 30 min before the injection of glucarpidase/placebo. If a patient experiences a grade 2-4 anaphylaxis, he/she will be removed from the study.

7.9 Guidelines on adjustment of folinic acid dose

$$\text{Total daily dose of folinic acid (mg)} = \frac{\text{Patient's serum MTX levels} \times \text{standard daily dose of folinic acid}^{*}}{\text{Upper limit of plasma MTX levels for day and time}^{**}}$$

* Standard daily dose is 60 mg/m^2

** Upper limits for plasma MTX levels are:

- at 24 h: 20 µmol/L
- at 48 h: 2 µmol/L
- at 72 h: 0.2 µmol/L

IMPORTANT NOTE: Apart from the first dose of folinic acid which is standard (15 mg/m^2 orally), all other folinic acid doses should be adjusted according to plasma MTX levels. For

M_1 and M_2 courses folinic acid doses should be adjusted according to plasma MTX levels measured by immunoassay. For $GluM_1$ and $GluM_2$ courses, for the first 48 hours after glucarpidase, folinic acid dose should be adjusted based upon the pre-glucarpidase plasma MTX levels measured by immunoassay. After that, the dose of folinic acid should be adjusted according to MTX plasma levels measured by HPLC. In patients who do not tolerate folinic acid orally (due to vomiting, nausea etc), the same dose should be given intravenously.

7.10 Guidelines on MTX induced renal failure and delayed MTX excretion

If plasma MTX concentration is ≥100μmol/L at 24 hours or ≥10μmol/L at 48 hours after MTX administration and there is a rise in serum creatinine of ≥100% within 24 hours of MTX administration, there is a risk that MTX elimination will be delayed. In this case intervention with glucarpidase should be considered in M_1 and M_2 courses.

8. "DAY 15" FITNESS CRITERIA

On day 15 of each cycle, patients will be assessed for their fitness to receive chemotherapy.

Patients will be considered fit to receive chemotherapy if **ALL** the following 8 criteria are fulfilled:

1. Neutrophils $\geq 0.75 \times 10^9$/L or WCC $\geq 2 \times 10^9$/L
2. Platelets $\geq 100 \times 10^9$/L
3. Bilirubin ≤1.5xULN
4. GFR (estimated) ≥70 ml/min/1.73m^2
5. Mucositis (clinical and functional): grade ≤1 (CTCAE v3.0)
6. No clinical evidence of infection
7. No pyrexia
8. Good overall clinical condition

9. PHARMACOKINETIC AND ANTIBODY STUDIES

9.1 MTX and DAMPA PHARMACOKINETIC ASSESSMENTS

DAMPA (4-deoxy-4-amino-N^{10}-methylpteroic acid), the catabolic product of glucarpidase action on MTX, is known to cross-react with MTX in most commercial immunological MTX assays[66-68]. Consequently MTX levels determined by commercial laboratories are unreliable following treatment with glucarpidase. Plasma MTX levels will be measured by both immunoassay and high performance liquid chromatography (HPLC). Plasma DAMPA levels will be measured by HPLC.

In order to minimise the potential for *ex vivo* conversion of MTX to DAMPA, all samples taken for HPLC analysis will be put on ice as soon as they are taken and spun to separate the plasma as soon as practical. The plasma will then be immediately transferred to tubes containing hydrochloric acid at a volume ratio of 10 parts plasma to 1 part acid.

9.1.1 Blood samples for analysis of MTX levels by immunoassay

The TDx/TDxFLx Methotrexate II assay (Abbott Laboratories), which utilizes Fluorescence Polarization Immunoassay (FPIA) technology, will be used to measure serum MTX levels.

Following each of the four HD-MTX doses (on days 1 & 8 of cycles 1 and 2), blood samples will be collected at the following timepoints: at 24 hours (prior to glucarpidase/placebo administration), 48 hours and 72 hours after starting MTX and then daily until serum MTX levels are <0.2µmol/L. At each time point, 5 mls of blood in a plain (red top) bottle will be sent for analysis to: Clinical Biochemistry UCL Hospitals, 60 Whitfield St, London, W1T 4EU, tel: 0845 155 5000, ext 2955, fax: 020 7380 9584.

9.1.2 Blood samples for analysis of MTX and DAMPA levels by HPLC

Following each of the four HD-MTX doses (on days 1 & 8 of cycles 1 and 2), blood samples will be collected for MTX and DAMPA HPLC analysis at the following timepoints: at 0 hours (prior to starting the MTX infusion), 4 hours (immediately prior to the end of the infusion), 24 hours (prior to glucarpidase/placebo administration), 24:20 hours (15 minutes after glucarpidase/placebo administration), 48 hours and 72 hours after starting MTX infusion and then daily until plasma MTX levels, measured by HPLC, are <0.2 µmol/L.

At each time point, 6 mls of blood will be collected in EDTA bottle. Patient's trial number, hospital number and date of birth will be documented on the sample label as well as date and time of sample collection. All samples should be immediately placed on ice and taken to the University College London Cancer Institute, The Paul O'Gorman Building, Gower Street, London, WC1E 6BT, for the attention of Janet Hartley or Dr Martha Perisoglou. The bottle will then be centrifuged at 2000 rpm for 10 minutes and the plasma aliquoted into 3x1 ml freezer vials containing hydrochloric acid at a volume ratio of 10 parts plasma to 1 part acid. Samples which will not be analysed straight away will be labelled and frozen at-80°C. Sample log will be kept for all stored samples.

The analysis by HPLC will be performed at the University College London, Cancer Institute, The Paul O'Gorman Building, Gower Street, London, WC1E 6BT. A validation report for the HPLC assay is available in the Trial Master File. The lab follows GLP standards.

9.2 ANTI-GLUCARPIDASE ANTIBODIES

9.2.1 Background

Glucarpidase is a recombinant bacterial protein and therefore has the potential to induce an immune response. In the PD, NCI, Berlin and PK Studies as described in section 1.5.2 of the protocol, the presence of antiglucarpidase IgG antibodies was determined by a validated qualitative ELISA assay. Serum samples containing anti-glucarpidase antibodies were evaluated for their ability to reduce the enzyme activity of glucarpidase by the use of a validated enzyme inhibition assay. Inhibition of the enzyme activity was considered to have occurred if the activity was reduced to less than 80% (ie ≥20% reduction in activity).

Across the four studies, 26/61 (43%) subjects developed anti-glucarpidase antibodies after administration of glucarpidase. Of the positive samples, 5/27 (19%) subjects inhibited the activity of glucarpidase. The reduction in enzyme activity in the five patients who had samples which caused a reduction were 35%, 49% and 84% for the PD Study, 23% for the Berlin Study and 44% for the PK Study. Only one of the positive samples in the PK Study inhibited the enzyme activity of glucarpidase by ≥20% (44%).

It is possible that greater antibody induction resulting in reduction in enzyme activity may be seen if glucarpidase is given in repeated MTX courses. In this study we will study the anti-glucarpidase antibody response after two doses of glucarpidase and evaluate its significance.

9.2.2 Blood sampling schedule

Blood samples for anti-glucarpidase antibody assessment and enzyme neutralisation assay will be collected on days 0, 1, 8 and 15 of cycle 1 and days 1, 8, 15 and 30 of cycle 2 and at 3 and 6 months after starting cycle 2.

9.2.3 Sampling procedures

At each time point, 5 mls of blood will be collected in plain tube (red top) and sent to the University College London Cancer Institute, The Paul O'Gorman Building, Gower Street, London, WC1E 6BT. Once the blood clots, the tube will be centrifuged at 2000 rpm for 10 minutes and 1 ml of serum put in a 1 ml freezer vial, labelled and frozen at -70°C. Patient's trial number, hospital number and date of birth will be documented on the sample label as well as date and time of sample collection. The samples will be sent on dry ice to Covance Laboratories Limited, Biotechnology Division, Otley Road, Harrogate, North Yorkshire, HG3 1PY. Following receipt at Covance, one serum aliquot will then be stored appropriately for anti-glucarpidase antibody neutralisation analysis and the second aliquot will be shipped to BioAnaLab Limited, Florey House, 3 Robert Robinson Avenue, The Oxford Science Park,

Oxford OX4 4GP for anti-glucarpidase antibody analysis. Following analysis of samples by BioAnaLab for anti-glucarpidase antibody, all positive samples will be identified to Covance who will then perform the anti-glucarpidase antibody neutralisation analysis using the second aliquot. A copy of the GLP certificate for Covance Laboratories Limited and BioAnaLab Limited will be submitted to the Regulatory Authorities and another copy will be filed in the Trial Master File.

10. ADVERSE EVENTS

10.1 Adverse Event

An adverse event (AE) is any untoward medical occurrence in a clinical trial subject administered a medicinal product, including occurences which are not necessarily caused by or related to the medicinal product.

An AE can, therefore, be any unfavourable and unintended sign (including an abnormal laboratory finding), symptom, or disease temporally associated with the use of a medicinal product, whether or not related to the medicinal product. AEs are documented on the CRF and graded for severity according to Common Terminology for Adverse Events v.3.0 (CTCAE).

The investigators and/or ITMC will judge whether or not an AE is related to glucarpidase, MTX or other therapeutic intervention. AEs related to glucarpidase will be recorded in the Glucarpidase Adverse Event form (part the CRF) and followed-up until their resolution. AEs that are not related to glucarpidase will be followed until the end of study, i.e. day 21 of cycle 2.

10.2 Adverse Reaction

An adverse reaction (AR) is any untoward and unintended response in a subject to an investigational medicinal product which is related to any dose administered to that subject.

10.3 Serious Adverse Event or Reaction

A serious adverse event (SAE) or serious adverse reaction (SAR) is an adverse event or adverse reaction that:

- results in death regardless of its cause (see 10.7)
- is life-threatening
- requires hospitalisation or prolongation of an existing hospitalisation. Not every hospitalisation constitutes a reportable serious adverse event. For exceptions see 10.7.
- results in persistent or significant disability or incapacity. For exceptions see 10.7.

- is a congenital anomaly or birth defect
- any other medically important condition such as abnormal biological or vital signs, pregnancy and secondary malignancies. For exceptions see 10.7.

10.4 Pregnancy

If a study participant becomes pregnant during the course of the trial the pregnancy data should be forwarded promptly (within 24 hours) to the sponsor using the sponsor's Pregnancy Reporting Form,, as it provides vital data to the overall knowledge concerning the IMP. These will be forwarded by the Joint UCLH/UCL Biomedical Research Unit (JBRU) to the MHRA and Ethics committee. All pregnancies that occur during the course of the trial will be followed to termination or to term.

10.5 Expected Adverse Events

The **expected adverse events associated with glucarpidase** are described in section 1.5.3, pages 31-33 of the protocol.

The **expected adverse events associated with methotrexate** are described in Appendix A6.1.3.

10.6 Suspected Unexpected Serious Adverse Reaction

A suspected unexpected serious adverse reaction (SUSAR) is a SAR, the nature or severity of which is **not** consistent with the applicable product information.

Examples include:

- an expected SAR with an unexpected outcome (e.g. fatal outcome)
- "acute renal failure" is an expected AR, a subsequent new report of "interstitial nephritis" is more specific and, therefore, unexpected
- an increase in the rate of occurrence of an expected AR which is judged to be clinically important is considered unexpected.

10.7 Clarifications and exceptions for GLU 1

- Any death during the study will be reported as an SAE, including death due to disease progression.
- The term "life-threatening" refers to an event where the patient is at immediate risk of death at the time of the event (e.g. requires immediate intensive care treatment). It does not refer to an event which hypothetically might cause death if it were more severe, e.g. drug induced hepatitis which, if resolved, is not life-threatening although it could lead to liver failure and death.

- Hospitalisation is defined as at least one unplanned overnight admission.
- Hospitalisation for HD-MTX is not reported as an SAE. In addition, expected methotrexate side-effects, listed in the product information (Appendix 6), will not be reported on an SAE form, unless they unexpectedly prolong hospitalisation or require intensive care therapy.
- Hospitalisation due to signs and symptoms associated with disease progression are not considered an SAE, unless outcome leads to death during the study.
- Elective hospitalization for pre-existing condition that has not worsened does not constitute a SAE.
- Disability is defined as a substantial disruption in a person's ability to conduct normal life functions. Disability directly due to osteosarcoma does not constitute a SAE.
- Other medically important conditions are important medical events which in the opinion of the investigator may not be immediately life-threatening or result in death or hospitalisation but may jeopardise the patient or may require intervention to prevent one of the other outcomes listed in the definition above (e.g. anaphylaxis). Expected serious adverse reactions (SAR) such as haematological toxicity or increase in liver enzymes after methotrexate which resolve are examples of SARs which are not considered reportable as SAE.

10.8 Reporting Serious Adverse Event

Collection, recording and reporting of adverse events (including serious and non-serious events and reactions) to the sponsor will be done according to the sponsor's SOP.

The investigators are responsible for the recording and reporting of SAEs. Each SAE must be evaluated for seriousness, causality, expectedness and severity by the investigators.

All SAEs regardless of causal relationship must be notified within 24 hours to the Joint UCLH/UCL Biomedical Research Unit (JBRU). Notification must be promptly followed by a detailed, written report using the Joint UCLH/UCL Serious Adverse Report Form which should be faxed to the JBRU on 020 7380 9937. An email will also be sent to the Lead trial co-ordinator in the JBRU. All SAE forms must also be emailed to Dr Karen Maubach, Clinical Development Executive, Protherics at karen.maubach@btgplc.com. In addition, the investigators must complete the UCLH NHS Foundation Trust incident form.

If the SAE is classified as a SUSAR the report will be sent to the MHRA within the required timeframe by the JBRU (7 days if the event was fatal or life-threatening with a further follow-up in 8 days or 15 days for all other events categorised as SUSARs). The report will also be sent by the JBRU to the appropriate research ethics committee. In addition, the investigators must send the report to Dr Karen Maubach at karen.maubach@btgplc.com. She will forward the information to Protherics Inc. (Nashville, USA). Protherics will ensure that the necessary reporting of SAEs is undertaken in the USA.

Depending on the SUSAR event, the investigators will be prompted by the Sponsor monthly or quarterly to complete a follow-up form requesting new or missing information of the event. However, if the investigator receives important information prior to the reminder, they should forward it on to the JBRU and to Protherics.

11. STATISTICAL ANALYSIS

11.1 Interim analysis

An interim analysis will be carried out following completion of both courses in both cycles of the first 14 participants (50% of sample size) in the study. The Independent Trial / Data Monitoring Committee (ITMC) will review interim analysis results considering the primary outcome, information on adverse events and study recruitment rates, in order to make a recommendation about study continuation at that time.

In particular termination of the study will be considered if:

i) Analysis of the primary outcome shows a statistically significant benefit of glucarpidase with $P<0.005$

ii) Treatment with glucarpidase and folinic acid is found to be significantly worse than standard treatment using an one-sided test with $P<0.05$

iii) Glucarpidase associated SAEs and SUSARs outweigh its benefit

iv) Recruitment rate appears inadequate to achieve the required sample size for the study.

In examining efficacy based on the primary outcome, we will use the O'Brien-Fleming method for judging significance of results from a McNemar's test[71]. A significance level of 0.005 will be used for this interim analysis and 0.048 for the final study analysis. To assess whether glucarpidase may be delaying further chemotherapy, the McNemar's test with a one sided significance level of 5% will be used.

The investigators and members of the Trial Steering Committee (TSC) will not be involved in the interim analysis or decisions regarding termination.

11.1.1 Outcome summary of Interim analysis and IDMC

The interim analysis was carried out following completion of both courses in both cycles of the first 16 study participants. In examining efficacy based on the primary outcome, the O'Brien-Fleming method for judging significance of results from a McNemar's test[71] was used. A significance level of 0.005 was used for this interim analysis. To assess whether

glucarpidase may be delaying further chemotherapy, the McNemar's test with a one sided significance level of 5% was used.

The investigators and members of the Trial Steering Committee (TSC) were not involved in the interim analysis or decisions regarding termination.

The Independent Data Monitoring Committee (IDMC) met on 26 May 2009 and reviewed the interim analysis results considering the primary outcome, information on adverse events and study recruitment rates. As per the IDMC report dated 26 May 2009 it is recommended that the study will continue since:

i) Analysis of the primary outcome did not show a statistically significant benefit of glucarpidase with $P<0.005$

ii) Treatment with glucarpidase and folinic acid was not found to be significantly worse than standard treatment using an one-sided test with $P<0.05$

iii) Glucarpidase associated SAEs and SUSARs did not outweigh its benefit. There were no AEs related to the IMP. Two SAEs were reported during this period, both unrelated to the IMP.

iv) Recruitment rate did not appear inadequate to achieve the required sample size for the study. However, the drop-out rate has been significantly higher than expected with only 11 out of 16 participants contributing to complete data set. The expected drop-out rate was 5% while the actual drop-out rate has been 31%. The trial sample size will be re-calculated assuming that the drop-out rate will remain around 30%.

11.2 Final analysis

Primary outcome:

Statistical analysis of the primary outcome will provide an estimate of the difference in proportions of patients ready to receive chemotherapy on day 15 of each chemotherapy cycle comparing standard rescue and glucarpidase+standard rescue. A 95% confidence interval will be calculated for this estimate and a McNemar's test carried out. A significance level of 0.048 will be used to allow for a single interim analysis[69]. The influence of period effects will be investigated using recommended methods[70]. All the analyses will be carried out by intention to treat.

Secondary outcomes:

The three secondary outcomes to be analysed will be incidence of grade ≥2 (CTCAE v3.0) mucositis, neutropaenia and renal toxicity considered to be caused by MTX. The statistical

methods used will be as for the primary outcome. These analyses will be considered purely as hypothesis generating.

Analysis of other outcomes will be descriptive using graphical or summary measures, as appropriate.

12. TREATMENT DISCONTINUATION

The investigators will make every reasonable effort to treat each patient with 4 courses of methotrexate. However, if the investigators remove any patients from the study or if a patient declines further participation, final assessments will be performed prior to any other therapeutic intervention and recorded on the End of Study form (part of the CRF). These assessments include physical examination, assessment for AEs, laboratory tests (FBC and white cell differential count, serum creatinine, urea, Na+, K+, Ca++, glucose, phosphate, magnesium, ALT, bilirubin, total protein, albumin and anti-glucarpidase antibody levels), performance status, mucositis and quality of life assessments.

13. PREMATURE TERMINATION OF THE TRIAL

If the trial is terminated before the concluding date then the appropriate MHRA form must be completed by the investigators immediately and emailed to the sponsor via the Lead trial co-ordinator in the JBRU. The JBRU will inform the appropriate research ethics committee and report this to the MHRA within 15 days.

The study will be terminated if:

- Glucarpidase is considered too toxic (e.g. grade 3 or 4 allergic reaction occurs despite premedication with hydrocortisone) in relation to its potential benefits to continue the study.
- Evidence has emerged that, in the opinion of the sponsor or the investigator or the TSC or the ITMC, makes the continuation of the study unnecessary or unethical.

14. CONCLUSION OF THE TRIAL

The trial will be completed will be completed after all 38 patients are recruited, treated and followed up. The conclusion of the trial should be reported to the sponsor on the appropriate MHRA form and emailed to the Lead trial co-ordinator at JBRU. The JBRU will inform the appropriate research ethics committee and report this to the MHRA within 90 days of the concluding date.

15. CONDITIONS FOR MODIFICATION OF THE TRIAL PROTOCOL

Protocol amendments to this trial that could potentially adversely affect the safety of participating patients, or alter the scope of the investigation, the scientific quality of the study, the experimental design, dosages, and duration of therapy, assessment variables or patient selection criteria must be made only after consultation with the investigators and other members of the TSC. Protocol amendments will be prepared and reviewed by the TSC.

Any amendments to the protocol that are made after receipt of the Ethics Committee (EC) approval must be submitted to the Sponsor, EC and Regulatory Authorities, before the changes can be implemented. Amendments to the protocol that eliminate an apparent hazard to patients do not need prior approval by the Ethics Committee. If protocol amendments are made, the investigators are responsible to inform all relevant trial staff.

Administrative amendments that do not affect the conduct of the study or patient safety and do not lead to a change in the patient information sheet, will be discussed and agreed by the TSC but will not be re-submitted for formal ethics reviews or regulatory approval.

16. ADMINISTRATION

16.1 Completion of the case record form (CRF)

Data collection will be conducted by the Bone Sarcoma research team. Patient-related data must be transcribed into the CRF from clinical source documents. Patients' details will remain confidential.

The CRF must be completed in black ink and corrections made by striking out any errors, with a single stroke. Correction fluid should not be used. All corrections must be initialled and dated.

16.2 Quality assurance and auditing

A representative from UCL/UCLH will regularly visit the investigators who should allow the representatives to inspect the various records of the study (CRFs and other pertinent data), provided that patient confidentiality is maintained. These records may also be reviewed by appropriate qualified personnel appointed by Protherics to audit the study, and by the Regulatory Authorities. The investigators agree to co-operate with above mentioned representatives, to ensure that any problems detected in the course of monitoring visits are resolved.

16.3 Retention of study documents

The investigators must maintain adequate and accurate records to enable the conduct of the study to be fully documented and the study data to be subsequently verified.

Study documents should not be destroyed without prior written agreement between the Sponsor, Protherics and the investigators.

All data relating to the study, including study master file contents, CRFs and source data must be retained for at least 15 years after the completion of the study, in order to comply with the ICH GCP guidelines and Data Protection Act 1998.

16.4 Ethical considerations

This protocol will be submitted to the Ethics Committee (EC), whose approval will be obtained before the start of the study. The investigators will inform the EC of subsequent protocol amendments and any serious adverse events which occur during the study.

The investigators must inform patients about the background to, and present knowledge of glucarpidase and HD-MTX with special reference to known activity and toxicity. The investigators must also ensure the following points are made:

- The patient must be provided with the Patient Information Sheet and Informed Consent form consistent with the protocol version used and approved by EC (Appendix 9 and 10). The patient must be informed that the study drug is new and that the exact degree of activity is at present unknown, but that treating him / her will contribute to further knowledge.
- The patient will be given sufficient time to discuss his / her participation in the study with the investigators and members of the family. Before the patient is entered into the study, the patient's written consent must be obtained using the Informed Consent form described above. The patient will retain a copy of the signed informed consent form and the original will be filed in the Trial's Master file.
- The patient may refuse treatment either before or at any time during the study. Refusal to participate will involve no penalty or loss of benefits to which the patient is otherwise entitled.
- An explanation on whom to contact for answers to pertinent questions about the research and research subject's rights, and who to contact in the event of a research-related injury to the subject must be given.

16.5 Publication policy

Results and information from the study will be submitted for peer review publication. Authorship will include the investigators and members of the TSC and ITMC. A report of the results of the study will be also provided to the study participants, if they wish one.

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APPENDIX 2. GLU 1 CLINICAL TRIAL, PATIENT AND PARENT INFORMATION SHEETS

The following information sheets were available:

- **Information sheet for participants aged 16+ with newly diagnosed osteosarcoma** (version 2.0, 2 April 2007)
- **Information sheet for participants aged 16+** (version 2.0, 2 April 2007) (for all other eligible patients aged 16+)
- **Parent information sheet (patients with newly diagnosed osteosarcoma)** (version 2.0, 2 April 2007)
- **Parent information sheet** (version 2.0, 2 April 2007)
  (all other eligible patients)
- **Information sheet for children aged 13 to 15 with newly diagnosed osteosarcoma** (version 2.0, 2 April 2007)
- **Information sheet for children aged 13 to 15** (version 2.0, 2 April 2007) (for all other eligible patients aged 13 to 15)
- **Information sheet for children aged 6 to 12** (version 2.0, 2 April 2007)
- **Information sheet for children aged 5** (version 2.0, 2 April 2007)

As an example, the information sheet for for participants aged 16+ with newly diagnosed osteosarcoma (version 2.0, 2 April 2007) was as follows:

University College London Hospitals NHS
NHS Foundation Trust

Cancer Clinical Trials Unit
University College Hospital
1st Floor, Central
250 Euston Road
London NW1 2PG

Direct Line: 020 7380 9320
Fax: 020 7380 9321
Hospital Switchboard: 0845 155 5000
Web-site: www.ucdh.org

GLU-1: Will the use of glucarpidase after methotrexate treatment allow more patients with bone sarcoma to have fewer side effects and receive their subsequent chemotherapy on time?

Chief Investigator: Dr Jeremy Whelan

Co-Investigator: Dr Martha Perisoglou

Information sheet for participant aged 16+ with newly diagnosed osteosarcoma (version 2.0, 2 April 2007)

You are being invited to take part in a research study, also called a clinical trial. This information leaflet explains what the study is all about.

Before you decide whether or not to take part, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

Thank you for taking time to read this information sheet.

1. What is the purpose of the study?

You have recently been diagnosed with a rare type of bone cancer called osteosarcoma. The standard treatment for patients with osteosarcoma is:

- MAP chemotherapy for about 10 weeks, then
- surgery, then
- MAP chemotherapy for another 18 weeks.

MAP stands for **M**ethotrexate, doxorubicin (also called **A**driamycin) and cis**P**latin.

One of the most effective chemotherapy drugs in the treatment of osteosarcoma is methotrexate. However, methotrexate frequently leads to sore mouth, tummy pain and increased risk of developing infections.

We try to save or "rescue" normal cells from the side effects of methotrexate by giving a drug called folinic acid. Folinic acid is started 24 hours after methotrexate and given regularly until methotrexate levels are really low and not dangerous to normal cells anymore. Despite this rescue, side effects are still a problem and many patients are not well enough to receive subsequent chemotherapy on time. Almost half of the planned chemotherapy cycles are not given on time due to methotrexate side effects.

In this study we will examine if adding a drug called glucarpidase to folinic acid is helpful. Glucarpidase is an enzyme that inactivates methotrexate in the blood stream. Lower methotrexate concentration in the blood stream leads to fewer side effects. We would like to see if glucarpidase helps patients to have their chemotherapy on time, by reducing the side effects of methotrexate.

2. Why have I been chosen?

Nearly 30 patients with bone sarcoma are being invited to take part in this study in University College Hospital.

3. Do I have to take part?

It is up to you to decide whether or not to take part in this study. If you decide that you want to take part you can keep this information sheet, and you will be asked to sign a consent form. You may keep a copy of the consent form. You are still free to withdraw from the study at any time without giving a reason. A decision not to take part, or later to withdraw, will not affect the standard of care you receive.

4. What will happen to me if I take part?

This study involves part of the chemotherapy you will be receiving before any planned surgery (also called pre-operative chemotherapy). The **schedule of standard pre-operative chemotherapy** is shown in table 1.

In this study you will receive the same drugs and doses as the standard pre-operative treatment. However the drug order will be different. Rather than receiving two cycles of doxorubicin & cisplatin followed by methotrexate, you will receive four courses of methotrexate first and then two courses of doxorubicin & cisplatin. This change in drug order will not alter the effect of your treatment. However, any surgery will be planned for week 13, two weeks later than what standard treatment suggests. We know that most patients having standard chemotherapy have surgery at about that time too, because of chemotherapy-associated side effects. **The pre-operative chemotherapy for patients on GLU-1 study** is shown in table 2.

Table1:

STANDARD PRE-OPERATIVE CHEMOTHERAPY

Week 1	Doxorubicin & cisplatin
Week 2	
Week 3	
Week 4	High-dose methotrexate
Week 5	High-dose methotrexate
Week 6	Doxorubicin & cisplatin
Week 7	
Week 8	
Week 9	High-dose methotrexate
Week 10	High-dose methotrexate
Week 11	SURGERY

Table 2:

GLU-1 PRE-OPERATIVE CHEMOTHERAPY

Week 1	High-dose methotrexate
Week 2	High-dose methotrexate
Week 3	
Week 4	High-dose methotrexate
Week 5	High-dose methotrexate
Week 6	
Week 7	Doxorubicin & cisplatin
Week 8	
Week 9	
Week 10	Doxorubicin & cisplatin
Week 11	
Week 12	
Week 13	SURGERY

5. What will the treatment involve?

If you join the study you will have 4 courses of high-dose methotrexate. High-dose methotrexate is normally given at weekly intervals, in blocks of two. You will have the first two courses on weeks 1 & 2; the second two courses on weeks 4 & 5. Two courses will be given with folinic acid rescue (standard high-dose methotrexate), and the other two will be given with glucarpidase rescue as well as folinic acid. This will enable us to compare whether there is any difference in side effects with and without glucarpidase and also how quickly patients recover from them.

Half of the patients will receive standard high-dose methotrexate on weeks 1 & 2 and high-dose methotrexate with glucarpidase on weeks 4 & 5 (**arm A**) and half of the patients will first have high-dose methotrexate with glucarpidase on weeks 1 & 2 and then standard high-dose methotrexate on weeks 4 & 5 (**arm B**). The two different treatment arms are shown in tables 3 and 4.

Table 3: **ARM A**

CYCLE 1	Week 1	High-dose methotrexate + folinic acid + **placebo**
	Week 2	High-dose methotrexate + folinic acid + **placebo**
	Week 3	
CYCLE 2	Week 4	High-dose methotrexate + folinic acid + **glucarpidase**
	Week 5	High-dose methotrexate + folinic acid + **glucarpidase**
	Week 6	

Table 4: **ARM B**

CYCLE 1	Week 1	High-dose methotrexate+ folinic acid + **glucarpidase**
	Week 2	High-dose methotrexate+ folinic acid + **glucarpidase**
	Week 3	
CYCLE 2	Week 4	High-dose methotrexate + folinic acid + **placebo**
	Week 5	High-dose methotrexate + folinic acid + **placebo**
	Week 6	

On the weeks you will not have glucarpidase, you will receive a "placebo". Placebo is a "dummy treatment", which looks like the genuine medicine but contains no active ingredient. Neither you nor your doctor will know whether you will be receiving glucarpidase with course 3 & 4 (arm A) or course 1 & 2 (arm B). This is so that neither you nor your doctor will be influenced one way or the other when assessing the side effects of methotrexate. You will be assigned to one of the two treatment arms by randomisation. Randomisation means that you are being put into a group by chance. You will have an equal chance of being placed in either group.

After week 6, your treatment will follow standard practice. However, you will need to have a blood test in approximately 1 month after starting the second chemotherapy cycle. Depending on the result you may need to have two more blood tests at 3 and 6 months after starting the second chemotherapy cycle. We will try to arrange these blood tests alongside your routine hospital visits.

Drugs

High-dose Methotrexate: High-dose methotrexate is given over 4 hours into a vein (intravenous). Lots of intravenous fluids are given before, during and after methotrexate, in order to help your body to get rid of the drug. You will normally be in hospital for 3-5 days for your methotrexate treatment.

Folinic acid: Folinic acid is given as a tablet or if you are sick, as an intravenous injection and started 26 hours after starting methotrexate, in order to protect normal cells from the side effects of methotrexate. You will have a blood test every day to see how much methotrexate remains in your body, so that we know when it has nearly gone. At that time the folinic acid rescue will stop and you can go home.

Glucarpidase / Placebo: The glucarpidase or placebo will be given as a short intravenous injection, 24 hours after starting methotrexate. This will be given just once, unlike folinic acid. Glucarpidase could potentially interact with folinic acid and reduce its activity. Therefore, it will be given 2 hours prior to starting folinic acid "rescue".

Central Line

For drugs to be given by vein, your doctor will likely recommend that you have a central venous line placed. This line is a type of tubing put into a large vein inside the chest by a surgeon or radiologist during a short operation. A central venous line is used to administer chemotherapy drugs and withdraw small amounts of blood for testing during treatment. You will be anaesthetised for this procedure and get pain relief medication afterwards to keep you comfortable. You will be given more information about this by the doctors and nurses looking after you.

Standard medical tests

If you take part in this study, you will have tests that are part of regular cancer care and may be done even if you do not join this study. These tests include blood tests, urine tests and nuclear medicine tests. However, if you join the study there will be some extra blood tests and we will also ask you to complete some questionnaires.

6. Additional research

a) All patients receiving methotrexate have daily blood tests to monitor the levels of methotrexate in their body, and monitor their kidney function. However, patients on this study will have **extra blood tests for chemotherapy drug levels and glucarpidase antibody levels**. During each hospital admission for chemotherapy, blood samples will be taken as follows:

Day 1: Just before starting methotrexate (extra blood test) and at the end of methotrexate infusion (extra blood test)

Day 2: 24 hours after starting methotrexate (routine blood test) and 20 minutes after the 24-hour blood test (i.e. just after the glucarpidase/placebo infusion) (extra blood test)

Day 3+: Routine daily blood tests until your body has got rid of the methotrexate

Extra blood samples will also be taken 15 days after starting each cycle and 1 month, and possibly 3 and 6 months, after starting the second cycle.

b) **Mucositis assessment questionnaires:** One of the common side-effects of methotrexate is sore mouth and tummy pain (mucositis). We would like you to complete a brief questionnaire on the severity and impact of mucositis and the degree to which it interferes with activities of daily life such as eating, swallowing, drinking, talking and sleeping. We would like you to complete this questionnaire in 6 occasions, i.e. prior to each chemotherapy course and 15 days after starting each cycle.

c) **Quality of life questionnaires:** As part of this study we think it is important to find out more about how patients feel both physically and emotionally during and after different treatments. In order to collect this information, questionnaires have been designed for you to complete. We would like you to complete questionnaires in 6 occasions, i.e. prior to each chemotherapy course and 15 days after starting each cycle. You can of course decline to complete the questionnaires at any time without affecting your relationship with your doctor.

7. How long will I be in this study?

You will be in this study for 6 weeks. However we will need to see you again for a blood test in approximately 1 month and possibly 3 months and 6 months after starting the

second chemotherapy cycle. We will try to arrange these tests alongside your routine hospital visits.

8. What are the alternatives for treatment?

Patients who do not want to take part in this study will receive the standard treatment with **MAP** chemotherapy as outlined earlier.

9. What are the side effects of the proposed treatment?

Side effects associated with **glucarpidase** are minor and include burning or tingling sensation, feeling hot, allergic reaction, flushing, itching or headache. These side effects are quite rare, each of them occurring in less than two out of every hundred patients. They all reverse and, except from infrequent use of an anti-histamine, do not need any treatment. However, there may be side effects that are not yet known. Your doctor will look after you carefully and treat any side effects that may occur.

Side effects associated with **methotrexate** include hair loss, loss of appetite, nausea (feeling sick), tiredness, risk of infection, and risk of bleeding. Some of these side effects can be controlled with drugs and your doctors and nurses will talk to you about how you can help to prevent some of these side effects. If you get an infection between courses of chemotherapy, you will need to receive antibiotic treatment in the hospital. Blood and platelet transfusions can also be given when necessary. Hair loss occurs but the hospital can help by providing you with a wig if you wish. Your hair will start to grow back as soon as your chemotherapy has finished.

High-dose methotrexate chemotherapy can be associated with sore mouth and tummy pain. It can also cause kidney damage. To prevent this happening, extra fluids are given with drugs to help "flush" it through the kidneys. You will be encouraged to drink plenty of water during your treatment. Blood tests to monitor your kidney function will be taken throughout your treatment and adjustments will be made to your chemotherapy if necessary. Very occasionally patients receiving high dose methotrexate get a rash on the soles of their feet or palms of their hands. If this occurs the doctors can prescribe anti-histamines (drugs used for allergies) to help control the rash, and it will resolve once your chemotherapy has finished.

As with many chemotherapy treatments it is possible that methotrexate could damage an unborn child and therefore women should not become pregnant during treatment. Men should also avoid unprotected sexual intercourse whilst on treatment since the drugs could damage the sperm. Methotrexate is excreted into breast milk and women

who are breastfeeding should not take part in this study. It is not known whether glucarpidase can appear in human breast milk. Any woman who finds she has become pregnant while taking part in the study should immediately tell her doctor.

10. What are the possible disadvantages and risks of taking part?

All cancer treatment is associated with side effects such as those described above, including potentially life-threatening problems such as infection. In particular, glucarpidase has the side effects described above.

11. Is this trial as safe as standard treatment?

Methotrexate has been used in the treatment of osteosarcoma and other cancers for many years. However, glucarpidase has only been used for patients with severe reactions to methotrexate, and has therefore been less widely used. We still try to learn about its side effects. To our knowledge glucarpidase administration has not been associated with significant side effects so far.

12. What are the possible benefits of taking part?

We hope that glucarpidase will reduce the side-effects of methotrexate and help patients to have their next chemotherapy on time. The information we will gain from this study may help us to treat future patients with bone sarcoma better, in just the same way that current treatment has been designed using information gained from previous clinical trials. We do not know whether glucarpidase will be better than the standard treatment yet.

13. What if new information becomes available?

Sometimes during the course of a research project, new information becomes available about the treatment that is being studied. If this happens, your doctor will tell you about it and discuss with you whether you want to continue in the study. If you decide to withdraw, your doctor will make arrangements for your treatment to continue according to the best available information at the time. If you decide to continue in the study you will be asked to sign another consent form.

It is also possible that on receiving new information about treatment your doctor might consider it to be in your best interest to withdraw from the study. Your doctor will explain the reasons and arrange for your care to continue.

14. What happens when the research stops?

If for any reasons the research study stops or the treatment programme needs to be changed, the reasons will be explained. Arrangements will be made for you to continue treatment according the best available information at the time.

15. What if something goes wrong?

If you are harmed as a result of your participation in this trial due to someone's negligence, then you may have grounds for legal action but you may have to pay for it. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been approached or treated during the course of this study, the normal NHS complaints mechanisms are available to you. University College Hospital continues to have a duty of care to you as a patient treated within the hospital whether in a trial or not.

If you are harmed as a result of your participation in this study and this is not due to negligence, University College London / University College London Hospitals (UCL/UCLH) would sympathetically consider any claim for compensation.

16. Will taking part in this study be kept confidential?

If you decide to take part in this study, information about you will be kept with the Co-Investigator who is managing the database for the study. Occasionally staff from UCL/UCLH, the regulatory authorities or Protherics, the company that makes glucarpidase, will need to visit the hospital to review your notes to check that the information being provided is correct. Your GP and the other doctors involved in your care will be kept fully informed, but otherwise all information about you and your treatment will remain confidential.

17. What will happen to the results of this study?

When the trial is completed the results will be published in a medical journal, and may be presented at conferences. No individual patients will be identified. We will also prepare a report of the results for patients who took part if they wish it.

18. Who is organising and funding the research?

This research project is being organised by University College Hospital and funded by Richard Scowcroft Foundation. Glucarpidase is being supplied free of charge by Protherics, the company that makes the drug. They are also providing nominal funding for the extra blood tests required in this study. The research has been reviewed by several cancer doctors expert in the field and also by an independent NHS Research Ethics Committee. Your doctor will not receive any payment for including you in the study.

All research that involves NHS patients or staff, information from NHS medical records or uses of NHS premises or facilities must be approved by a NHS Research Ethics Committee before it goes ahead. Approval does not guarantee that you will not come into any harm if you take part. However, approval means that the Committee is satisfied that your rights will be respected, that any risks have been reduced to a minimum and balanced against possible benefits, and that you have been given sufficient information on which to make an informed decision to take part or not.

19. Contact for further information

If you have any further questions about your disease or clinical trials, please discuss them with your doctor. You may also find it helpful to contact CancerBACUP, an independent patient advisory group (freephone: 0808 800 1234; address: 3 Bath Place, Rivington Street, London, EC2A 3JR; web site www.cancerbacup.org.uk).

If you have private medical insurance we recommend that you contact your health insurance company about your participation before agreeing to take part.

20. Investigators' names and telephone numbers

Dr Jeremy Whelan, Consultant Medical Oncologist, 020 7380 9346

Dr Martha Perisoglou, Clinical Research Fellow in Oncology, 020 7380 9346

21. What if I have any concerns?

If you have any concerns or other questions about this study or the way it has been carried out, you could contact Dr Martha Perisoglou, or you may contact the University College Hospital complaints department.

APPENDIX 3. GLU 1 CLINICAL TRIAL, GENERAL PRACTITIONER INFORMATION SHEETS

The following information sheets were available for participants' general practitioners:

- **General Practitioner information sheet for patients with newly diagnosed osteosarcoma** (version 2.0, 2 April 2007)
- **General Practitioner information sheet for all other eligible patients** (version 2.0, 2 April 2007)

As an example, the General Practitioner information sheet for patients with newly diagnosed osteosarcoma (version 2.0, 2 April 2007) was as follows:

University College London Hospitals NHS
NHS Foundation Trust

Cancer Clinical Trials Unit
University College Hospital
1st Floor, Central
250 Euston Road
London NW1 2PG

Direct Line: 020 7380 9320
Fax: 020 7380 9321
Hospital Switchboard: 0845 155 5000
Web-site: www.uclh.org

GLU-1: Single centre, double-blind, randomised, cross-over phase II study, to investigate the efficacy and safety of glucarpidase for routine use after high dose methotrexate in patients with bone sarcoma.

Chief Investigator: Dr Jeremy Whelan

Co-Investigator: Dr Martha Perisoglou

General Practitioner Information Sheet for patients with newly diagnosed osteosarcoma (version 2.0, 2 April 2007)

Dear Dr

As you are aware, your patient..... has been diagnosed with osteosarcoma. I am writing to inform you that he/she has been entered into a University College Hospital study, aiming to investigate the efficacy and safety of glucarpidase in routine rescue after high-dose methotrexate (HD-MTX) for bone sarcomas.

Osteosarcoma is the most common bone tumour in children and adolescents, with a long-term survival of approximately 50-60%. The standard treatment for osteosarcoma includes 10 weeks of pre-operative chemotherapy with Doxorubicin (**A**driamycin), Cis**P**latin and high-dose **M**ethotrexate (**MAP**), followed by surgery, followed by 18 weeks of post-operative chemotherapy with MAP. The **standard schedule of pre-operative chemotherapy** is shown in table 1.

In this study patients will receive the same drugs and doses as in standard treatment. However, the drug order will be different. The change in drug order does not alter the effect of the treatment. Nevertheless, surgery will be planned for week 13, two weeks later than what standard treatment suggests. We know that most patients having standard chemotherapy have surgery at about that time too, because of chemotherapy-associated side effects.

The pre-operative chemotherapy for patients on GLU-1 study is shown in table 2. GLU-1 refers to the first 6 weeks of pre-operative chemotherapy, consisting of 4 courses of high-dose methotrexate.

Table 1:

STANDARD PRE-OPERATIVE CHEMOTHERAPY

Week 1	Doxorubicin & cisplatin
Week 2	
Week 3	
Week 4	High-dose methotrexate
Week 5	High-dose methotrexate
Week 6	Doxorubicin & Cisplatin
Week 7	
Week 8	
Week 9	High-dose methotrexate
Week 10	High-dose methotrexate
Week 11	SURGERY

Table 2:

GLU-1 PRE-OPERATIVE CHEMOTHERAPY

Week 1	High-dose methotrexate
Week 2	High-dose methotrexate
Week 3	
Week 4	High-dose methotrexate
Week 5	High-dose methotrexate
Week 6	
Week 7	Doxorubicin & Cisplatin
Week 8	
Week 9	
Week 10	Doxorubicin & Cisplatin
Week 11	
Week 12	
Week 13	SURGERY

HD-MTX, at a dose of 12gr/m2, is an essential component of osteosarcoma treatment. It is administered with vigorous patient hydration and urinary alkalinisation along with a pharmacokinetically guided folinic acid "rescue" (FAR) schedule. Folinic acid replenishes the intracellular source of reduced folates. Although FAR may decrease the degree of methotrexate toxicity, patients will remain at risk as long as elevated concentration of methotrexate persists in circulation. Moreover, if extracellular methotrexate levels are very high, FAR may prove inadequate.

Despite current supportive measures, methotrexate induced toxicity (myelosuppression, mucositis, hepatic and renal toxicity) still occurs and results in increased morbidity, patient discomfort, increased costs and possibly decrease in treatment efficacy. A review of 56 osteosarcoma patients treated in our institution revealed that methotrexate-induced toxicity resulted in delays in 47% of chemotherapy cycles.

Several studies reported that in osteosarcoma treatment the fewer chemotherapy delays, the better the outcome. Therefore, it is important to optimise rescue following treatment with methotrexate and maintain chemotherapy intensity.

Glucarpidase (formerly known as Carboxypeptidase G2,), inactivates extracellular methotrexate. It is currently used effectively to treat patients with methotrexate-induced renal dysfunction. Administration of a single dose of glucarpidase after treatment with methotrexate results in reduction of serum methotrexate levels to non-toxic range within minutes without causing toxicity. Glucarpidase has a much higher affinity for methotrexate than folinic acid so even high circulating folinic acid levels are unlikely to interfere with extracellular methotrexate inactivation. Moreover, glucarpidase is a high molecular weight protein and it does not gain intracellular access. Therefore it will not counteract the anti-tumour effect of methotrexate, offering a promising rescue following methotrexate treatment.

In this study we will investigate the role of glucarpidase in the routine rescue after HD-MTX in osteosarcoma treatment. Patients will be randomised into arm A or B as shown in table 3 and 4. All patients will have 4 courses of HD-MTX. Two of them will be given as per standard practice, i.e. with folinic acid rescue, and the other two will be given with glucarpidase rescue as well as folinic acid. Patients tolerate methotrexate differently. For each patient we will compare methotrexate-associated side effects with and without glucarpidase and also how quickly they recover from them.

Glucarpidase is a recombinant bacterial protein and as such it has the potential to induce an immune response. We will also investigate the formation of anti-glucarpidase antibodies.

After completion of week 6, your patient's treatment will follow standard practice. However we will need to see him/her again in approximately 1 month and possibly 3 and 6 months after starting the second chemotherapy cycle. We will try to arrange these reviews alongside their routine hospital visits.

Table 3: **Arm A**

CYCLE 1	Week 1	High-dose methotrexate + folinic acid + **placebo**
	Week 2	High-dose methotrexate + folinic acid + **placebo**
	Week 3	
CYCLE 2	Week 4	High-dose methotrexate + folinic acid + **glucarpidase**
	Week 5	High-dose methotrexate + folinic acid + **glucarpidase**
	Week 6	

Table 4: **Arm B**

CYCLE 1	Week 1	High-dose methotrexate+ folinic acid + **glucarpidase**
	Week 2	High-dose methotrexate+ folinic acid + **glucarpidase**
	Week 3	
CYCLE 2	Week 4	High-dose methotrexate + folinic acid + **placebo**
	Week 5	High-dose methotrexate + folinic acid + **placebo**
	Week 6	

Side effects of methotrexate include mucositis, elevated transaminases, nephrotoxicity and occasionally rash (usually on soles and palms) and encephalopathy. Side effects of glucarpidase are short lived and include flushing (<2%), burning sensation (1%), paraesthesia (1%), and less commonly headache, hot feeling, tremor, elevation of aspartate aminotransferase, allergic dermatitis, hypertension, pruritus and pyrexia.

As with many chemotherapy treatments it is possible that the drugs could damage an unborn child and therefore women should not become pregnant during treatment. Men should also avoid unprotected sexual intercourse whilst on treatment since the drugs could damage the sperm. Methotrexate is excreted into breast milk and women who are breastfeeding should not take part in this study. It is not known whether glucarpidase can appear in human breast milk.

Should your patient develop any evidence of infection whilst on treatment, please contact University College Hospital immediately.

We will keep you informed about your patient's progress. Should you require any further information about this study, please do not hesitate to contact us. We would be happy to answer any questions you may have.

Yours sincerely

APPENDIX 4. GLU 1 CLINICAL TRIAL, PATIENTS' CONSENT FORMS

- **Consent form for patients aged 16+** (version 2.0, 2 April 2007)
- **Parent consent form (**version 2.0, 2 April 2007)
- **Assent form for children** (version 2.0, 2 April 2007)

University College London Hospitals NHS
NHS Foundation Trust

Cancer Clinical Trials Unit
University College Hospital
1st Floor, Central
250 Euston Road
London NW1 2PG

Direct Line: 020 7380 9320
Fax: 020 7380 9321
Hospital Switchboard: 0845 155 5000
Web-site: www.uclh.org

GLU-1: Will the use of glucarpidase after methotrexate treatment allow more patients with bone sarcoma to have fewer side effects and receive their subsequent chemotherapy on time?

(**GLU** stands for glucarpidase)

Chief Investigator: Dr Jeremy Whelan
Co-Investigator: Dr Martha Perisoglou

Study: GLU-1 (version 2.0, 2 April 2007)

Patient Study Number: ☐☐

CONSENT FORM FOR PATIENTS AGED 16+ (version 2.0, 2 April 2007)

Please initial box

1. I confirm that I have read and understand the information sheet (version 2.0, 2 April 2007) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐

3. I understand that relevant sections of any of my medical notes and data collected during the study may be looked at by responsible individuals from University College London Hospitals Foundation NHS Trust and University College London, from the regulatory authorities or Protherics, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records. ☐

4. I agree to my GP being informed of my participation in the study. ☐

5. I agree to take part in the above study. ☐

Name of patient	Signature	Date
Name of person taking consent	Signature	Date

When completed, 1 for patient; 2 for researcher site file; 3 (original) to be kept in medical notes

University College London Hospitals NHS
NHS Foundation Trust

Cancer Clinical Trials Unit
University College Hospital
1st Floor, Central
250 Euston Road
London NW1 2PG

Direct Line: 020 7380 9320
Fax: 020 7380 9321
Hospital Switchboard: 0845 155 5000
Web-site: www.uclh.org

GLU-1: Will the use of glucarpidase after methotrexate treatment allow more patients with bone sarcoma to have fewer side effects and receive their subsequent chemotherapy on time?

(**GLU** stands for glucarpidase)

Chief Investigator: Dr Jeremy Whelan
Co-Investigator: Dr Martha Perisoglou

Study: GLU-1 (version 2.0, 2 April 2007)

Patient Study Number: ☐☐

PARENT CONSENT FORM (version 2.0, 2 April 2007)

Please initial box

1. I confirm that I have read and understand the information sheet (version 2.0, 2 April 2007) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐

2. I understand that my child's participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my child's medical care or legal rights being affected. ☐

3. I understand that relevant sections of any of my child's medical notes and data collected during the study may be looked at by responsible individuals from University College London Hospitals Foundation NHS Trust and University College London, from the regulatory authorities or Protherics, where it is relevant to my child's taking part in this research. I give permission for these individuals to have access to my child's records. ☐

4. I agree to my child's GP being informed of my child's participation in the study. ☐

5. I agree for my child to take part in the above study. ☐

Name of parent (state relationship)	Signature	Date
Name of person taking consent	Signature	Date

When completed, 1 for patient; 2 for researcher site file; 3 (original) to be kept in medical notes

University College London Hospitals NHS
NHS Foundation Trust

Cancer Clinical Trials Unit
University College Hospital
1st Floor, Central
250 Euston Road
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Direct Line: 020 7380 9320
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Web-site: www.uclh.org

GLU-1: Will the use of glucarpidase after methotrexate treatment allow more patients with bone sarcoma to have their subsequent chemotherapy on time? (**GLU** stands for glucarpidase)

Chief Investigator: Dr Jeremy Whelan
Co-Investigator: Dr Martha Perisoglou

Study: GLU-1 (version 2.0, 2 April 2007)

Patient Study Number: ☐☐

ASSENT FORM FOR CHILDREN

(version 2.0, 2 April 2007)

(To be completed by the child and their parent/guardian)

Please tick the box if your answer is yes

1. Have you read about the project? ☐
2. Has somebody else told you about the project? ☐
3. Do you understand what the project is about? ☐
4. Have you asked any questions you want? ☐

5. Have you had your questions answered? ☐

6. Are you happy to take part? ☐

- If you **don't** want to take part, **don't** sign your name!
- If you do want to take part, please write your name and today's date. If you don't like writing, you can draw a smiley face instead. Someone else can fill in your name for you.

Your name.....

Date.....

Your parent or guardian must write their name here too if they are happy for you to do the project. This is called giving consent.

Print Name.....

Sign.....

Date.....

The person who explained this project to you needs to sign too:

Print Name.....

Sign.....

Date.....

When completed, 1 for patient; 2 for researcher site file; 3 (original) to be kept in medical notes

APPENDIX 5. REGISTRATION FORM FOR GLU 1 CLINICAL TRIAL

University College London Hospitals NHS
NHS Foundation Trust

UCL HOSPITALS

REGISTRATION NUMBER

PATIENT'S INITIALS:

HOSPITAL:

PATIENT HOSPITAL NUMBER:

DATE OF REGISTRATION:

FIRST STUDY TREATMENT (DATE):

UCL

APPENDIX 6. CRF FOR GLU 1 CLINICAL TRIAL

CRF for GLU 1 clinical trial

Patient's Initials ☐☐☐☐ Patient's Trial Number ☐☐

University College London Hospitals NHS
NHS Foundation Trust

UCL HOSPITALS

GLU 1

A SINGLE CENTRE, DOUBLE-BLIND, RANDOMISED, CROSS-OVER, PHASE II STUDY, TO INVESTIGATE THE EFFICACY AND SAFETY OF GLUCARPIDASE FOR ROUTINE USE AFTER HIGH DOSE METHOTREXATE IN PATIENTS WITH OSTEOSARCOMA OR SPINDLE CELL SARCOMA OF BONE

CHIEF INVESTIGATOR: DR JEREMY WHELAN

CO-INVESTIGATOR: DR MARTHA PERISOGLOU

SPONSOR: UCLH/UCL

EudraCT number: 2006-003203-40

UCL

CASE RECORD FORM

Patient's Initials ☐☐☐☐ Patient's Trial Number ☐☐

REGISTRATION FORM

Date of CONSENT	
Diagnosis	
Date of diagnosis	
UCH Hospital Number	
DOB	
Histology	
Previous treatments (chemotherapy, surgery. radiotherapy)	
Concomitant Medications at trial entry	

Patient's Initials ☐☐☐☐ Patient's Trial Number ☐☐

PATIENT ELIGIBILITY

Eligibility criteria	Tick
1. Signed informed consent of patient or parent/guardian	
2. Diagnosis of high grade osteosarcoma, primary or metastatic or both **or** high grade osteosarcoma as a second maligancy **or** spindle cell sarcoma of bone **or** relapsed high grade osteosarcoma	
3. Age: 5-50 years at registration	
4. Patient able to comply with study and follow up procedures	
5. No concomitant anti-cancer or investigational drugs during the study and complete resolution of toxicity related to previous treatment	
6. Life expectancy of at least 3 months	
7. Absolute neutrophil count ≥1 $x10^9$/L	
8. Platelets ≥100 $x10^9$/L	
9. Bilirubin: ≤1.5 x ULN, ALT ≤1.5 x ULN, Albumin ≥ ULN	
10. GFR (radioisotope) ≥70 ml/min/1.73m^2	
11. **No** previous treatment with glucarpidase	
12. Patient **not** pregnant or lactating	
13. **No** concomitant treatment with agents which interact with methotrexate metabolism or excretion	
14. **No** Serous effusions or other "3rd space"	

Date information sheet given	
Version of information sheet given	

INVESTIGATOR'S SIGNATURE..... DATE.....

Patient's Initials ☐☐☐☐ Patient's Trial Number ☐☐

PAST & CURRENT MEDICAL HISTORY

Any past medical history?	YES		NO	
Category	Clinically Significant Medical History			
General Appearance and Skin				
Head, Eyes, Ears, Nose, Throat				
Cardiovascular System				
Haematological				
Respiratory System				
Abdominal and Gastrointestinal System				
Musculoskeletal and Nervous System				
Genitourinary System				
Endocrine				
Allergies and Drug Sensitivities				
Other				

INVESTIGATOR'S SIGNATURE..... DATE.....

Patient's Initials ☐☐☐☐ Patient's Trial Number ☐☐

BLOOD TESTS COURSE 1a

	Day 0	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Date							
Haemoglobin g/dL							
WBC 10^9/L							
Platelets 10^9/L							
Neutrophils 10^9/L							
Sodium mmol/L							
Potassium mmol/L							
Urea mmol/L							
Creatinine µmol/L							
Calcium mmol/L							
ALP IU/L							
Phosphate mmol/L							
Magnesium mmol/L							
GFR (radionucleotide)							
Bilirubin µmol/L							
ALT IU/L							
Total Protein g/L							
Albumin g/L							
If there are multiple samples on the same calendar day, please document the most abnormal values							

INVESTIGATOR'S SIGNATURE..... DATE.....

Patient's Initials ☐☐☐☐ Patient's Trial Number ☐☐

PHYSICAL EXAMINATION: DAY 0 or 1 OF CYCLE 1

Date (dd/mm/yyyy) | | | | | | | | |

Height (cm)	Weight (kg)	BSA (m^2)	Blood Pressure (mmHg)	Pulse (bpm)	Respiratory rate (bpm)	Temperature (°C)	Performance Status

Not done	Category	Normal	Details if different from normal
	General Appearance and Skin		
	Head, Eyes, Ears, Nose, Throat		
	Cardiovascular System		
	Respiratory System		
	Abdominal and Gastrointestinal System		
	Musculoskeletal and Nervous System		
	Genitourinary System		
	Endocrine		
	Allergies and Drug Sensitivities		
	Concomitant medications and doses		
	Other		

INVESTIGATOR'S SIGNATURE..... DATE.....

CRF for GLU 1 clinical trial

Patient's Initials ☐☐☐☐ Patient's Trial Number ☐☐

ASSESSMENT: DAY 1 CYCLE 1

Date (dd/mm/yyyy) | | | | | | | | |

	Yes	No		Yes	No
Neutrophils ≥1x10^9/L			Mucositis (clinical)		
Platelets ≥1OOx10^9/L			Mucositis (functional)		
Bilirubin ≤1.5 x ULN			Infection		
Albumin ≥ ULN			Pyrexia		
ALT ≤1.5 x ULN			Serous effusions or "3rd space"		
GFR(radioisotope) 70ml/min/1.73m^2			urine output > 1ml/kg/hr		
Diarrhoea			Urinary pH >7.0 immediately prior to MTX		

MUCOSITIS ASSESSMENT

Tools	Description					
WHO	Grade ☐					
OAG	swallow		mucous membrane			
	lips, mouth corners		gingiva			
	tongue		teeth			
	saliva		voice			
OMWQ	1 ☐ 2 ☐ 3 ☐ 4 ☐ 5 ☐ 6 ☐					
CTCAE	Mucositis (clinical)	GRADE		Colitis	GRADE	
	Mucositis (functional)			Enteritis		
	Diarrhoea			Abdominal distension		

INVESTIGATOR'S SIGNATURE..... DATE.....

Patient's Initials ☐☐☐☐ Patient's Trial Number ☐☐

BLOOD TESTS COURSE 1b

	Day 0	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Date							
Haemoglobin g/dL							
WBC 10^9/L							
Platelets 10^9/L							
Neutrophils 10^9/L							
Sodium mmol/L							
Potassium mmol/L							
Urea mmol/L							
Creatinine µmol/L							
Calcium mmol/L							
ALP IU/L							
Phosphate mmol/L							
Magnesium mmol/L							
GFR (radionucleotide)							
Bilirubin µmol/L							
ALT IU/L							
Total Protein g/L							
Albumin g/L							
If there are multiple samples on the same calendar day, please document the most abnormal values							

INVESTIGATOR'S SIGNATURE..... DATE.....

Patient's Initials ☐☐☐☐ Patient's Trial Number ☐☐

PHYSICAL EXAMINATION: DAY 8 OF CYCLE 1

Date (dd/mm/yyyy) ☐☐☐☐☐☐☐☐

Height (cm)	Weight (kg)	BSA (m^2)	Blood Pressure (mmHg)	Pulse (bpm)	Respiratory rate (bpm)	Temperature (°C)	Performance Status

Not done	Category	Normal	Details if different from normal
	General Appearance and Skin		
	Head, Eyes, Ears, Nose, Throat		
	Cardiovascular System		
	Respiratory System		
	Abdominal and Gastrointestinal System		
	Musculoskeletal and Nervous System		
	Genitourinary System		
	Endocrine		
	Allergies and Drug Sensitivities		
	Concomitant medications and doses		
	Other		

INVESTIGATOR'S SIGNATURE..... DATE.....

CRF for GLU 1 clinical trial

Patient's Initials ☐☐☐☐ Patient's Trial Number ☐☐

ASSESSMENT: DAY 8 CYCLE 1

Date (dd/mm/yyyy) | | | | | | | | |

	Yes/No		Yes/No
Neutrophils ≥0.25x10^9/L		Mucositis (clinical)	
Platelets ≥50x10^9/L		Mucositis (functional)	
Bilirubin ≤1.5 x ULN		Infection	
GFR(estimated) 70ml/min/1.73m^2		Pyrexia	
Serous effusions or "3rd space"		Urine output > 1ml/kg/hr immediately prior to MTX	
Urinary pH >7.0 immediately prior to MTX			

MUCOSITIS ASSESSMENT

Tools	Description					
WHO	Grade ☐					
OAG	swallow		mucous membrane			
	lips, mouth corners		gingiva			
	tongue		teeth			
	saliva		voice			
OMWQ	1 ☐ 2 ☐ 3 ☐ 4 ☐ 5 ☐ 6 ☐					
CTCAE	Mucositis (clinical)	GRADE		Colitis	GRADE	
	Mucositis (functional)			Enteritis		
	Diarrhoea			Abdominal distension		

INVESTIGATOR'S SIGNATURE..... DATE.....

Patient's Initials ☐☐☐☐ Patient's Trial Number ☐☐

ASSESSMENT: DAY 8 CYCLE 1 (continued)

Record worst grade for the last 7 days:
A= CTCAE v3.0 grade (see Appendix 5)
B= Caused delay to chemotherapy (0=No, 1=Yes)

A	B	
		Neutrophils
		Platelets
		Febrile Neutropaenia
		Infection with neutropaenia
		Infection with normal ANC
		Bilirubin
		Creatinine
		ALT
		Hypophosphataemia
		Mucositis (clinical)
		Mucositis (functional)
		GFR
		Other
		Administrative

INVESTIGATOR'S SIGNATURE..... DATE.....

Patient's Initials ☐☐☐☐ Patient's Trial Number ☐☐

PHYSICAL EXAMINATION: DAY 15 OF CYCLE 1

Date (dd/mm/yyyy) | | | | | | | | |

Height (cm)	Weight (kg)	BSA (m^2)	Blood Pressure (mmHg)	Pulse (bpm)	Respiratory rate (bpm)	Temperature (°C)	Performance Status

Not done	Category	Normal	Details if different from normal
	General Appearance and Skin		
	Head, Eyes, Ears, Nose, Throat		
	Cardiovascular System		
	Respiratory System		
	Abdominal and Gastrointestinal System		
	Musculoskeletal and Nervous System		
	Genitourinary System		
	Allergies and Drug Sensitivities		
	Concomitant medications and doses		
	Other		
	Other		

INVESTIGATOR'S SIGNATURE..... DATE.....

Patient's Initials ☐☐☐☐ Patient's Trial Number ☐☐

BLOOD TESTS DAY 15 OF CYCLE 1

Haemoglobin g/dL	
WBC 10^9/L	
Platelets 10^9/L	
Neutrophils 10^9/L	
Sodium mmol/L	
Potassium mmol/L	
Urea mmol/L	
Creatinine µmol/L	
Calcium mmol/L	
ALP IU/L	
Phosphate mmol/L	
Magnesium mmol/L	
GFR (estimated)	
Bilirubin µmol/L	
ALT IU/L	
Total Protein g/L	
Albumin g/L	
If there are multiple samples on day 15 of cycle 1, please document the most abnormal values	

INVESTIGATOR'S SIGNATURE..... DATE.....

CRF for GLU 1 clinical trial

Patient's Initials ☐☐☐☐ Patient's Trial Number ☐☐

ASSESSMENT: DAY 15 CYCLE 1

Date (dd/mm/yyyy) | | | | | | | | |

	Yes/No		Yes/No
Neutrophils ≥0.75x10^9/L **or** WCC ≥2x10^9/L		Mucositis (clinical)	
Platelets ≥75x10^9/L		Mucositis (functional)	
Bilirubin ≤1.5 x ULN		Infection	
GFR(estimated) 70ml/min/1.73m^2		Pyrexia	
General condition satisfactory for subsequent chemotherapy (If no, describe why)			

MUCOSITIS ASSESSMENT

Tools	Description					
WHO	Grade ☐					
OAG	swallow		mucous membrane			
	lips, mouth corners		gingiva			
	tongue		teeth			
	saliva		voice			
OMWQ	1 ☐ 2 ☐ 3 ☐ 4 ☐ 5 ☐ 6 ☐					
CTCAE	Mucositis (clinical)	GRADE		Colitis	GRADE	
	Mucositis (functional)			Enteritis		
	Diarrhoea			Abdominal distension		

"DAY 15" FITNESS CRITERIA FULLFILLED (tick): **YES**☐ **NO**☐

INVESTIGATOR'S SIGNATURE..... DATE.....

Patient's Initials ☐☐☐☐ Patient's Trial Number ☐☐

ASSESSMENT: DAY 15 CYCLE 1 (continued)

Record worst grade for the last 7 days:
A= CTCAE v3.0 grade (see Appendix 5)
B= Caused delay to chemotherapy (0=No, 1=Yes)

A	B	
		Neutrophils
		Platelets
		Febrile Neutropaenia
		Infection with neutropaenia
		Infection with normal ANC
		Bilirubin
		Creatinine
		ALT
		Hypophosphataemia
		Mucositis (clinical)
		Mucositis (functional)
		GFR
		Other

INVESTIGATOR'S SIGNATURE..... DATE.....

Patient's Initials ☐☐☐☐ Patient's Trial Number ☐☐

BLOOD TESTS COURSE 2a

	Day 0	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Date							
Haemoglobin g/dL							
WBC 10^9/L							
Platelets 10^9/L							
Neutrophils 10^9/L							
Sodium mmol/L							
Potassium mmol/L							
Urea mmol/L							
Creatinine µmol/L							
Calcium mmol/L							
ALP IU/L							
Phosphate mmol/L							
Magnesium mmol/L							
GFR (radionucleotide)							
Bilirubin µmol/L							
ALT IU/L							
Total Protein g/L							
Albumin g/L							
If there are multiple samples on the same calendar day, please document the most abnormal values							

INVESTIGATOR'S SIGNATURE..... DATE.....

Patient's Initials ☐☐☐☐ Patient's Trial Number ☐☐

PHYSICAL EXAMINATION: DAY 0 or 1 OF CYCLE 2

Date (dd/mm/yyyy) | | | | | | | | |

Height (cm)	Weight (kg)	BSA (m^2)	Blood Pressure (mmHg)	Pulse (bpm)	Respiratory rate (bpm)	Temperature (°C)	Performance Status

Not done	Category	Normal	Details if different from normal
	General Appearance and Skin		
	Head, Eyes, Ears, Nose, Throat		
	Cardiovascular System		
	Respiratory System		
	Abdominal and Gastrointestinal System		
	Musculoskeletal and Nervous System		
	Genitourinary System		
	Endocrine		
	Allergies and Drug Sensitivities		
	Concomitant medications and doses		
	Other		

INVESTIGATOR'S SIGNATURE..... DATE.....

CRF for GLU 1 clinical trial

Patient's Initials ☐☐☐☐ Patient's Trial Number ☐☐

ASSESSMENT: DAY 1 CYCLE 2

Date (dd/mm/yyyy) | | | | | | | | |

	Yes	No		Yes	No
Neutrophils ≥1x10^9/L			Mucositis (clinical)		
Platelets ≥1OOx10^9/L			Mucositis (functional)		
Bilirubin ≤1.5 x ULN			Infection		
Albumin ≥ ULN			Pyrexia		
ALT ≤1.5 x ULN			Serous effusions or "3rd space"		
GFR(radioisotope) 70ml/min/1.73m^2			urine output > 1ml/kg/hr		
Diarrhoea			Urinary pH >7.0 immediately prior to MTX		

MUCOSITIS ASSESSMENT

Tools	Description					
WHO	Grade ☐					
OAG	swallow		mucous membrane			
	lips, mouth corners		gingiva			
	tongue		teeth			
	saliva		voice			
OMWQ	1 ☐ 2 ☐ 3 ☐ 4 ☐ 5 ☐ 6 ☐					
CTCAE	Mucositis (clinical)	GRADE		Colitis	GRADE	
	Mucositis (functional)			Enteritis		
	Diarrhoea			Abdominal distension		

INVESTIGATOR'S SIGNATURE..... DATE.....

Patient's Initials ☐☐☐☐ Patient's Trial Number ☐☐

BLOOD TESTS COURSE 2b

	Day 0	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Date							
Haemoglobin g/dL							
WBC 10^9/L							
Platelets 10^9/L							
Neutrophils 10^9/L							
Sodium mmol/L							
Potassium mmol/L							
Urea mmol/L							
Creatinine µmol/L							
Calcium mmol/L							
ALP IU/L							
Phosphate mmol/L							
Magnesium mmol/L							
GFR (radionucleotide)							
Bilirubin µmol/L							
ALT IU/L							
Total Protein g/L							
Albumin g/L							
If there are multiple samples on the same calendar day, please document the most abnormal values							

INVESTIGATOR'S SIGNATURE..... DATE.....

Patient's Initials ☐☐☐☐ Patient's Trial Number ☐☐

PHYSICAL EXAMINATION: DAY 8 OF CYCLE 2

Date (dd/mm/yyyy) ☐☐☐☐☐☐☐☐

Height (cm)	Weight (kg)	BSA (m^2)	Blood Pressure (mmHg)	Pulse (bpm)	Respiratory rate (bpm)	Temperature (°C)	Performance Status

Not done	Category	Normal	Details if different from normal
	General Appearance and Skin		
	Head, Eyes, Ears, Nose, Throat		
	Cardiovascular System		
	Respiratory System		
	Abdominal and Gastrointestinal System		
	Musculoskeletal and Nervous System		
	Genitourinary System		
	Endocrine		
	Allergies and Drug Sensitivities		
	Concomitant medications and doses		
	Other		

INVESTIGATOR'S SIGNATURE..... DATE.....

CRF for GLU 1 clinical trial

Patient's Initials ☐☐☐☐ Patient's Trial Number ☐☐

ASSESSMENT: DAY 8 CYCLE 2

Date (dd/mm/yyyy) | | | | | | | | |

	Yes/No		Yes/No
Neutrophils ≥0.25x10^9/L		Mucositis (clinical)	
Platelets ≥50x10^9/L		Mucositis (functional)	
Bilirubin ≤1.5 x ULN		Infection	
GFR(estimated) 70ml/min/1.73m^2		Pyrexia	
Serous effusions or "3rd space"		Urine output > 1ml/kg/hr immediately prior to MTX	
Urinary pH >7.0 immediately prior to MTX			

MUCOSITIS ASSESSMENT

Tools	Description						
WHO	Grade ☐						
OAG	swallow		mucous membrane				
	lips, mouth corners		gingiva				
	tongue		teeth				
	saliva		voice				
OMWQ	1 ☐ 2 ☐ 3 ☐ 4 ☐ 5 ☐ 6 ☐						
CTCAE	Mucositis (clinical)	GRADE		Colitis	GRADE		
	Mucositis (functional)			Enteritis			
	Diarrhoea			Abdominal distension			

INVESTIGATOR'S SIGNATURE..... DATE.....

Patient's Initials ☐☐☐☐ Patient's Trial Number ☐☐

ASSESSMENT: DAY 8 CYCLE 2 (continued)

Record worst grade for the last 7 days:
A= CTCAE v3.0 grade (see Appendix 5)
B= Caused delay to chemotherapy (0=No, 1=Yes)

A	B	
		Neutrophils
		Platelets
		Febrile Neutropaenia
		Infection with neutropaenia
		Infection with normal ANC
		Bilirubin
		Creatinine
		ALT
		Hypophosphataemia
		Mucositis (clinical)
		Mucositis (functional)
		GFR
		Other
		Administrative

INVESTIGATOR'S SIGNATURE..... DATE.....

Patient's Initials ☐☐☐☐ Patient's Trial Number ☐☐

PHYSICAL EXAMINATION: DAY 15 OF CYCLE 2

Date (dd/mm/yyyy) | | | | | | | | |

Height (cm)	Weight (kg)	BSA (m^2)	Blood Pressure (mmHg)	Pulse (bpm)	Respiratory rate (bpm)	Temperature (°C)	Performance Status

Not done	Category	Normal	Details if different from normal
	General Appearance and Skin		
	Head, Eyes, Ears, Nose, Throat		
	Cardiovascular System		
	Respiratory System		
	Abdominal and Gastrointestinal System		
	Musculoskeletal and Nervous System		
	Genitourinary System		
	Allergies and Drug Sensitivities		
	Concomitant medications and doses		
	Other		
	Other		

INVESTIGATOR'S SIGNATURE..... DATE.....

Patient's Initials ☐☐☐☐ Patient's Trial Number ☐☐

BLOOD TESTS DAY 15 OF CYCLE 2

Haemoglobin g/dL	
WBC 10^9/L	
Platelets 10^9/L	
Neutrophils 10^9/L	
Sodium mmol/L	
Potassium mmol/L	
Urea mmol/L	
Creatinine µmol/L	
Calcium mmol/L	
ALP IU/L	
Phosphate mmol/L	
Magnesium mmol/L	
GFR (estimated)	
Bilirubin µmol/L	
ALT IU/L	
Total Protein g/L	
Albumin g/L	
If there are multiple samples on day 15 of cycle 2, please document the most abnormal values	

INVESTIGATOR'S SIGNATURE..... DATE.....

CRF for GLU 1 clinical trial

Patient's Initials ☐☐☐☐ Patient's Trial Number ☐☐

ASSESSMENT: DAY 15 CYCLE 2

Date (dd/mm/yyyy) | | | | | | | | |

	Yes/No		Yes/No
Neutrophils ≥0.75x10^9/L **or** WCC ≥2x10^9/L		Mucositis (clinical)	
Platelets ≥75x10^9/L		Mucositis (functional)	
Bilirubin ≤1.5 x ULN		Infection	
GFR(estimated) 70ml/min/1.73m^2		Pyrexia	
General condition satisfactory for subsequent chemotherapy (If no, describe why)			

MUCOSITIS ASSESSMENT

Tools	Description					
WHO	Grade ☐					
OAG	swallow		mucous membrane			
	lips, mouth corners		gingiva			
	tongue		teeth			
	saliva		voice			
OMWQ	1 ☐ 2 ☐ 3 ☐ 4 ☐ 5 ☐ 6 ☐					
CTCAE	Mucositis (clinical)	GRADE		Colitis	GRADE	
	Mucositis (functional)			Enteritis		
	Diarrhoea			Abdominal distension		

"DAY 15" FITNESS CRITERIA FULLFILLED (tick): **YES**☐ **NO**☐

INVESTIGATOR'S SIGNATURE..... DATE.....

Patient's Initials ☐☐☐☐ Patient's Trial Number ☐☐

ASSESSMENT: DAY 15 CYCLE 2 (continued)

Record worst grade for the last 7 days:
A= CTCAE v3.0 grade (see Appendix 5)
B= Caused delay to chemotherapy (0=No, 1=Yes)

A	B	
		Neutrophils
		Platelets
		Febrile Neutropaenia
		Infection with neutropaenia
		Infection with normal ANC
		Bilirubin
		Creatinine
		ALT
		Hypophosphataemia
		Mucositis (clinical)
		Mucositis (functional)
		GFR
		Other

INVESTIGATOR'S SIGNATURE..... DATE.....

Patient's Initials ☐☐☐☐ Patient's Trial Number ☐☐

METHOTREXATE AND DAMPA CUMULATIVE RESULTS COURSE 1a

Time of starting MTX infusion:

Time of completing MTX infusion:

HPLC results

Time (hours)	Date and time sample taken	Methotrexate (µmol/L)	DAMPA (µmol/L)
0			
4			
24			
24:20			
48			
72			
96			
120			

Immunoassay results

Time (hours)	Methotrexate (µmol/L)
24	
48	
72	
96	
120	

INVESTIGATOR'S SIGNATURE..... DATE.....

Patient's Initials ☐☐☐☐ Patient's Trial Number ☐☐

METHOTREXATE AND DAMPA CUMULATIVE RESULTS COURSE 1b

Time of starting MTX infusion:

Time of completing MTX infusion:

HPLC results

Time (hours)	Date and time sample taken	Methotrexate (µmol/L)	DAMPA (µmol/L)
0			
4			
24			
24:20			
48			
72			
96			
120			

Immunoassay results

Time (hours)	Methotrexate (µmol/L)
24	
48	
72	
96	
120	

INVESTIGATOR'S SIGNATURE..... DATE.....

Patient's Initials ☐☐☐☐ Patient's Trial Number ☐☐

METHOTREXATE AND DAMPA CUMULATIVE RESULTS COURSE 2a

Time of starting MTX infusion:

Time of completing MTX infusion:

HPLC results

Time (hours)	Date and time sample taken	Methotrexate (µmol/L)	DAMPA (µmol/L)
0			
4			
24			
24:20			
48			
72			
96			
120			

Immunoassay results

Time (hours)	Methotrexate (µmol/L)
24	
48	
72	
96	
120	

INVESTIGATOR'S SIGNATURE..... DATE.....

Patient's Initials ☐☐☐☐ Patient's Trial Number ☐☐

METHOTREXATE AND DAMPA CUMULATIVE RESULTS COURSE 2b

Time of starting MTX infusion:

Time of completing MTX infusion:

HPLC results

Time (hours)	Date and time sample taken	Methotrexate (µmol/L)	DAMPA (µmol/L)
0			
4			
24			
24:20			
48			
72			
96			
120			

Immunoassay results

Time (hours)	Methotrexate (µmol/L)
24	
48	
72	
96	
120	

INVESTIGATOR'S SIGNATURE..... DATE.....

Patient's Initials ☐☐☐☐ Patient's Trial Number ☐☐

TREATMENT SUMMARY

COURSE	MTX DOSE (mg)	DATE (dd/mm/yyyy)	GLUCARPIDASE /PLACEBO DOSE (units)	DATE
1a				
1b				
2a				
2b				

INVESTIGATOR'S SIGNATURE..... DATE.....

Patient's Initials ☐☐☐☐ Patient's Trial Number ☐☐

ANTI-GLUCARPIDASE ANTIBODIES: CUMULATIVE RESULTS

Cycle	Day	Date	Anti-glucarpidase antibody levels
1	1		
	8		
	15		
2	1		
	8		
	15		
	30		
	90		
	180		

INVESTIGATOR'S SIGNATURE..... DATE.....

CRF for GLU 1 clinical trial

Patient's Initials ☐☐☐☐ Patient's Trial Number ☐☐

END OF STUDY FORM

course	date	Total folinic acid dose per course (mg)
1a		
1b		
2a		
2b		

Reason for terminating study (circle as appropriate):

1. Study completed
2. Excessive toxicity related to methotrexate
3. Excessive toxicity related to glucarpidase
4. Treatment refusal
5. Inappropriate enrolment
6. Non-compliance with protocol schedule
7. Other, specify.....

Number of nights in hospital during cycle 1: ☐☐☐☐

Number of nights in hospital during cycle 2: ☐☐☐☐

Quality of life Questionnaires received (tick as appropriate):

CYCLES	DAY	QLQ-C30	FACT-G	PedsQL 3.0
CYCLE 1	0			
	8			
	15			
CYCLE 2	1			
	8			
	15			

INVESTIGATOR'S SIGNATURE..... DATE.....

APPENDIX 7. PERFORMANCE STATUS FOR GLU 1 STUDY

	Lansky score (1-16 years)		**Karnofsky score (> 16 years)**
100	Fully active, normal.	100	Normal; no complaints; no evidence of disease.
90	Minor restrictions in physically strenuous activity.	90	Able to carry on normal activities; minor signs or symptoms of disease.
80	Active, but tires more quickly.	80	Normal activity with effort; some signs and symptoms of disease.
70	Both greater restriction of, and less time spent in, active play.	70	Cares for self but unable to carry on normal activity or to do work.
60	Up and around, but minimal active play; keeps busy with quieter activities.	60	Requires occasional assistance but is able to care for most of personal needs.
50	Gets dressed, but lies around much of the day; no active play; able to participate in all quiet play and activities.	50	Requires frequent assistance and medical care.
40	Mostly in bed; participates in quiet activities.	40	Disabled; requires special care and assistance.
30	In bed; needs assistance even for quiet play.	30	Severely disabled; hospitalization is indicated though death not imminent.
20	Often sleeping; play entirely limited to very passive activity.	20	Very ill; hospitalization and active supportive care necessary.
10	No play, does not get out of bed. Moribund.	10	Moribund, fatal processes progressing rapidly.
0	Unresponsive. Dead.	0	Unresponsive. Dead.

WHO PERFORMANCE SCALE	
0	Asymptomatic
1	Symptomatic but completely ambulant
2	Symptomatic, <50% in bed during the day
3	Symptomatic, >50% in bed during the day
4	Bed bound
5	Death

APPENDIX 8. GLU 1 CLINICAL TRIAL, DRUG INTERACTIONS WITH MTX

The following are contraindicated in patients receiving methotrexate:

- Salicylates, sulfonamides, probenecid, cephalothin, penicillins (carbenicillin, ticarcillin) in high concentrations, omeprazole: known to compete with methotrexate for membrane transport, and thus may reduce renal tubular secretion.
- Salicylates, sulfonamides, phenylbutazone, hypoglycaemics, diphenylhydantoins, tetracyclins, chloramphenicol, p-aminobenzoic acid and acidic anti-inflammatory drugs: known to displace methotrexate from its binding sites on plasma proteins, and thus may increase free methotrexate levels in plasma
- Theophylline or methionine could enhance methotrexate cytotoxicity
- NSAIDs reduce the tubular secretion of methotrexate
- Co-trimoxazole, nitrous oxide and trimethoprime are folate-antagonists and may increase methotrexate cytotoxicity
- Chloramphenicol and tetracycline could interfere with the enterohepatic circulation

APPENDIX 9. GLU 1 CLINICAL TRIAL, MEASURING RENAL FUNCTION

The suggested formulae for GFR estimation are:

Schwartz Formula [age 1-18][1]

$$\text{Estimated creatinine clearance (ml/min/1.73m}^2\text{)} = \frac{\text{F x Height [cm] x 88.4}}{\text{Serum creatinine (}\mu\text{mol/L)}}$$

Where **F** is proportional to body muscle mass, hence depending on age and gender:

- Infants (< 1 year of age) **F** = 0.45
- Male, 1-16 years **F** = 0.55
- Female, 1-21 years **F** = 0.55
- Male, 16-21 years **F** = 0.70

Normal values [ml/min/1.73m²]: mean value =120, normal range 90-150

Cockcroft – Gault Formula (>18 years) [2]

$$\text{Estimated creatinine clearance for females (ml/min/1.73m}^2\text{)} = \frac{\text{1.05 x [140 - age (yrs)] x wt (kg)}}{\text{serum creatinine (}\mu\text{mol/L)}}$$

$$\text{Estimated creatinine clearance for males (ml/min/1.73m}^2\text{)} = \frac{\text{1.25 x [140 - age (yrs)] x wt (kg)}}{\text{serum creatinine (}\mu\text{mol/L)}}$$

Note: the accuracy of these formulae have been incompletely evaluated in patients receiving repeated cycles of intensive chemotherapy or in adolescents. Renal function may be over-estimated by these methods.

REFERENCES

1. Schwartz GJ et al. The use of plasma creatinine concentration for estimating glomerular filtration rate in infants, children, and adolescents. Pediatr Clin North Am, 1987; 34(3):571-90.
2. Cockcroft DW et al. Prediction of creatinine clearance from serum creatinine. Nephron, 1976; 16(1):31-41.

APPENDIX 10. GLU 1 CLINICAL TRIAL, QUALITY OF LIFE QUESTIONNAIRES

For patients aged 16 and over at registration, quality of life will be assessed using the EORTC (European Organisation for Research and Treatment of Cancer) **QLQ-C30 version 3** [1-2] and the **FACT-G** (Functional Assessment of Cancer Therapy Scale-General)[3] questionnaires.

The QLQ-C30 questionnaire is a reliable and valid measure of the quality of life of cancer patients in multicultural clinical research settings. It incorporates nine multi-item scales: five functional scales (physical, role, cognitive, emotional and social); three symptom scales (fatigue, pain, nausea and vomiting); and a global health and quality of life scale. The average time required to complete the questionnaire is 11 minutes, and most patients will not require assistance[1].

The FACT-G is a 33-item questionnaire that can be easily completed in 5 minutes, usually without assistance[3]. It meets all requirements for use in an oncology clinical trial including ease of administration, brevity, reliability, validity and responsiveness to clinical change. It includes questions on physical, social/family, emotional and functional well-being along with questions on relationship with the doctor.

For patients aged 15 and under, Quality of Life will be assessed using the "**PedsQL 3.0 Cancer Module Acute Version** questionnaire". It encompasses 8 scales: pain and hurt (2 items), nausea (5 items), procedural anxiety (3 items), treatment anxiety (3 items), worry (3 items), cognitive problems (5 items), perceived physical appearance (3 items), and communication (3 items). PedsQL[4] has questionnaires for different age groups including 5-7 years, 8-12 years and 13-18 years. There are self-report questionnaires and parent proxy reports. Both the self-reporting and the parent proxy

questionnaire will be completed. Patients who become 16 years old during the course of the trial should continue to use the paediatric instrument for further assessments, as PedsQL has been validated up to the age of 18.

The QLQ-C30 English and PedsQL Cancer module are widely available.

FACT-G (version 4) questionnaire

Below is a list of statements that other people with your illness have said are important. **By circling one number per line, please indicate how true each statement has been for you during the past 7 days.**

	PHYSICAL WELL-BEING	Not at all	A little bit	Some-what	Quite a bit	Very much
GP1	**I have a lack of energy**	0	1	2	3	4
GP2	I have nausea	0	1	2	3	4
GP3	Because of my physical condition, I have trouble meeting the needs of my family	0	1	2	3	4
GP4	I have pain	0	1	2	3	4
GP5	I am bothered by side effects of treatment	0	1	2	3	4
GP6	I feel ill	0	1	2	3	4
GP7	**I am forced to spend time in bed**	0	1	2	3	4
	SOCIAL/FAMILY WELL-BEING	**Not at all**	**A little bit**	**Some-what**	**Quite a bit**	**Very much**
GS1	I feel close to my friends	0	1	2	3	4
GS2	I get emotional support from my family	0	1	2	3	4
GS3	I get support from my friends	0	1	2	3	4
GS4	My family has accepted my illness	0	1	2	3	4
GS5	I am satisfied with family communication about my illness	0	1	2	3	4
GS6	I feel close to my partner (or the person who is my main support)	0	1	2	3	4
Q1 GS7	**Regardless of your current level of sexual activity, please answer the following question. If you prefer not to answer it, please check this box □ and go to the next section**					
	I am satisfied with my sex life	**0**	1	2	3	4

By circling one number per line, please indicate how true each statement has been for you during the past 7 days.

	EMOTIONAL WELL-BEING	Not at all	A little bit	Some-what	Quite a bit	Very much
GE1	**I feel sad**	0	1	2	3	4
GE2	I am satisfied with how I am coping with my illness	0	1	2	3	4
GE3	I am losing hope in the fight against my illness	0	1	2	3	4
GE4	I feel nervous	0	1	2	3	4
GE5	I worry about dying	0	1	2	3	4
GE6	I worry that my condition will get worse	0	1	2	3	4
	FUNCTIONAL WELL-BEING	**Not at all**	**A little bit**	**Some-what**	**Quite a bit**	**Very much**
GF1	I am able to work (include work at home)	0	1	2	3	4
GF2	My work (include work at home) is fulfilling	0	1	2	3	4
GF3	I am able to enjoy life	0	1	2	3	4
GF4	I have accepted my illness	0	1	2	3	4
GF5	I am sleeping well	0	1	2	3	4
GF6	I am enjoying the things I usually do for fun	0	1	2	3	4
GF7	I am content with the quality of my life right now	0	1	2	3	4

REFERENCES

1. Aaronson NK et al. The European Organization for Research and Treatment of Cancer QLQ-C30: a quality of life instrument for use in international clinical trials in oncology. Journal of the National Cancer Institute 1993, 85, 365-376.
2. Fayers PM et al. EORTC QLQ-C30 Scoring Manual. Brussels: EORTC, 1995
3. Cella D et al. The Functional Assessment of Cancer Therapy Scale: development and validation of the general measure. J Clin Oncol, 1993, 11:570-579

4. Varni JW et al. The PedsQL in pediatric cancer: reliability and validity of the Pediatric Quality of Life Inventory Generic Core Scales, Multidimensional Fatigue Scale, and Cancer Module. *Cancer* 2002, 94, 2090-2106.

APPENDIX 11. GLU 1 CLINICAL TRIAL, MUCOSITIS ASSESSMENT TOOLS

Oral and gastro-intestinal mucositis is a frequent complication of treatment with high-dose methotrexate. Prolonged or profound mucositis leads to significant pain and morbidity, excess costs for supportive care and hospitalisation, increased frequency of infection, and chemotherapy delays and dose reductions[1,2]. In an analysis of 3 studies involving 132 patients receiving methotrexate chemotherapy, 23% developed grade 3-4 oral mucositis[3].

It is important to be able to describe precisely, classify objectively and measure reproducibly the severity of mucosal damage. In an analysis of approximately 400 trials, as a component of the evidence-based review for the Multidisciplinary Association of Supportive Care in Cancer (MASCC) clinical practice guidelines, it was determined that most of the studies utilised the National Cancer Institute (NCI) (43%) or World Health Organization (WHO) (38%) scales. A further 10% of the studies utilised a study-specific scale. 5% of the studies used a cooperative group scale, such as those used by the Radiation Therapy Oncology Group (RTOG)[3, 4].

No single scale meets all requirements of a mucositis assessment tool or is universally accepted. Therefore, in this study four different assessment tools will be used:

1. National Cancer Institute Common Terminology criteria for Adverse Events, version 3.0 (CTCAE v 3.0)[5]
2. World Health Organisation Toxicity Criteria for Oral Mucositis[6]
3. Oral Assessment Guide[7]
4. Oral Mucositis Weekly Questionnaire[8]

The NCI CTCAE v3.0 and WHO scoring scales have been chosen as assessment tools in view of their widespread use in other studies studying mucositis. Both the scales measure anatomical, symptomatic and functional components of oral mucositis. In

addition, the NCI CTCAE v3.0 scale, also grades enteritis, colitis, diarrhoea and abdominal distension /bloating.

The Oral Assessment Guide is a validated instrument, which has been extensively used in adults with cancer. It is designed to objectively assess the physiological changes of the oral cavity following the administration of chemotherapy and radiotherapy. An adapted version has now been validated for paediatric use (by Gibson F et al, awaiting publication in Eur J Cancer).

The Oral Mucositis Weekly Questionnaire has been adapted from the Oral Mucositis Daily Questionnaire, which is developed as a mucositis specific questionnaire to assess patient-reported outcomes[9]. It is designed to assess the severity and impact of oral mucositis by evaluating mouth and throat soreness and the degree to which these interfere with activities of daily life such as eating, swallowing, drinking, talking and sleeping. It also assesses the overall health of the patient and the severity of diarrhoea. It has been shown to be a feasible, reliable, valid and responsive patient-reported measure of oral mucositis toxicity[8].

WHO TOXICITY CRITERIA: ORAL MUCOSITIS [6]

0	1	2	3	4
None	Soreness, erythema	Eryrhema, Ulcers, ability to eat solids	Ulcers, requires liquid diet	Alimentation not possible

Grade 1 may include buccal mucosa scalloping with or without erythema. No ulcers. Patient can swallow solid diet.

Grade 2 must include ulcers with or without erythema. Patient can swallow solid diet.

Grade 3 must include ulcers with or without (extensive) erythema. Patient is able to swallow liquid, but not solid diet.

Grade 4 means mucositis to the extent that alimentation is not possible. If total parenteral nutrition was started for reasons other that mucositis, a determination of the subject's ability to swallow must be made using the above criteria.

EXCERPT FROM COMMON TERMINOLOGY CRITERIA FOR ADVERSE EVENTS (CTCAE), v 3.0: GASTRO-INTESTINAL TOXICITY SCALE (Published December 12, 2003) [5]

GASTROINTESTINAL					
	Grade				
ADVERSE EVENT	**1**	**2**	**3**	**4**	**5**
Diarrhea	Increase of < 4 stools per day over baseline; mild increase in ostomy output compared to baseline	Increase of 4-6 stools per day over baseline; IV fluids indicated<24 hrs; moderate increase in ostomy output compared to baseline; not interfering with ADL*	Increase of ≥7 stools per day over baseline; incontinence; IV fluids ≥24 hrs; hospitalization; severe increase in ostomy output compared to baseline; interfering with ADL*	Life-threatening consequences (e.g. hemodynamic collapse)	Death
Remark: Diarrhea includes diarrhea of small bowel or colonic origin, and/or ostomy diarrhea					

EXCERPT FROM COMMON TERMINOLOGY CRITERIA FOR ADVERSE EVENTS (CTCAE), v 3.0: GASTRO-INTESTINAL

TOXICITY SCALE (Published December 12, 2003) [5]

GASTROINTESTINAL					
	Grade				
ADVERSE EVENT	**1**	**2**	**3**	**4**	**5**
Mucositis/stomatitis (clinical exam) -Select: - Anus - Esophagus - Large Bowel - Larynx - Oral Cavity - Pharynx - Rectum - Small Bowel - Stomach - Trachea	Erythema of the mucosa	Patchy ulcerations or pseudomembranes	Confluent ulcerations or pseudomembranes; bleeding with minor trauma	Tissue necrosis; significant spontaneous bleeding; life-threatening consequences	Death

EXCERPT FROM COMMON TERMINOLOGY CRITERIA FOR ADVERSE EVENTS (CTCAE), v 3.0: GASTRO-INTESTINAL TOXICITY SCALE (Published December 12, 2003) [5]

GASTROINTESTINAL					
	Grade				
ADVERSE EVENT	**1**	**2**	**3**	**4**	**5**
Mucositis/stomatitis (functional/ symptomatic) -Select: - Anus - Esophagus - Large Bowel - Larynx - Oral Cavity - Pharynx - Rectum - Small Bowel - Stomach - Trachea	Upper aerodigestive tract sites: Minimal symptoms, normal diet; minimal respiratory symptoms but not interfering with function Lower GI** sites: Minimal discomfort, intervention not indicated	Upper aerodigestive tract sites: Symptomatic but can eat and swallow modified diet; respiratory symptoms interfering with function but not interfering with ADL* Lower GI** sites: Symptomatic, medical intervention indicated but not interfering with ADL*	Upper aerodigestive tract sites: Symptomatic and unable to adequately aliment or hydrate orally; respiratory symptoms interfering with ADL* Lower GI**sites: Stool incontinence or other symptoms interfering with ADL*	Symptoms associated with life-threatening consequences	Death
Colitis	Asymptomatic, pathologic or radiographic findings only	Abdominal pain; mucus or blood in stool	Abdominal pain,fever, change in bowel habits with ileus; peritoneal signs	Life-threatening consequences (e.g. perforation, bleeding, ischaemia, necrosis, toxic megacolon)	Death

EXCERPT FROM COMMON TERMINOLOGY CRITERIA FOR ADVERSE EVENTS (CTCAE), v 3.0: GASTRO-INTESTINAL

TOXICITY SCALE (Published December 12, 2003) [5]

GASTROINTESTINAL					
	Grade				
ADVERSE EVENT	**1**	**2**	**3**	**4**	**5**
Enteritis (inflammation of the small bowel)	Asymptomatic, pathologic or radiographic findings only	Abdominal pain; mucus or blood in stool	Abdominal pain, fever, change in bowel habits with ileus; peritoneal signs	Life-threatening consequences (e.g. perforation, bleeding, ischaemia, necrosis)	Death
Distension /bloating, abdominal	Asymptomatic	Symptomatic, but not interfering with GI** function	Symptomatic, interfering with GI** function	–	–

* ADL: Activities of Daily Living
** GI: Gastro-intestinal

ORAL ASSESSMENT GUIDE FOR CHILDREN AND YOUNG PEOPLE

Oral assessment guide adapted from Eilers J et al. 19887, by F. Gibson et al (awaiting publication)

CATEGORY	METHOD OF ASSESSMENT	1	2	3
SWALLOW	Ask the child to swallow or observe the swallowing process. Ask the parent if there are any notable changes.	Normal. Without difficulty	Difficulty in swallowing	Unable to swallow at all. Pooling, dribbling of secretions
LIPS AND CORNER OF MOUTH	Observe appearance of tissue	Normal. Smooth, pink and moist	Dry, cracked or swollen	Ulcerated or bleeding
TONGUE	Observe the appearance of the tongue using a pen-torch to illuminate the oral cavity	Normal. Firm without fissures (cracking or splitting) or prominent papillae. Pink and moist	Coated or loss of papillae with a shiny appearance with or without redness and/or oral Candida	Ulcerated, sloughing or cracked
SALIVA	Observe consistency and quality of saliva	Normal. Thin and watery	Excess amount of saliva, drooling	Thick, ropy or absent
MUCOUS MEMBRANE	Observe the appearance of tissue using a pen-torch to illuminate the oral cavity	Normal. Pink and moist	Reddened or coated without ulceration and/or oral Candida	Ulceration and sloughing, with or without bleeding
GINGIVA	Observe the appearance of tissue using a pen-torch to illuminate the oral cavity	Normal. Pink or coral with a stippled (dotted) surface. Gum margins tight and well defined, no	Oedematous with or without redness, smooth	Spontaneous bleeding
TEETH (IF NO TEETH SCORE 1)	Observe the appearance of teeth using a pen-torch to illuminate the oral cavity	Normal. Clean and no debris	Plaque or debris in localised areas	Plaque or debris generalised along gum line
VOICE	Talk and listen to the child. Ask the parent if there are any notable changes	Normal tone and quality when talking or crying	Deeper or raspy	Difficult to talk, cry or not talking at all

ORAL ASSESSMENT GUIDE FOR ADULTS [7]

CATEGORY	TOOLS FOR ASSESSMENT	METHOD OF ASSESSMENT	1	2	3
VOICE	Auditory	Converse with patient	Normal	Deeper or raspy	Difficulty talking or painful
SWALLOW	Observation	Ask patient to swallow. To test gag reflux, gently place blade on back of tongue and depress	Normal swallow	Some pain on swallow	Unable to swallow
LIPS	Visual/palpatory	Observe and feel tissue	Smooth and pink and moist	Dry or cracked	Ulcerated or bleeding
TONGUE	Visual/palpatory	Feel and observe appearance of tissue	Pink and moist and papillae present	Coated or loss of papillae with a shiny appearance with or without redness	Blistered or cracked
SALIVA	Tongue blade	Insert blade into mouth, touching the centre of the tongue and the floor of the mouth	Watery	Thick or ropy	Absent
MUCOUS MEMBRANES	Visual	Observe appearance of tissue	Pink and moist	Reddened or coated (increased whiteness without ulceration)	Ulcerations with or without bleeding
GINGIVA	Tongue blade and visual	Gently press tissue with tip of blade	Pink and stippled and firm	Oedematous with or without redness	Spontaneous bleeding or bleeding with pressure
TEETH	Visual	Observe appearance of teeth or denture bearing area	Clean and no debris	Plaque or debris in localized areas (between teeth if present)	Plaque or debris generalized along gum line or denture bearing area

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ORAL MUCOSITIS WEEKLY QUESTIONNAIRE (OMWQ)

(adapted from the Oral Mucositis Daily Questionnaire (OMDQ)[8]

1. How would you rate your OVERALL HEALTH during the PAST WEEK?
(Circle one number)

|---|---|---|---|---|---|---|---|---|---|---|
0 1 2 3 4 5 6 7 8 9 10

worst possible — half way between worst possible and perfect health — perfect health

2. During the PAST WEEK, how much MOUTH AND THROAT SORENESS did you have?
(Circle one number)

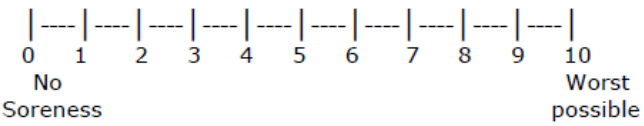
No soreness.....0 → if you circled 0, please skip to question 5
A little soreness.....1
Moderate soreness.....2
Quite a lot of soreness.....3
Extreme soreness.....4

3. During the PAST WEEK, how much did MOUTH AND THROAT SORENESS limit you in each of the following activities? (circle one number)

	Not limited	Limited a little	Limited some	Limited a lot	Unable to do

a) Swallowing.....0 1 2 3 4
b) Drinking.....0 1 2 3 4
c) Eating.....0 1 2 3 4
d) Talking.....0 1 2 3 4
e) Sleeping.....0 1 2 3 4

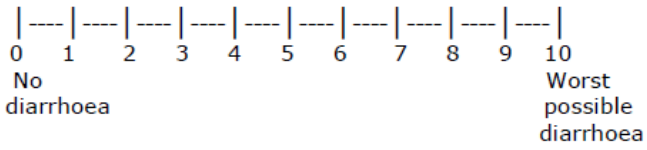
4. On a scale of 1 to 10, how would you rate your OVERALL MOUTH and THROAT SORENESS, during the PAST WEEK?



5. During the PAST WEEK, how much diarrhoea did you have? (circle one number)

No diarrhoea.....0 → if you circled 0, STOP here
A little diarrhoea.....1
Moderate diarrhoea.....2
Quite a lot of diarrhoea.....3
Severe diarrhoea.....4

6. On a scale of 1 to 10, how would you rate your OVERALL DIARRHOEA during the PAST WEEK? (circle one number)



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APPENDIX 12. GLU 1 CLINICAL TRIAL, TREATMENT EXPERIENCE FOR EACH PARTICIPANT

HEOL-01	
Date of birth	05 July 1983 (24 years), male
Date of diagnosis	10 October 2006
Diagnosis	High grade osteosacoma of right proximal humerous, osteoblastic type, with multiple bilateral small pulmonary metastases
Other medical problems	Already received 1 course of cisplatin 120 mg/m^2 and doxorubicin 75 mg/ m^2, 6 courses of ifosfamide 14gr/m^2 and etoposide 500 mg/ m^2 and 8 doses of methotrexate 12 gr/m^2 05 February 2007: right forequarter amputation
Date of consent	13 June 2007
Concomitant medication at trial entry	Trimovate cream, topically on inguinal creases, routine mouthcare with Nystatin and Corsodyl mouthwash
Examination at trial entry	Unremarkable apart from some settling erythema on inguinal creases and right forequarter amputation
GFR prior to trial entry	119 ml/min/1.73 m^2 on 11 June 2007

HEOL started treatment on the the GLU 1 clinical trial on 03 July 2007. He was randomised to arm B and received glucarpidase in cycle 1.

CYCLE 1

His clinical and laboratory (FBC, renal and liver function) assessments on day 1 of cycle 1 were unremarkable apart from Hb of 10.1 gr/dl (CTCAE toxicity grade 1).

MTX dose 1: HEOL had his first dose of methotrexate on 03 July 2007. He had glucarpidase on 04 July 2007. Folinic acid rescue was started on the same day. Methotrexate plasma levels at 24 hours were 5.34 µmol/L. Methotrexate was cleared within 48 hours post dose. His serum creatinine prior to first methotrexate dose was 59 µmol/L and remained within normal range post methotrexate (CTCAE toxicity grade 0). His serum phosphate and bilirubin remained within normal range. ALT peaked at 244 IU/L on day 4 post methotrexate (CTCAE toxicity grade 2). No further bone marrow suppression was noted following the first dose of methotrexate. There were no adverse events related to glucarpidase.

His clinical examination on day 8 of cycle 1 revealed no changes and he had no mucositis. He remained on Trimovate cream topically on his inguinal creases. A swab from the inguinal creases taken on 02 July 2007 did not reveal any pathogens. He was also on laxatives. Creatinine was 56 µmol/L (CTCAE toxicity grade 0) and calculated GFR was 207 ml/min/1.73 m^2. ALT was elevated at 176 IU/L (CTCAE toxicity grade 2). His phosphate was normal at 1.09 mmol/L. His haemoglobin dropped to 9.6 gr/dl (CTCTAE toxicity grade 2). He was fit to proceed to his second dose of methotrexate on time.

MTX dose 2: HEOL had his second dose of methotrexate on 10 July 2007. He had glucarpidase on 11 July 2007. Folinic acid rescue was started on the same day. Methotrexate plasma levels at 24 hours were 7.02 µmol/L. Methotrexate was cleared within 48 hours post dose. His serum creatinine, phosphate and bilirubin remained within normal range post methotrexate. ALT peaked at 275 IU/L on day 2 post methotrexate (CTCAE toxicity grade 3). No further bone marrow suppression was noted following the second dose of methotrexate. There were no adverse events related to glucarpidase.

Assessment on day 15 of cycle 1 took place on 17 July 2007. His clinical examination was unremarkable. In particular there was no mucositis. He remained on Trimovate cream and mouthcare. Calculated GFR and creatinine were within normal range at 193 ml/min/1.73 m^2 and 60µmol/L respectively. ALT was elevated at 195 IU/L (CTCAE toxicity grade 2). His serum phosphate and bilirubin were within normal range. His HB was stable at 10.6 gr/dl (CTCAE toxicity grade 1) although his platelets dropped to 148 x 10^9/L (CTCAE toxicity grade 1). **"Day 15" criteria were met.**

CYCLE 2

Clinical assessment on day 1 of cycle 2 on 23 July 2007 was unremarkable. He remained on Trimovate cream topically. Creatinine was 69 µmol/L (CTCAE toxicity grade 0) and calculated GFR was within normal range at 176 ml/min/1.73 m^2. His ALT was mildly raised at 95 IU/L (CTCAE toxicity grade 1). His serum phosphate and bilirubin were within normal range. His FBC was stable with haemoglobin of 12 gr/dl (CTCAE toxicity grade 1) and platelets of 182 x 10^9/L (CTCAE toxicity grade 0). He was fit to proceed to his third dose of methotrexate on time.

MTX dose 3: HEOL had the third dose of methotrexate on 24 July 2007. Folinic acid rescue was started on 15 July 2007. Methotrexate plasma levels at 24 hours were 15.57 µmol/L. Methotrexate was cleared within 72 hours post dose. His serum creatinine and phosphate remained within normal range. His bilirubin peaked at 46 µmol/L (CTCAE toxicity grade 2) on day 2 post methotrexate. ALT peaked at 515 IU/L on day 2 post methotrexate (CTCAE toxicity grade 3). No further bone marrow suppression was noted following the third dose of methotrexate.

On clinical assessment on day 8 of cycle 2, HEOL had two oral ulcers. He complained of sore throat but he was able to eat and drink although less than normally (CTCAE mucositis clinical toxicity grade 2, CTCAE mucositis functional toxicity grade 1). Serum creatinine and phosphate were within normal range although his bilirubin was slightly raised at 26 µmol/L (CTCAE toxicity grade 1). His calculated GFR was 187ml/min/1.73 m^2. ALT was elevated at 155 IU/L (CTCAE toxicity grade 2). He was fit to receive his fourth dose of methothrexate on time.

MTX dose 4: HEOL received his fourth dose of methotrexate on 31 July 2007. Folinic acid rescue was started on 01 August 2007. Methotrexate plasma levels at 24 hours were 13.01 µmol/L. Methotrexate was cleared within 72 hours post dose. His serum creatinine and phosphate remained within normal range. His bilirubin peaked at 56µmol/L (CTCAE toxicity grade 2) on day 2 post methotrexate. His ALT remained stable (maximum of 155 IU/L on day 3 post methotrexate, CTCAE toxicity grade 2). His Hb remained stable although platelets dropped to 62 $x10^9$/L (CTCAE toxicity grade 2).

Assessment on day 15 of cycle 2 took place on 07 August 2007, after four doses of methotrexate. HEOL was at his local hospital, attending for transfusion of red packed cell and platelets. He had mucositis (CTCAE mucositis clinical toxicity grade 3, CTCAE mucositis functional toxicity grade 2). His calculated GFR was 182 ml/min/1.73 m^2. His serum creatinine was within normal range. His ALT was raised at 99 IU/L (CTCAE toxicity grade 1). His bilirubin was also raised at 40 µmol/L (CTCAE toxicity grade 2). He was anaemic with Hb of 7.7 gr/dl (CTCAE toxicity grade 3), thrombocytopenic with platelets of 37 $x10^9$/L (CTCAE toxicity grade 3) and neutropenic with neutrophils of 0.9 $x10^9$/L (CTCAE toxicity grade 3). In view of his mucositis and bone marrow suppression, **"day 15" criteria were not met.**

Antiglucarpidase antibodies were negative on days 1, 8 and 15 of cycle 1 and also negative on days 1, 8, 15 of cycle 2.

ANME-02	
Date of birth	20 November 1988 (19 years), male
Date of diagnosis	24 January 2007
Diagnosis	High grade intramedullary osteosacoma of left distal femur with multiple bilateral pulmonary metastases
Other medical problems	Already received 4 courses of cisplatin 120 mg/m^2, 6 courses of doxorubicin 75 mg/ m^2 and 8 doses of methotrexate 12 gr/m^2 Distal femoral replacement on 17/04/2007, good response to pre-operative chemotherapy
Date of consent	13 August 2007
Concomitant medication at trial entry	nil
Examination at trial entry	Unremarkable apart from distal femoral replacement on 17/04/2007
GFR prior to trial entry	73 ml/min/1.73 m^2 on 06 June 2007

ANME started treatment on GLU 1 clinical trial on 03 September 2007. He was randomised to arm A and received glucarpidase in cycle 2.

CYCLE 1

His clinical and laboratory (FBC, renal and liver function) assessments on day 1 of cycle 1 were unremarkable apart from haemoglobin of 10 gr/dl (CTCAE toxicity grade 1) and platelets of 104 x 10^9/l (CTCAE toxicity grade 1).

MTX dose 1: ANME had his first dose of methotrexate on 03 September 2007. Folinic acid rescue was started on 04 September 2007. Methotrexate plasma levels at 24 hours were 22.79 µmol/L. Methotrexate was cleared within 72 hours post dose. His serum creatinine prior to first methotrexate dose was 69 µmol/L and remained within normal range post methotrexate (CTCAE toxicity grade 0). Phosphate dropped to 0.59mmol/L on day 4 post methotrexate (CTCAE toxicity grade 3). ALT peaked at 210 IU/L on day 2 post methotrexate (CTCAE toxicity grade 2). No further bone marrow suppression was noted following the first dose of methotrexate.

Assessment on day 8 of cycle 1 revealed one ulcer on lower lip (CTCAE mucositis clinical toxicity grade 2, CTCAE mucositis functional toxicity grade 0). The patient did not need analgesia. Creatinine was 67 µmol/L (CTCAE toxicity grade 0) and calculated

GFR was 142.97 ml/min/1.73 m^2. ALT was elevated at 128 IU/L (CTCAE toxicity grade 1). His phosphate was normal at 1.42 mmol/L. He was fit to proceed to his 2nd dose of methotrexate on time.

MTX dose 2: ANME had his 2nd dose of methotrexate on 10 September 2007. Folinic acid rescue was started on 11 September 2007. Methotrexate plasma levels at 24 hours were 20.47 µmol/L. Methotrexate was cleared within 72 hours post dose. His serum creatinine remained within normal range post methotrexate. Phosphate dropped to 0.4 mmol/L on day 3 post methotrexate (CTCAE toxicity grade 3) and he received phosphate oral supplements for three days. ALT peaked at 201 IU/L on day 3 post methotrexate (CTCAE toxicity grade 2). No bone marrow suppression was noted following the second dose of methotrexate.

Assessment on day 15 of cycle 1 took place on 17 September 2007, after two doses of methotrexate. ANME had multiple ulcers on his lower lip although they were not painful (CTCAE mucositis clinical toxicity grade 2, CTCAE mucositis functional toxicity grade 0). He was able to eat and drink as normal and required no analgesia. Calculated GFR and creatinine were within normal range at 126 ml/min/1.73 m^2 and 76 µmol/L respectively. ALT was elevated at 116 IU/L (CTCAE toxicity grade 1). His serum phosphate and bilirubin were within normal range. No bone marrow suppression was noted throughout the first cycle of treatment. In view of his mucositis on day 15 of cycle 1, **"day 15" criteria were not met.**

CYCLE 2

Clinical assessment on day 1 of cycle 2 on 01 October 2007 was unremarkable. He was on no medication. Creatinine was 77 µmol/L (CTCAE toxicity grade 0) and calculated GFR was within normal range at 156 ml/min/1.73 m^2. His ALT, serum phosphate and bilirubin were all within normal range. His FBC was stable although he had and haemoglobin of 11.5 gr/dl (CTCAE toxicity grade 1) He was fit to proceed to his third dose of methotrexate.

MTX dose 3: ANME had the third dose of methotrexate on 01 October 2007, delayed by 7 days in view of his mucositis. He had glucarpidase on 02 October 2007. Folinic acid rescue was started on the same day. Methotrexate plasma levels at 24 hours were

32.29 µmol/L. Methotrexate was cleared within 48 hours post dose. His serum creatinine and bilirubin remained within normal range. His phosphate dropped to 0.59 mmol/L (CTCAE toxicity grade 3) on day 4 post methotrexate. ALT peaked at 115 IU/L on day 2 post methotrexate (CTCAE toxicity grade 1). No further bone marrow suppression was noted following the third dose of methotrexate. There were no adverse events related to glucarpidase.

Clinical assessment on day 8 of cycle 2 was unremarkable. In particular, there was no mucositis. Serum creatinine, phosphate and bilirubin were within normal range. His calculated GFR was 136ml/min/1.73 m^2. ALT was slightly elevated at 66 IU/L (CTCAE toxicity grade 1). He was fit to receive his fourth dose of methothrexate on time.

MTX dose 4: ANME received his fourth dose of methotrexate on 08 October 2007. He had glucarpidase on 09 October 2007. Folinic acid rescue was started on the same day. Methotrexate plasma levels at 24 hours were 23.74 µmol/L. Methotrexate was cleared within 48 hours post dose. His serum creatinine and bilirubin remained within normal range. His phosphate dropped to 0.69 mmol/L (CTCAE toxicity grade 2) on day 4 post methotrexate. ALT peaked at 121 IU/L on day 2 post methotrexate (CTCAE toxicity grade 3). His Hb and Plts dropped to 10.8 gr/dl (CTCTAE toxicity grade 1) and 142 $x10^9$/L (CTCAE toxicity grade 1) respectively. There were no adverse events related to glucarpidase.

Assessment on day 15 of cycle 2 took place on 15 October 2007, after four doses of methotrexate. He had 1 small healing ulcer over the fraenulum. Otherwise his clinical examination was unremarkable and he required no analgesia. His calculated GFR was 141 ml/min/1.73 m^2. His serum creatinine was within normal range. The blood sample taken for bilirubin, phosphate and ALT was slightly haemolysed and therefore there are no results. His haemoglobin and platelets peaked up at to 12.8 gr/dl (CTCTAE toxicity grade 1) and 152 $x10^9$/L (CTCAE toxicity grade 0) respectively. **"Day 15" criteria were met.**

Antiglucarpidase antibodies were negative on days 1, 8 and 15 of cycle 1 and 1, 8 and 15 of cycle 2.

JACA-03	
Date of birth	03 December 1959 (37 years), female
Date of diagnosis	23 July 2007
Diagnosis	High grade intramedullary osteosacoma of 7th right rib, osteoblastic type
Other medical problems	Surgical excision of 7th right rib prior to starting treatment for osteosarcoma
Date of consent	25 September 2007
Concomitant medication at trial entry	Oxycontin 20 mg BD PO, Oxynorm 5 mg 2-4 hourly PRN PO, Paracetamol, 1gr QDS PO, Cyclizine 50 mg QDS PO, Tolterodine Tartate 4 mg OD PO (muscarinic receptor antagonist, to treat overactive bladder -frequency and urgency).
Examination at trial entry	Unremarkable apart from healing scar from surgical excision of 7th right rib
GFR prior to trial entry	94 ml/min/1.73 m^2 on 19 July 2007

JACA started treatment on the GLU 1 clinical trial on 25 September 2007. She was randomised to arm B and received glucarpidase in cycle 1.

CYCLE 1

Her clinical and laboratory (FBC, renal and liver function) assessments on day 1 of cycle 1 were unremarkable.

MTX dose 1: JACA had the first dose of methotrexate on 25 September 2007 with glucarpidase rescue on 26 September 2007. Folinic acid rescue was started on 26 September 2007. Methotrexate plasma levels at 24 hours were 9.12 µmol/L. Methotrexate was cleared within 48 hours post dose. Creatinine prior to the first methotrexate dose was 57 µmol/L and peaked at 91 µmol/L, on day 4 post first dose of methotrexate (CTCAE toxicity grade 0). Phosphate dropped to 0.41 mmol/L on day 2 post methotrexate (CTCAE toxicity grade 3). ALT peaked at 736 IU/L on day 2 post methotrexate (CTCAE toxicity grade 4). No bone marrow suppression was noted following the first dose of methotrexate. There were no adverse events related to glucarpidase.

Assessment on day 8 of cycle 1 revealed slight erythema at the back of her throat. The patient did not need analgesia. Creatinine was 75 µmol/L (CTCAE toxicity grade 0)

and calculated GFR was acceptable at 75 ml/min/1.73 m^2. ALT was elevated at 155 IU/L (CTCAE toxicity grade 1). Her phosphate was normal at 1.14 mmol/L. She was fit to proceed to her second dose of methotrexate on time.

MTX dose 2: JACA had the second dose of methotrexate on 02 October 2007 with glucarpidase rescue on 03 October 2007. Folinic acid rescue was started on 03 October 2007. Methotrexate plasma levels at 24 hours were 63.46 µmol/L. Methotrexate was cleared within 48 hours post dose. Calculated GFR prior to her second dose of methotrexate was 75 ml/min/1.73 m^2. Creatinine prior to her second methotrexate dose was 75 µmol/L and remained stable with max of 89 µmol/L, on day 2 post methotrexate (CTCAE toxicity grade 0). Phosphate dropped to 0.46 mmol/L on day 3 post methotrexate (CTCAE toxicity grade 3). ALT peaked at 654 IU/L on day 2 post methotrexate (CTCAE toxicity grade 3). Her bilirubin peaked at 31 µmol/L on day 2 post methotrexate (CTCAE toxicity grade 2). No bone marrow suppression was noted following the second dose of methotrexate. There were no adverse events related to glucarpidase.

Assessment on day 15 of cycle 1 took place on 09 October 2007, post two doses of methotrexate. There were two to three small ulcers on her lips (CTCAE for mucositis clinical, toxicity grade 2). JACA felt nauseated although she was able to eat and drink. Calculated GFR and creatinine were within normal range at 79.25 ml/min/1.73 m^2 and 69µmol/L respectively. ALT was elevated at 386 IU/L (CTCAE toxicity grade 3). No bone marrow suppression was noted throughout the first cycle of treatment. In view of her mucositis on day 15 of cycle 1, **"day 15" criteria were not met.**

CYCLE 2

Assessment on day 1 of cycle 2 on 15 October 2007 revealed no lip ulcers; however there were some residual changes on her left cheek and some throat erythema (CTCAE toxicity grade 0-1).Eating and drinking was not compromised. Creatinine was 79 µmol/L (CTCAE toxicity grade 0) and calculated GFR was almost acceptable at 69.2 ml/min/1.73 m^2. ALT was elevated at 342 IU/L (CTCAE toxicity grade 3). Her phosphate was normal at 1.19mmol/L. She was fit to proceed to her third dose of methotrexate.

MTX dose 3: JACA had the third dose of methotrexate on 16 October 2007, delayed by 7 days in view of her mucositis. She had glucarpidase rescue on 17 October 2007. Folinic acid rescue was started on 17 October 2007. Methotrexate plasma levels at 24 hours were 24.45 µmol/L. Methotrexate clearance was delayed and methotrexate plasma levels were <0.2µmol/L at 144 hours post dose. Her creatinine peaked at 111 µmol/L, on day 4 post third dose of methotrexate (CTCAE toxicity grade 1). Phosphate dropped to 0.56 mmol/L on day 2 post methotrexate (CTCAE toxicity grade 3). ALT peaked at 658 IU/L on day 2 post methotrexate (CTCAE toxicity grade 3). Her bilirubin remained within normal range. No bone marrow suppression was noted following the third dose of methotrexate. There were no adverse events related to glucarpidase.

Assessment on day 8 of cycle 2 revealed no mucositis. Creatinine was 95 µmol/L (CTCAE toxicity grade 1) and calculated GFR dropped to 57.56 ml/min/1.73 m^2. ALT was elevated at 248 IU/L (CTCAE toxicity grade 3). Her phosphate was normal at 1.3 mmol/L. She was tearful and felt rather depressed. In view of her decreased GFR, her fourth dose of methotrexate was postponed by 7 days.

Assessment on day 15 of cycle 2 took place on 29 October 2007, after three doses of methotrexate. There was no mucositis and the rest of her clinical examination was unremarkable. Calculated GFR dropped further at 50 ml/min/1.73 m^2 and her creatinine was increased at 108 µmol/L (CTCAE toxicity grade 1). ALT was elevated at 139 IU/L (CTCAE toxicity grade 2). No bone marrow suppression was noted throughout her second cycle of treatment. In view of impaired renal function on day 15 of cycle 2, **"day 15" criteria were not met.** Radionucleotide GFR on 31 October 2007 was 55 ml/min/1.73 m^2; therefore it was decided not to receive further methotrexate.

Antiglucarpidase antibodies were negative on days 1, 8 and 15 of cycle 1. There were also negative on days 1, 15 and 120 of cycle 2.

NIFI-04	
Date of birth	25 March 1960 (47 years), male
Date of diagnosis	24 July 2007
Diagnosis	High grade intramedullary chondrosarcoma of left pelvis grade 2 and 3. In addition, there is a high grade spindle cell intravascular component regarded as de-differentiated chondrosarcoma Post surgery, bulky intravascular (left femoral and external iliac vein) and local recurrence (on MR pelvis 28/09/07)
Other medical problems	Total internal hemipelvectomy on 24 July 2007 and insertion of femoral prosthesis prior to starting chemotherapy/trial treatment IVC filter inserted 28 September 2007
Date of consent	28 September 2007
Concomitant medication at trial entry	MST 10 mg mane, 20mg nocte, PO, Amitryptiline, Pregabalin, Tramadol, Ranitidine, Lactulose, Senna, Fragmin
Examination at trial entry	Total internal hemipelvectomy and insertion of femoral prosthesis on 24 July 2007, left leg sensation normal but left foot drop and some pain in left foot
GFR prior to trial entry	103 ml/min/1.73 m^2 on 26 September 2007

NIFI started treatment on the GLU 1 clinical trial on 01 October 2007. He was randomised to arm B and received glucarpidase in cycle 1.

CYCLE 1

On clinical examination on day 1 of cycle 1 NIFI had left foot drop and was wearing a brace. There was also some pain at his left foot. His laboratory (FBC, renal and liver function) assessment on day 1 of cycle 1 was unremarkable apart from haemoglobin of 10.4 gr/dl (CTCAE grade 1).

MTX dose 1: NIFI had his first dose of methotrexate on 01 October 2007. Glucarpidase was given on 02 October 2007. Folinic acid rescue was started on 02 October 2007. Methotrexate plasma levels at 24 hours were 17.44 µmol/L (immunoassay). Methotrexate was cleared within 48 hours post dose. His serum creatinine prior to first methotrexate dose was 56 µmol/L and peaked at 95 µmol/L on day 3 post methotrexate, remaining within normal range (CTCAE toxicity grade 0). Phosphate remained within normal range. ALT peaked at 891 IU/L on day 2 post methotrexate (CTCAE toxicity grade 3). His serum bilirubin remained within normal range. No bone

marrow suppression was noted following the first dose of methotrexate. There were no adverse events related to glucarpidase.

Assessment on day 8 of cycle 1 revealed no mucositis. There was left leg and left sided pedal oedema but no other new problems. He remained on MST 30 mg OD, PO, Amitryptilline 75mg OD nocté, Pregabalin 75mg am, 150mg pm, PO and Fragmin 15.000 units OD, SC. His creatinine was 100 µmol/L (CTCAE toxicity grade 0) but calculated GFR dropped to 72ml/min/1.73 m^2. ALT was elevated at 267 IU/L (CTCAE toxicity grade 3). His phosphate and bilirubin remained within normal range. He was fit to proceed to his second dose of methotrexate on time.

MTX dose 2: NIFI had his second dose of methotrexate on 08 October 2007. Glucarpidase was given on 09 October 2007. Folinic acid rescue was started on 09 October 2007. Methotrexate plasma levels at 24 hours were high at 70.16 µmol/L. He had delayed methotrexate elimination. Methotrexate was eventually cleared at 216 hours post second dose. His serum creatinine peaked at 127 µmol/L on day 3 post methotrexate (CTCAE toxicity grade 1). Phosphate and bilirubin remained within normal range. ALT remained elevated and peaked at 470 IU/L on day 2 post methotrexate (CTCAE toxicity grade 3). Mild bone marrow suppression was noted following the second dose of methotrexate. His haemoglobin dropped to 8.8 gr/dl (CTCAE toxicity grade 2) on day 7 post second methotrexate and he received a blood transfusion on 14 October 2007. His platelets dropped to 98 (CTCAE toxicity grade 1) and neutrophils to 1.55 (CTCAE toxicity grade 1) on day 8 post second methotrexate.

Assessment on day 15 of cycle 1 took place on 15 October 2007, after two doses of methotrexate. NIFI did not clear his second methotrexate dose until 17 October 2007; 10 days post his second dose. His mouth remained healthy although his fluid intake was not brilliant. He remained on intravenous hydration in view of delayed methotrexate elimination. He had no diarrhoea. His creatinine was 102 µmol/L (CTCAE toxicity grade 0). His calculated GFR was 90 ml/min/1.73 m^2. ALT was elevated at 139 IU/L (CTCAE toxicity grade 2). His serum phosphate was normal at 1.35 mmol/L. Mild bone marrow suppression was noted following the second dose of methotrexate. His haemoglobin dropped down to 8.8 gr/dl (CTCAE toxicity grade 2) post second methotrexate and he received a blood transfusion on 14 October 2007. His platelets dropped to 98 (CTCAE

toxicity grade 1) and neutrophils to 1.55 (CTCAE toxicity grade 1) post second methotrexate. In view of delayed methotrexate elimination, on day 15 of cycle 1, **“day 15” criteria were not met.**

Antiglucarpidase antibodies were negative on days 1, 8, 15 and 30 of cycle 1.

SHHI-05	
Date of birth	08 July 1977 (30 years), male
Date of diagnosis	February 1998: high grade osteosarcoma of left proximal tibia April 2007: single left sided pulmonary metastasis October 2007: metastatic osteosarcoma of sacrum, with small volume pulmonary metastases
Diagnosis	February 1998: high grade osteosarcoma of left proximal tibia, managed with doxorubicin and cisplatin (BO06 clinical trial, randomised to receive GCSF) and proximal tibial replacement, treatment completed in June 1998 April 2007: single left sided pulmonary metastasis, managed with surgical excision on 27 June 2007 October 2007; metastatic high grade osteosarcoma of sacrum, fibroblastic type with small volume pulmonary metastases
Other medical problems	nil
Date of consent	8 October 2008
Concomitant medication at trial entry	Tramadol 100 mg QDS PO, Paracetamol 1 gr QDS PO, Morphine Sulphate M/R 20 mg am & 30 mg pm, PO,Lansoprazole 30 mg OD PO, Lactulose 10 mg BD PO, Diclofenac 75 mg BD PO (discontinued prior to starting methotrexate)
Examination at trial entry	Previous left proximal tibial replacement Persistent back pain settling with analgesia
GFR prior to trial entry	82 ml/min/1.73 m^2 on 05/10/07

SHHI started treatment on the GLU 1 clinical trial on 09 October 2007. He was randomised to arm A; therefore he did not receive glucarpidase in cycle 1.

His clinical and laboratory assessments on day 1 of cycle 1 were unremarkable.

MTX dose 1: SHHI had his first dose of methotrexate on 09 October 2007. Folinic acid rescue was started on 10 October. Methotrexate plasma levels at 24 hours were 33.22 µmol/L. Methotrexate plasma levels at 72 hours were 0.44 µmol/L. His creatinine was 82 µmol/L prior to methotrexate. It remained within normal range on days 2 and 3 post methotrexate. However, it started to escalate from 13 October 2007, day 4 onwards. The patient developed oliguria. His creatinine was 17 7 µmol/L on day 4, 353 µmol/L on day 5, 464 µmol/L on day 6, 556 µmol/L on day 7, 631 µmol/L on day 8 and peaked at 677µmol/L on day 9 post first dose of methotrexate (CTCAE toxicity grade 3). His urine output gradually improved. Phosphate went also up from day 5 onwards and peaked at 2.57 mmol/L on day 9 post methotrexate (CTCAE toxicity grade 1). ALT peaked at 289

IU/L on day 2 post methotrexate (CTCAE toxicity grade 3). In view delayed methotrexate clearance and impaired renal function his treatment arm was unblinded as per protocol. He was found the have been randomised to arm A; therefore he had not receive glucarpidase at 24 hours post methotrexate, He received emergency treatment with glucarpidase (50 units/kg) on 13 October 2007 as per protocol. Methotrexate plasma levels dropped to < 0.2 µmol/L immediately post glucarpidase and remained < 0.2 µmol/L thereafter. There were no adverse events related to glucarpidase.

In view of acute renal failure post methotrexate, it was decided not to receive further methotrexate. He was started on doxorubicin with dexrazoxane protection on 23 October i.e. prior to day 15 of cycle 1.

Assessment on day 15 of cycle 1 took place on 22 September 2008, after only one dose of methotrexate. There was no mucositis. Calculated GFR and creatinine were abnormal at 44 ml/min/1.73 m^2 and 301 µmol/L respectively. ALT was normal at 35 IU/L (CTCAE toxicity grade 0). Phosphate levels were back to normal. No bone marrow suppression was noted throughout the first cycle of treatment. In view of acute renal failure related to methoterexate **"day 15" criteria were not met.**

Radionucleotide GFR was 84 ml/min/1.73 m^2 on 27 November 2007. His creatinine was back to normal when measured on 05 December 2007.

Antiglucarpidase antibodies were not assessed as this patient did not receive glucarpidase as part of the trial and only received one dose glucarpidase as emergency treatment.

SAWI-06	
Date of birth	04 May 1992 (15 years), male
Date of diagnosis	12 September 2007
Diagnosis	High grade intramedullary osteosacoma of left proximal humerus, largely osteoblastic type with some chondroblastic differentiation with metastases in skull and T9
Other medical problems	Already received 3 cycles of MAP (methotrexate 3 doses of 12 gr/m^2, adriamycin and cisplatin) December 2007: left forequarter amputation
Date of consent	28 January 2008
Concomitant medication at trial entry	Gabapentin 300 mg TDS PO Fentanyl patch 12 mcg/hr for 72 hours Sodium Docusate 100 mg TDS PO
Examination at trial entry	Unremarkable apart from left fore quarter amputation
GFR prior to trial entry	116 ml/min/1.73 m^2 on 07 January 2008

SAWI started treatment on the GLU 1 clinical trial on 28 January 2008. He was randomised to arm A and received glucarpidase in cycle 2.

CYCLE 1

His clinical and laboratory (FBC, renal and liver function) assessments on day 1 of cycle 1 were unremarkable apart from slightly raised ALT at 71 IU/L (CTCAE toxicity grade 1).

MTX dose 1: SAWI had his first dose of methotrexate on 28 January 2008. Folinic acid rescue was started on 29 January 2008. Methotrexate plasma levels at 24 hours were 12.55 µmol/L. Methotrexate was cleared within 72 hours post dose. His serum creatinine prior to first methotrexate dose was 39 µmol/L and remained within normal range post methotrexate (CTCAE toxicity grade 0). Phosphate dropped to 0.69 mmol/L on day 4 post methotrexate (CTCAE toxicity grade 2). ALT peaked at 446 IU/L on day 8 post methotrexate (CTCAE toxicity grade 3). No bone marrow suppression was noted following the first dose of methotrexate.

Assessment on day 8 of cycle 1 revealed two tiny oral ulcers (CTCAE mucositis clinical toxicity grade 2) although the patient could eat and drink normally (CTCAE mucositis functional toxicity grade 0). The patient did not need analgesia. Creatinine

was 45 µmol/L (CTCAE toxicity grade 0) and calculated GFR was 111.58 ml/min/1.73 m^2. ALT was elevated at 446 IU/L (CTCAE toxicity grade 3). His phosphate was normal at 1.8 mmol/L. He was fit to proceed to his second dose of methotrexate on time.

MTX dose 2: SAWI had his second dose of methotrexate on 04 February 2008. Folinic acid rescue was started on 05 February 2008. Methotrexate plasma levels at 24 hours were 3.55 µmol/L. Methotrexate was cleared within 72 hours post dose. His serum creatinine remained within normal range post methotrexate. Phosphate dropped to 0.65 mmol/L on day 2 post methotrexate (CTCAE toxicity grade 2). ALT dropped to 430 IU/L on day 2 post methotrexate (CTCAE toxicity grade 3) and dropped further to 317 IU/L on day 2 post methotrexate (CTCAE toxicity grade 3). However, it climbed up to 1056 IU/L 8 days post methotrexate (CTCAE toxicity grade 4). No bone marrow suppression was noted following the second dose of methotrexate.

Assessment on day 15 of cycle 1 took place on 11 February 2008, after two doses of methotrexate. SAWI had severe mucositis and was not able to open his mouth to be examined due to pain (CTCAE for mucositis clinical, toxicity grade 3). He could not eat or drink (CTCAE for mucositis functional, toxicity grade 3) and was tachycardic due to pain and dehydration. He was mildly hypertensive with BP of 128/85 mmHg. He required analgesia. Calculated GFR and creatinine were within normal range at 199.3 ml/min/1.73 m^2 and 40 µmol/L respectively. ALT was elevated at 1056 IU/L (CTCAE toxicity grade 4). His serum phosphate and bilirubin were within normal range. No bone marrow suppression was noted throughout the first cycle of treatment. In view of his severe mucositis on day 15 of cycle 1, **"day 15" criteria were not met.**

CYCLE 2

Clinical assessment on day 1 of cycle 2 on 25 February 2008 was unremarkable apart from his left forequarter amputation and a bit of erythema around the entry site of his PICC line for which he was on iv Teicoplanin. He was off Gabapentin and continued on Sodium Docusate. Creatinine was 56 µmol/L (CTCAE toxicity grade 0) and calculated GFR was within normal range at 142 ml/min/1.73 m^2. ALT was elevated at 102 IU/L (CTCAE toxicity grade 1). His serum phosphate and bilirubin were within normal range. He was fit to proceed to his third dose of methotrexate.

MTX dose 3: SAWI had the third dose of methotrexate on 25 February 2008, delayed by 7 days in view of his mucositis. He had glucarpidase on 26 February 2008. Folinic acid rescue was started on 26 February 2008. Methotrexate plasma levels at 24 hours were 12.76 µmol/L. Methotrexate was cleared within 48 hours post dose. His serum creatinine, phosphate and bilirubin remained within normal range. ALT peaked at 317 IU/L on day 2 post methotrexate (CTCAE toxicity grade 3). No bone marrow suppression was noted following the third dose of methotrexate. There were no adverse events related to glucarpidase.

Clinical assessment on day 8 of cycle 2 was unremarkable. In particular, there was no mucositis. Serum creatinine, phosphate and bilirubin were within normal range. His calculated GFR was 153.34 ml/min/1.73 m^2. ALT was elevated at 373 IU/L (CTCAE toxicity grade 3). He was fit to receive his fourth dose of methothrexate on time.

MTX dose 4: SAWI's PICC line required repositioning and he received his fourth dose of methotrexate on 04 March 2008, delayed by one day. He had glucarpidase on 06 March 2008. Folinic acid rescue was started on 06 March 2008. Methotrexate plasma levels at 24 hours were 14.74 µmol/L. Methotrexate was cleared within 72 hours post dose. His serum creatinine, phosphate and bilirubin remained within normal range. ALT peaked at 604 IU/L on day 2 post methotrexate (CTCAE toxicity grade 3). No bone marrow suppression was noted following the fourth dose of methotrexate. There were no adverse events related to glucarpidase.

Assessment on day 15 of cycle 2 took place on 11 March 2008, after four doses of methotrexate. There was no mucositis and the rest of his clinical examination was unremarkable. His calculated GFR was 100.9 ml/min/1.73 m^2. His serum creatinine, phosphate and bilirubin were within normal range. ALT was elevated at 592 IU/L (CTCAE toxicity grade 3). No bone marrow suppression was noted throughout the second cycle of treatment. **"Day 15" criteria were met.**

Antiglucarpidase antibodies were negative on days 1, 8 and 15 of cycle 1. There were negative on days 1, 8, 15, 30 and 90 of cycle 2.

IFOK-07	
Date of birth	03 November 1990 (17 years), male
Date of diagnosis	05 September 2007
Diagnosis	High grade osteosarcoma of left proximal tibia (telangiectatic type)
Other medical problems	Already received 6 courses of methotrexate at a dose of 12 gr/m^2/course, 4 courses of adriamycin at a dose of 75 mg/m^2/course and 4 courses of cisplatin at a dose of120 mg/m^2/course December 2007: excision of tumour and left tibial replacement
Date of consent	17 February 2008
Concomitant medication at trial entry	nil
Examination at trial entry	Unremarkable apart from scar from proximal tibial replacement
GFR prior to trial entry	129 ml/min/1.73 m^2 on 07 January 2008

IFOK started treatment on the GLU 1 clinical trial on 18 February 2008. He was randomised to arm B and received glucarpidase in cycle 1.

CYCLE 1

His clinical and laboratory (FBC, renal and liver function) assessments on day 1 of cycle 1 were unremarkable apart from Hb of 9.9 gr/dl (CTCAE toxicity grade 2).

MTX dose 1: IFOK had his first dose of methotrexate on 18 February 2008. Glucarpidase was given on 19 February 2008. Folinic acid rescue was started on 19 February 2008. Methotrexate plasma levels at 24 hours were rather high at 95.92µmol/L. Methotrexate was cleared within 72 hours post dose. His serum creatinine prior to first methotrexate dose was 79 µmol/L and peaked at 127 µmol/L on day 3 post methotrexate (CTCAE toxicity grade 1). Phosphate dropped to 0.71mmol/L on day 5 post methotrexate (CTCAE toxicity grade 2). ALT peaked at 729 IU/L on day 2 post methotrexate (CTCAE toxicity grade 3). His haemoglobin dropped to 8.8 gr/dl on days 4 and 5 post methotrexate (CTCAE toxicity grade 2). There was neither thrombocytopenia nor neutropenia following the first dose of methotrexate. There were no adverse events related to glucarpidase.

His second dose of methotrexate was due on 25 February 2008; however this was delayed to 28 February 2008 due to oral mucositis. **Clinical assessment on 28 February 2008, "day 8" of cycle 1,** was unremarkable. Prior to starting his second dose of methotrexate on trial, his creatinine was 90 µmol/L (CTCAE toxicity grade 0) and calculated GFR was 176 ml/min/1.73 m^2. ALT was slightly elevated at 66 IU/L (CTCAE toxicity grade 1). His haemoglobin was low at 8.8 gr/dl (CTCAE toxicity grade 2) but the rest of his bone marrow function was unremarkable. He was fit to proceed to his second dose of methotrexate with a 3 day delay in view of mucositis.

MTX dose 2: IFOK had his second dose of methotrexate on 28 February 2008. Glucarpidase was given on 29 February 2008. Folinic acid rescue was started on 29 February 2008. Methotrexate plasma levels at 24 hours were elevated at 141.69 µmol/L. However, methotrexate was cleared within 96 hours post dose. His serum creatinine peaked at 139 µmol/L on day 3 post methotrexate (CTCAE toxicity grade 1). Phosphate dropped to 0.58 mmol/L on day 2 post methotrexate (CTCAE toxicity grade 3). ALT peaked at 1070 IU/L on day 2 post methotrexate (CTCAE toxicity grade 4) No bone marrow suppression was noted following the second dose of methotrexate, although haemoglobin remained between 8.8 and 9.1 gr/dl. There were no adverse events related to glucarpidase.

Assessment on day 15 of cycle 1 took place on 03 March 2008, after two doses of methotrexate. He remained on intravenous hydration and folinic acid rescue as he did not clear his methotrexate until the following day, 4th March 2008. He was also on Movicol and domperidone. IFOK did not have mucositis and the rest of his clinical examination was unremarkable. Calculated GFR and creatinine were within normal range at 103 ml/min/1.73 m^2 and 108µmol/L respectively. ALT was elevated at 275 IU/L (CTCAE toxicity grade 3). His serum phosphate was low at 0.58 mmol/L (CTCAE toxicity grade 3). His bilirubin was within normal range. No bone marrow suppression was noted throughout the first cycle of treatment although Hb remained low at 9.1 gr/dl (CTCAE grade 2). In view of the delay in methotrexate elimination, **"day 15" criteria were not met.**

CYCLE 2

Clinical assessment on day 0 of cycle 2 on 16 March 2008 was unremarkable. He was on no medication. Creatinine was 98 µmol/L (CTCAE toxicity grade 0) and calculated GFR was within normal range at 115 ml/min/1.73 m^2. ALT and serum phosphate were within normal range. His haemoglobin remained low at 9.3 gr/dl (CTCAE toxicity grade 2). He was fit to proceed to his third dose of methotrexate.

MTX dose 3: IFOK had the third dose of methotrexate on 17 March 2008. Folinic acid rescue was started on 18 March 2008. Methotrexate plasma levels at 24 hours were high at 149.78 µmol/L. Methotrexate was cleared within 144 hours post dose. His serum creatinine peaked at 136 µmol/L on day 3 post methotrexate (CTCAE toxicity grade 1). Phosphate dropped to 0.57 mmol/L on day 3 post methotrexate (CTCAE toxicity grade 3) and he received phosphate supplements. ALT peaked at 684 IU/L on day 2 post methotrexate (CTCAE toxicity grade 4). His Hb remained stable and no further bone marrow suppression was noted following the third dose of methotrexate.

In view of the delay in the elimination of IFOK's third dose of methotrexate, he did not receive his fourth dose until the 27th March 2008. **Clinical assessment prior to his fourth dose** was unremarkable. In particular, there was no mucositis. He was on no medication. Serum creatinine, phosphate and bilirubin were within normal range. His calculated GFR was 125.8 ml/min/1.73 m^2. ALT was slightly elevated at 66 IU/L (CTCAE toxicity grade 1).

MTX dose 4: IFOK had his fourth dose of methotrexate on 27th March 2007. Folinic acid rescue was started on 27 March 2008. Methotrexate plasma levels at 24 hours were elevated at 114.83 µmol/L. Methotrexate was cleared within 120 hours post dose. His serum creatinine peaked at 125 µmol/L on day 2 post methotrexate (CTCAE toxicity grade 1). Phosphate dropped to 0.29mmol/L on day 5 post methotrexate (CTCAE toxicity grade 4) and he received phosphate supplements. ALT peaked at 1241 IU/L on day 2 post methotrexate (CTCAE toxicity grade 4). His Hb remained stable (minimum Hb 8.8.gr/dl, CTCTAE grade 2) and no further bone marrow suppression was noted following the forth dose of methotrexate.

Assessment on day 15 of cycle 2 took place on 01 April 2008, after four doses of methotrexate. There was no mucositis and the rest of his clinical examination was unremarkable. His calculated GFR was 115.5 ml/min/1.73 m^2. His serum creatinine and

bilirubin were within normal range. His serum phosphate was 0.98mmol/L and he remained on phosphate supplements. ALT was elevated at 222 IU/L (CTCAE toxicity grade 2). His haemoglobin remained low at 8.2 gr/dl (CTCAE grade 2) but no further bone marrow suppression was noted throughout the second cycle of treatment. **"Day 15" criteria were not met** as IFOK was still clearing his forth methotrexate dose**.**

Antiglucarpidase antibodies were negative on days 1, 8 and 15 of cycle 1. There were positive on day 1 of cycle 2, negative on day 8 of cycle 2 and then again positive on days 15 and 23 of cycle 2.

PAHE-08	
Date of birth	17 August 1990 (17 years), female
Date of diagnosis	23 January 2008
Diagnosis	High grade osteosarcoma of left inferior pelvis with large volume pulmonary metastases
Other medical problems	Already received 1 course of cisplatin 120 mg/m^2 and doxorubicin 75 mg/ m^2 on 25 January 2008
Date of consent	17 February 2008
Concomitant medication at trial entry	Paracetamol 1gr QDS, PO, Oxycontin 10mg BD,PO, Gabapentin 300mg TDS, PO, Norethisterone 5 mg tds,po, Fragmin 9000 units BD, SC, Lanzoprazole 30mg, OD,PO, Dexamethasone 4mg OD,PO reducing dose
Examination at trial entry	Dry skin, very swollen left leg, on treatment dose of fragmin, reduction of left hip flexion 2/5, left hip extension 3/5, left knee flexion and extension 3/5, left ankle flexion ¾ and left ankle extension 4/5, due to compressive symptoms of her large tumour
GFR prior to trial entry	106 ml/min/1.73 m^2 on 11 February 2008

PAHE started treatment on the GLU 1 clinical trial on 18 February 2008. She was randomised to arm B and received glucarpidase in cycle 1.

CYCLE 1

Her laboratory (FBC, renal and liver function) assessments on day 1 of cycle 1 were unremarkable apart from haemoglobin of 8.4 gr/dl (CTCAE toxicity grade 2). She had dry skin, very swollen left leg (on treatment dose of fragmin), reduction of left hip flexion 2/5, left hip extension 3/5, left knee flexion and extension 3/5, left ankle flexion 3/4 and left ankle extension 4/5, due to compressive symptoms of her large tumour.

MTX dose 1: PAHE had her first dose of methotrexate on 18 February 2008. She had glucarpidase on 19 February 2008. Folinic acid rescue was started on the same day. Methotrexate plasma levels at 24 hours were 4.49 µmol/L. Methotrexate was cleared within 72 hours post dose. Her serum creatinine prior to first methotrexate dose was 42 µmol/L and remained within normal range post methotrexate (CTCAE toxicity grade 0). Serum phosphate levels remained within normal range. ALT peaked at 307 IU/L on day 2 post methotrexate (CTCAE toxicity grade 3). Her haemoglobin dropped further to 7.8 gr/dl (CTCAE toxicity grade 3) and she was transfused with red packed cells. No further

bone marrow suppression was noted following the first dose of methotrexate. There were no adverse events related to glucarpidase.

Day 8 treatment was delayed by 1 day due to issues with patient's transport. On clinical examination "on day 8" of cycle 1 on 26 February 2008, she was found to have oral thrush. Her skin remained dry and flaky. Clinical examination of her right leg remained unchanged. PAHE had no mucositis. She was on Oxycontin 10mg BD,PO, Oxynorm 2.4 mg QDS,PO, Gabapentin 300mg TDS, PO, Norethisterone 5 mg tds,po, Fragmin 9000 units BD, SC, Fluconazole, 5mls, BD, PO and sucralfate 5mls, BD, PO. She remained in pain despite Oxycontin and regular Oxynorm. The dose of oxycontin was increased to 15mg, BD, PO and Paracetamol was added regularly.

Creatinine was 47 µmol/L (CTCAE toxicity grade 0) and calculated GFR was 189 ml/min/1.73 m^2. ALT was only slightly elevated at 48 IU/L (CTCAE toxicity grade 1). Her phosphate was normal at 1.39 mmol/L. She was fit to proceed to her second dose of methotrexate.

MTX dose 2: PAHE had her second dose of methotrexate on 26 February 2008, delayed by one day due to issues with hospital transport. She had glucarpidase on 27 February 2008. Folinic acid rescue was started on the same day. Methotrexate plasma levels at 24 hours were 9.06 µmol/L. Methotrexate was cleared within 48 hours post dose. Her serum creatinine remained within normal range post methotrexate. Phosphate dropped to 0.7 mmol/L on day 2 post methotrexate (CTCAE toxicity grade 2). ALT peaked at 211 IU/L on day 2 post methotrexate (CTCAE toxicity grade 3). No further bone marrow suppression was noted following the second dose of methotrexate. There were no adverse events related to glucarpidase.

Assessment on day 15 of cycle 1 took place on 03 March 2008, after two doses of methotrexate. PAHE had dry skin as before. Clinical assessment of her right leg remained unchanged. Her oral thrush had settled and she had no mucositis. Calculated GFR and creatinine were within normal range at 187 ml/min/1.73 m^2 and 44 µmol/L respectively. ALT was slightly elevated at 51 IU/L (CTCAE toxicity grade 1). Her serum phosphate and bilirubin were within normal range. No further bone marrow suppression was noted throughout the first cycle of treatment. "Day 15" criteria were met.

CYCLE 2

Clinical assessment on day 1 of cycle 12 March 2008 was unremarkable apart from the unchanged issues with her swollen right leg. She remained on Oxycontin 15mg BD, PO, Gabapentin 300mg TDS, PO, Norethisterone 5 mg, TDS, PO, Paracetamol 1gr, QDS, PO and Fragmin 9000 units BD, SC. Creatinine was 40 µmol/L (CTCAE toxicity grade 0) and calculated GFR was within normal range at 205 ml/min/1.73 m^2. Her ALT remained slightly elevated at 55 IU/L (CTCAE toxicity grade 1). Her serum phosphate and bilirubin remained within normal range. Her haemoglobin remained stable at 8 gr/dl (CTCAE toxicity grade 2). She was fit to proceed to third dose of methotrexate on time.

MTX dose 3: PAHE had her third dose of methotrexate on 13 March 2008. Folinic acid rescue was started on 14 March 2008. Methotrexate plasma levels at 24 hours were 5.57 µmol/L. Methotrexate was cleared within 72 hours post dose. Her serum creatinine, phosphate and bilirubin remained within normal range. ALT peaked at 185 IU/L (CTCAE toxicity grade 3) on day 2 post methotrexate. No further bone marrow suppression was noted following the third dose of methotrexate. On 19 March she was found to have thrush and was prescribed fluconazole, PO.

Clinical assessment on day 8 of cycle 2 was unremarkable. In particular, there was no mucositis. Serum creatinine, phosphate and bilirubin were within normal range. Her calculated GFR was 195 ml/min/1.73 m^2. ALT was elevated at 63 IU/L (CTCAE toxicity grade 1). She was fit to receive her fourth dose of methothrexate on time.

MTX dose 4: PAHE received her fourth dose of methotrexate on 19 March 2008, one day early in view of Easter Holidays. Folinic acid rescue was started on 20 March 2008. Methotrexate plasma levels at 24 hours were 5.16 µmol/L. Methotrexate was cleared within 72 hours post dose. Her serum creatinine and bilirubin remained within normal range. Her phosphate dropped to 0.74 mmol/L (CTCAE toxicity grade 2) on day 3 post methotrexate. ALT peaked at 193 IU/L (CTCAE toxicity grade 3) on day 2 post methotrexate. No further bone marrow suppression was noted following the fourth dose of methotrexate.

Assessment on day 15 of cycle 2 took place on 27 March 2008, after four doses of methotrexate. She had no mucositis and examination of her right leg remained

unchanged. She remained on Oxycontin 15mg BD, PO, Gabapentin 300mg TDS, PO, Norethisterone 5 mg, TDS, PO and Fragmin 9000 units BD, SC. Her calculated GFR was 187 ml/min/1.73 m^2. Her serum creatinine, bilirubin and phosphate were all within normal range. Her ALT was slightly elevated at 56 IU/L (CTCAE toxicity grade 1). No further bone marrow suppression was noted following the forth dose of methotrexate. “Day 15” criteria were met.

PAHE developed right sided tension pneumothorax (CTCAE v3.0, grade 4) during her hospital admission for her forth dose of methotrexate. She required admission to Intensive Care Unit. In view of that, the event was reported as Serious Adverse Event as per trial protocol. It was thought unrelated to trial medication and related to patient’s extensive pulmonary disease.

Antiglucarpidase antibodies were negative on days 1, 8 and 15 of cycle 1. There were positive on day 1 of cycle 2 but negative on days 8, 15, 30 and 90 of cycle 2.

PEFR-09	
Date of birth	30 October 1994 (13 years), male
Date of diagnosis	14 March 2008
Diagnosis	Localised high grade osteosarcoma of the sphenoid bone with diffuse chondroblastic differentiation likely secondary to radiation therapy for low grade glioma in May 1997
Other medical problems	1997: Low grade glioma, received radical radiotherapy, 50 Gy in 30 fractions, between 17 June and 29 July 1997 Prior to starting GLU 1 clinical trial, already received 2 courses of cisplatin 120 mg/m^2 and doxorubicin 75 mg/ m^2
Date of consent	23 April 2008
Concomitant medication at trial entry	Lansoprazole 30mg, OD,PO, and magnesium glycerophosphate 4 mls, TDS, PO
Examination at trial entry	Erythema on buttocks, managed with sudocream PRN III and XI cranial nerve palsy on right side, right sided hemiparesis, hyperreflexia, more in lower limbs, improving right sided ptosis with emerging diplopia
GFR prior to trial entry	174.6 ml/min/1.73 m^2 calculated at trial entry

PEFR started treatment on the GLU 1 clinical trial on 12 May 2008. He was randomised to arm A and received glucarpidase in cycle 2.

CYCLE 1

His laboratory (FBC, renal and liver function) assessments on day 1 of cycle 1 were unremarkable apart from haemoglobin of 8.1 gr/dl (CTCAE toxicity grade 2). He had some erythema on his buttocks, managed with sudocream PRN. He also had III and XI cranial nerve palsy on the right side, improving right sided ptosis with emerging diplopia, right sided hemiparesis and hyperreflexia, more in lower limbs,

MTX dose 1: PEFR had his first dose of methotrexate on 12 May 2008. Folinic acid rescue was started on 13 May 2008. Methotrexate plasma levels at 24 hours were 7.26 µmol/L. Methotrexate was cleared within 72 hours post dose. His serum creatinine prior to first methotrexate dose was 49 µmol/L and remained within normal range post methotrexate (CTCAE toxicity grade 0). His serum phosphate and bilirubin remained within normal range. No further bone marrow suppression was noted following the first dose of methotrexate.

Assessment on day 8 of cycle 1 revealed no mucositis although the patient complained of sore throat (CTCAE mucositis clinical toxicity grade 0, CTCAE mucositis functional toxicity grade 0) and some epigastric discomfort. The rest of his clinical examination remained unchanged. PEFR remained on the same medication that he was at the beginning of the trial treatment. Creatinine was 49 µmol/L (CTCAE toxicity grade 0) and calculated GFR was 162 ml/min/1.73 m^2. ALT was mildly elevated at 116 IU/L (CTCAE toxicity grade 1). His phosphate was normal at 1.4 mmol/L. He was fit to proceed to his second dose of methotrexate on time.

MTX dose 2: PEFR had his second dose of methotrexate on 19 May 2008. Folinic acid rescue was started on 20 May 2008. Methotrexate plasma levels at 24 hours were 7.6 µmol/L. Methotrexate was cleared within 72 hours post dose. His serum creatinine, phosphate and bilirubin remained within normal range post methotrexate. ALT peaked at 124 IU/L on day 2 post methotrexate (CTCAE toxicity grade 1). No further bone marrow suppression was noted following the first dose of methotrexate.

Assessment on day 15 of cycle 1 took place on 27 May 2008, after two doses of methotrexate. His clinical examination was unremarkable apart from mild bilateral blepharitis. The patient remained on the same medication that he was at the beginning of the trial treatment. Calculated GFR and creatinine were within normal range at 204 ml/min/1.73 m^2 and 42 µmol/L respectively. ALT was elevated at 164 IU/L (CTCAE toxicity grade 2). His serum phosphate and bilirubin were within normal range. His haemoglobin was 9.2 gr/dl (CTCAE toxicity grade 2). **"Day 15" criteria were met.**

CYCLE 2

On clinical examination on day 1 of cycle 2 on 02 June 2008, his blepharitis was still present and both eyes were red and sticky. Eye swabs taken on 28 May 2008 grew staphylococcus aureus and haemophilus influenza and PEFR was started on chlorampenicol eye drops. He remained on Lansoprazole 30mg, OD, PO, and magnesium glycerophosphate 4 mls, TDS, PO. Creatinine was 48 µmol/L (CTCAE toxicity grade 0) and calculated GFR was within normal range at 117 ml/min/1.73 m^2. His serum ALT, phosphate and bilirubin were within normal range. His haemoglobin was stable at 9.5gr/dl (CTCAE toxicity grade 2). He was fit to proceed to his third dose of methotrexate on time.

MTX dose 3: PEFR had the third dose of methotrexate on 02 June 2008. He had glucarpidase on 03 June 2008. Folinic acid rescue was started on the same day. Methotrexate plasma levels at 24 hours were 8.4 µmol/L. Methotrexate was cleared within 48 hours post dose. His serum creatinine, phosphate and bilirubin remained within normal range. ALT peaked at 91 IU/L on day 2 post methotrexate (CTCAE toxicity grade 1). No further bone marrow suppression was noted following the third dose of methotrexate. There were no adverse events related to glucarpidase.

Clinical assessment on day 8 of cycle 2 revealed III and IX cranial nerve palsy, brisk reflexes on right upper and lower extremities, mild dysdiachokinesia and past pointing bilaterally more pronounced on the right side. His eyes cleared and he had no mucositis. Serum creatinine, phosphate and bilirubin were within normal range. His calculated GFR was stable at 126 ml/min/1.73 m^2. ALT was elevated at 278 IU/L (CTCAE toxicity grade 3). His haemoglobin was 10.4 gr/dl (CTCAE toxicity grade 1). He was fit to receive his fourth dose of methothrexate on time.

MTX dose 4: PEFR received his fourth dose of methotrexate on 09 June 2008. He had glucarpidase on 10 June 2008. Folinic acid rescue was started on the same day. Methotrexate plasma levels at 24 hours were 9.44 µmol/L. Methotrexate was cleared within 48 hours post dose. His serum creatinine and bilirubin remained within normal range. His phosphate dropped to 0.8 mmol/L (CTCAE toxicity grade 1) on day 3 post methotrexate. His ALT did not go further up post methotrexate. No further bone marrow suppression was noted following the forth dose of methotrexate. There were no adverse events related to glucarpidase.

Assessment on day 15 of cycle 2 took place on 16 June 2008, after four doses of methotrexate. PEFR described intermittent soreness at his perianal area, although this was not visible on clinic examination. He had some dizziness on walking, blurred vision on the right, minor medial, upward and downward movement of his right eye, no pupil reaction on the right, minor right-sided facial palsy and right-sided hearing impairment. There was some pus around his Hickman line exit site. He had no mucositis. His calculated GFR was 171 ml/min/1.73 m^2. His serum creatinine, phosphate and bilirubin were within normal range. No further bone marrow suppression was noted following the fourth dose of methotrexate. **"Day 15" criteria were met.**

Antiglucarpidase antibodies were negative on days 1, 8 and 15 of cycle 1. They were negative on day 8 of cycle 2, positive on day 15 of cycle 2 and again negative on days 30 and 90 of cycle 2. No results are available from blood sample taken on day 1 of cycle 2, on 02 June 2008.

CHWC-10	
Date of birth	05 March 1978 (29 years), male
Date of diagnosis	21 January 2008
Diagnosis	Intramedullary osteosacoma of left first metatarsal on the background of giant cell tumour of bone
Other medical problems	1999: Giant cell tumour of left first metatarsal, treated with curettage, fibula strut graft and adjuvant radiotherapy (50 Gy in 25 fractions) 2006 & 2007: pain 2008 Intramedullary osteosacoma of left first metatarsal on the background of giant cell tumour of bone Already received 1 course of adriamycin and cisplatin on 14 April 2008
Date of consent	23 May 2008
Concomitant medication at trial entry	Codeine phosphate as required, nil else
Examination at trial entry	Unremarkable
GFR prior to trial entry	98 ml/min/1.73 m^2 on 19 May 2008

CHWC started treatment on the GLU 1 clinical trial on 16 May 2008. He was randomised to arm A and received glucarpidase in cycle 2.

CYCLE 1

His clinical and laboratory (FBC, renal and liver function) assessments on day 1 of cycle 1 were unremarkable apart from haemoglobin of 8.6 gr/dl (CTCAE toxicity grade 2).

MTX dose 1: CHWC had his first dose of methotrexate on 26 May 2008. Folinic acid rescue was started on 27 May 2008. Methotrexate plasma levels at 24 hours were 28.42 µmol/L. Methotrexate was cleared within 72 hours post dose. His serum creatinine prior to first methotrexate dose was 77 µmol/L and remained within normal range post methotrexate (CTCAE toxicity grade 0). Phosphate dropped to 0.53 mmol/L on day 4 post methotrexate (CTCAE toxicity grade 3). ALT peaked at 531 IU/L on day 2 post methotrexate (CTCAE toxicity grade 3). No further bone marrow suppression was noted following the first dose of methotrexate.

Assessment on day 8 of cycle 1 revealed two to three erythematous areas on oral mucous membranes with a small ulcer (CTCAE mucositis clinical toxicity grade 1-2,

CTCAE mucositis functional toxicity grade 0). The patient needed Difflam mouthwash for analgesia. Creatinine was 79 µmol/L (CTCAE toxicity grade 0) and calculated GFR was 123.57 ml/min/1.73 m^2. ALT was elevated at 141 IU/L (CTCAE toxicity grade 2). His phosphate was normal at 1.47mmol/L. He was fit to proceed to his second dose of methotrexate on time.

MTX dose 2: CHWC had his second dose of methotrexate on time on 02 June 2008. Folinic acid rescue was started on 03 June 2008. Methotrexate plasma levels at 24 hours were 22.77 µmol/L. Methotrexate was cleared within 72 hours post dose. His serum creatinine peaked at 144 µmol/L on day 3 post second dose of methotrexate (CTCAE toxicity grade 1). Phosphate remained within normal range. ALT peaked at 601 IU/L on day 2 post methotrexate (CTCAE toxicity grade 3). His haemoglobin remained stable at 9.2 gr/dl (CTCAE toxicity grade 2) although his platelets dropped to 132 $x10^9$/L (CTCAE toxicity grade 1).

Assessment on day 15 of cycle 1 took place on 09 June 2008, after two doses of methotrexate. CHWC had confluent ulcers in his oral cavity (CTCAE for mucositis clinical, toxicity grade 3). He was still able to drink although his solid intake was compromised (CTCAE for mucositis functional, toxicity grade 2) His mouth pain was not controlled on regular codeine phosphate and paracetamol and kept him awake at night. He had no abdominal pain. Calculated GFR and creatinine were within normal range at 90.39 ml/min/1.73 m^2 and 108 µmol/L respectively. ALT was elevated at 320 IU/L (CTCAE toxicity grade 3). His serum phosphate and bilirubin were within normal range. His haemoglobin and platelets remained stable at 9 gr/dl (CTCAE toxicity grade 2) and 106 $x10^9$/L respectively (CTCAE toxicity grade 1). His neutrophils were 1.83 $x10^9$/L (CTCAE toxicity grade 1) on day 15 of cycle 1 on 09 June 2010 but dropped further to 0.79 $x10^9$/L (CTCAE toxicity grade 3) on 23 June 2008, 20 days post his second methotrexate dose.

In view of his mucositis on day 15 of cycle 1, **"day 15" criteria were not met.**

CYCLE 2

Clinical assessment on day 1 of cycle 2 on 26 June 2008 was unremarkable CHWC was on no medication. Creatinine was 101 µmol/L (CTCAE toxicity grade 0) and

calculated GFR was within normal range at 95 ml/min/1.73 m^2. His ALT, serum phosphate and bilirubin were within normal range. His neutrophils were 1.28 $x10^9$/L (CTCAE toxicity grade 2). He was fit to proceed to his third dose of methotrexate.

MTX dose 3: CHWC had his third dose of methotrexate on 26 June 2008, delayed by 10 days in view of his mucositis and neutropenia. He had glucarpidase on 27 June 2008. Folinic acid rescue was started on 27 June 2008. Methotrexate plasma levels at 24 hours were 41.23 µmol/L. Methotrexate was cleared within 72 hours post dose. His serum creatinine remained within normal range. His phosphate dropped to 0.72 mmol/L (CTCAE toxicity grade 2) on day 2 post methotrexate. His bilirubin and ALT peaked at 32 µmol/L (CTCAE toxicity grade 2) and 801 IU/L (CTCAE toxicity grade 3) on day 2 post methotrexate. His Plts dropped at 103 $x10^9$/L (CTCAE toxicity grade 1) on day 8 post third dose of methotrexate. No further bone marrow suppression was noted. There were no adverse events related to glucarpidase.

Clinical assessment on day 8 of cycle 2 was unremarkable. In particular, there was no mucositis. Serum creatinine, phosphate and bilirubin were within normal range. His calculated GFR was 109.3 ml/min/1.73 m^2. ALT was elevated at 368 IU/L (CTCAE toxicity grade 3). His haemoglobin remained stable at 10.1 gr/dl (CTCAE toxicity grade 2) although his platelets dropped to 103 $x10^9$/L (CTCAE toxicity grade 1).

He was fit to receive his fourth dose of methothrexate on time.

MTX dose 4: CHWC received his fourth dose of methotrexate on 03 July 2008. He had glucarpidase on 04 July 2008. Folinic acid rescue was started on 04 July 2008. Methotrexate plasma levels at 24 hours were 55.76 µmol/L. Methotrexate was cleared within 96 hours post dose. His serum creatinine peaked at 145 µmol/L (CTCAE toxicity grade 1) on day 4 post methotrexate. His phosphate remained within normal range. His bilirubin and ALT peaked at 25 µmol/L (CTCAE toxicity grade 1) and 580 IU/L (CTCAE toxicity grade 3) on day 2 post methotrexate. His platelets dropped further to 82$x10^9$/L (CTCAE toxicity grade 1) on day 5 post fourth dose of methotrexate. There were no adverse events related to glucarpidase.

Assessment on day 15 of cycle 2 took place on 10 July 2008, after four doses of methotrexate. There was slight erythema on his buccal mucosa but no ulcers (CTCAE

for mucositis clinical, toxicity grade 0, CTCAE for mucositis functional, toxicity grade 0). The rest of his clinical examination was unremarkable. His calculated GFR was 72.3 ml/min/1.73 m^2. His serum creatinine was elevated at 135 µmol/L (CTCAE toxicity grade 1). Phosphate and bilirubin were within normal range. ALT was elevated at 273 IU/L (CTCAE toxicity grade 3). His haemoglobin and platelets remained stable at 11.9 gr/dl (CTCAE toxicity grade 1) and 89 $x10^9$/L (CTCAE toxicity grade 1) respectively.

“Day 15” criteria were met.

Antiglucarpidase antibodies were negative on days 1, 8 and 15 of cycle 1 and remained negative on days 1, 8, 15 and 90 of cycle 2.

JASH-11	
Date of birth	10 December 1991 (17 years), male
Date of diagnosis	09 February 2008
Diagnosis	Localised high grade telangiectatic ostesarcoma of left distal tibia
Other medical problems	Already received 3 courses of cisplatin 120 mg/m^2 and doxorubicin 75 mg/ m^2 and 3 courses of methotrexate 12gr/m^2 Below knee amputation, April 2008
Date of consent	27 May 2008
Concomitant medication at trial entry	Gabapentin 300mg, BD,PO, Lansoprazole 30mg, OD,PO
Examination at trial entry	Unremarkable apart from amputation of left foot
GFR prior to trial entry	111 ml/min/1.73 m^2 on 23 April 2008

JASH started treatment on the GLU 1 clinical trial on 27 May 2008. He was randomised to arm A and received glucarpidase in cycle 2.

CYCLE 1

His clinical and laboratory assessments on day 1 of cycle 1 were unremarkable apart from left below knee amputation and haemoglobin of 10.5 gr/dl (CTCAE toxicity grade 1).

MTX dose 1: JASH had his first dose of methotrexate on 27 May 2008. Folinic acid rescue was started on 28 May 2008. Methotrexate plasma levels at 24 hours were 28.03 µmol/L. Methotrexate was cleared within 72 hours post dose. His serum creatinine prior to first methotrexate dose was 43 µmol/L and remained within normal range post methotrexate (CTCAE toxicity grade 0). Phosphate dropped to 0.47 mmol/L on day 3 post methotrexate (CTCAE toxicity grade 3). ALT peaked at 1384 IU/L on day 2 post methotrexate (CTCAE toxicity grade 4). His serum bilirubin remained within normal range. No further bone marrow suppression was noted following the first dose of methotrexate.

Assessment on day 8 of cycle 1 revealed no mucositis but loose bowel motions, 2-3 times per day. The patient remained on the same medication that he was at the beginning of the trial treatment. Creatinine was 48 µmol/L (CTCAE toxicity grade 0) and calculated GFR was 228 ml/min/1.73 m^2. ALT was elevated at 258 IU/L (CTCAE toxicity

grade 3). His phosphate was normal at 1.41 mmol/L. He was fit to proceed to his seconddose of methotrexate on time.

MTX dose 2: JASH had his second dose of methotrexate on 03 June 2008. Folinic acid rescue was started on 04 June 2008. Methotrexate plasma levels at 24 hours were 11.05 µmol/L. Methotrexate was cleared within 72 hours post dose. His serum creatinine remained within normal range post methotrexate. Phosphate dropped to 0.58 mmol/L on day 3 post methotrexate (CTCAE toxicity grade 3). ALT peaked at 1410 IU/L on day 2 post methotrexate (CTCAE toxicity grade 4). His platelets dropped to 123 x 10^9/L on day 3 post methotrexate (CTCAE toxicity grade 1).

Assessment on day 15 of cycle 1 took place on 10 June 2008, after two doses of methotrexate. His clinical examination was unremarkable. The patient remained on the same medication that he was at the beginning of the trial treatment. Calculated GFR and creatinine were within normal range at 215 ml/min/1.73 m^2 and 51 µmol/L respectively. ALT was elevated at 164 IU/L (CTCAE toxicity grade 2). His serum bilirubin was within normal range. There was no documentiation of his serum phosphate. His haemoglobin was 9.9 gr/dl (CTCAE toxicity grade 2) and platelets 129 x 10^9/L (CTCAE toxicity grade 1). **"Day 15" criteria were met.**

CYCLE 2

Clinical assessment on day 1 of cycle 2 on 15 June 2008 was unremarkable. He remained on Gabapentin 300mg, BD,PO, Lansoprazole 30mg, OD,PO. Creatinine was 41 µmol/L (CTCAE toxicity grade 0) and calculated GFR was within normal range at 264 ml/min/1.73 m^2. His ALT remained elevated at 71 IU/L (CTCAE toxicity grade 1). His serum phosphate and bilirubin were within normal range. His Hb was stable at 11.5gr/dl (CTCAE toxicity grade 1) but his platelets normalised. He was fit to proceed to his third dose of methotrexate on time.

MTX dose 3: JASH had the third dose of methotrexate on 16 June 2008. He had glucarpidase on 17 June 2008. Folinic acid rescue was started on the same day. Methotrexate plasma levels at 24 hours were 16.94 µmol/L. Methotrexate was cleared within 48 hours post dose. His serum creatinine, phosphate and bilirubin remained within normal range. ALT peaked at 1710 IU/L on day 2 post methotrexate (CTCAE

toxicity grade 4). His platelets dropped to 175 x 10^9/L (CTCAE toxicity grade 1). There were no adverse events related to glucarpidase.

Clinical assessment on day 8 of cycle 2 was unremarkable. In particular, there was no mucositis. Serum creatinine, phosphate and bilirubin were within normal range. His calculated GFR was 249ml/min/1.73 m^2. ALT was elevated at 296 IU/L (CTCAE toxicity grade 3). His platelets remained slightly low at 188 x 10^9/L (CTCAE toxicity grade 1). He was fit to receive his fourth dose of methothrexate on time.

MTX dose 4: JASH received his fourth dose of methotrexate on 23 June 2008. He had glucarpidase on 24 June 2008. Folinic acid rescue was started on the same day. Methotrexate plasma levels at 24 hours were 20.44 µmol/L. Methotrexate was cleared within 72 hours post dose. His serum creatinine and bilirubin remained within normal range. His phosphate dropped to 0.72 mmol/L (CTCAE toxicity grade 1) on day 3 post methotrexate. ALT peaked at 466 IU/L on day 2 post methotrexate (CTCAE toxicity grade 3). No further bone marrow suppression was noted following the forth dose of methotrexate. There were no adverse events related to glucarpidase.

Assessment on day 15 of cycle 2 took place on 30 June 2008, after four doses of methotrexate. JASH was tired and had a productive cough. His chest X-ray was normal. He had no mucositis. He remained on Gabapentin 300mg, BD,PO, Lansoprazole 30mg, OD,PO. His calculated GFR was 228 ml/min/1.73 m^2. His serum creatinine and bilirubin were within normal range. His serum phosphate was not measured. No further bone marrow suppression was noted following the forth dose of methotrexate. **"Day 15" criteria were met.**

Antiglucarpidase antibodies were negative on days 1, 8 and 15 of cycle 1 and on days 1, 8, 15, 30 and 90 of cycle 2. Blood sample was also collected on 30 January 2009 (day 180 of cycle 2) but was not processed as antiglucarpidase antibodies were found to be negative on day 90 of cycle 2.

SAPH-12	
Date of birth	03 August 1991 (17 years), male
Date of diagnosis	03 April 2008
Diagnosis	Localised high grade osteoblastic osteosacoma of T12 2 right sided pulmonary nodules (<5mm) of uncertain significance
Other medical problems	Initially he received 2 courses of adriamycin and cisplatin, following which he developed cord compression and required vertebrectomy (24 May 2008). Following the above chemotherapy he received 2 courses of Ifosfamide and etoposide prior to starting treatment on GLU 1 clinical trial
Date of consent	07 August 2008
Concomitant medication at trial entry	MST 30mg BD, PO Ibuprofen 400 mg BD, PO, stopped prior to starting methotrexate Omeprazole 20 mg OD, PO Fragmin18.000 units OD, SC
Examination at trial entry	Morbidly obese Minimal pulmonary effusion , unlikely to interfere with methotrexate clearance
GFR prior to trial entry	77 ml/min/1.73 m^2 on 01 August 2008

SAPH started treatment on the GLU 1 clinical trial on 07 August 2008. He was randomised to arm B and received glucarpidase in cycle 1.

CYCLE 1

On clinical examination on day 1 of cycle 1 SAPH was found to be morbidly obese. He had extensive steroid induced striae and two pressure induced blisters on his back. His laboratory (FBC, renal and liver function) assessment on day 1 of cycle 1 was unremarkable apart from mildly decreased albumin of 30 g/L (CTCAE toxicity grade 1).

MTX dose 1: SAPH had his first dose of methotrexate on 07 August 2008. Glucarpidase was given on 08 August 2008. Folinic acid rescue was started on 08 August 2008. Methotrexate plasma levels at 24 hours were 52.05 µmol/L. Samples taken for HPLC at T=0 hours, 4 hours, 24 hours, 24.20 hours and 48 hours became jelly like with the addition of HCL as per protocol. Therefore they were unsuitable for HPLC analysis. His plasma methotrexate levels measured by HPLC were <0.2 µmol/L at 72 hours post methotrexate. Since there were no previous samples that were measured with HPLC to confirm a trend in plasma methotrexate excretion, folinic acid

was continued and the dose was adjusted based on plasma methotrexate levels at 72 hours as measured by immunoassay. His plasma methotrexate levels measured by HPLC were 0.27 µmol/L at 96 hours post methotrexate, so folinic acid rescue was continued as appropriate. Methotrexate was cleared within 120 hours post dose. His serum creatinine prior to first methotrexate dose was 41 µmol/L and peaked at 112 µmol/L on day 4 post methotrexate, although it remained within normal range (CTCAE toxicity grade 0). His potassium was up to 5.2 mmol/L on day 4 post methotrexate but settled subsequently (CTCAE toxicity grade 1). His albumin remained normal post methotrexate. Phosphate dropped to 0.72 mmol/L on day 7 post methotrexate (CTCAE toxicity grade 1). ALT peaked at 285 IU/L on day 6 post methotrexate (CTCAE toxicity grade 3). Bilirubin peaked at 34 µmol/L on day 8 post methotrexate (CTCAE toxicity grade 2). No bone marrow suppression was noted following the first dose of methotrexate. There were no adverse events related to glucarpidase.

Assessment on day 8 of cycle 1 revealed no mucositis or other new problems. He remained on MST 30 mg OD, PO, Omeprazole 20 mg OD, PO and Fragmin 18.000 units OD, SC. His creatinine was 105 µmol/L (CTCAE toxicity grade 0) and calculated GFR was 103.13ml/min/1.73 m^2. ALT was elevated at 268 IU/L (CTCAE toxicity grade 3). His phosphate was low at 0.72 mmol/L (CTCAE toxicity grade 2). His bilirubin was elevated at 34 µmol/L (CTCAE toxicity grade 2) as noted above. He was fit to proceed to his second dose of methotrexate on time.

MTX dose 2: SAPH had his second dose of methotrexate on 14 August 2008. Glucarpidase was given on 15 August 2008. Folinic acid rescue was started on 15 February 2008. In view of the problems with the addition of HCL in his plasma samples post his first methotrexate dose, it was decided to process his HPLC samples with no HCL post second dose. Methotrexate plasma levels at 24 hours were really high at 253.54 µmol/L. The patient's treatment arm was unblinded as per trial protocol and it became clear that he had received glucarpidase at 24 hours post methotrexate. High dose folinic acid was started. Methotrexate was eventually cleared at 312 hours post second dose. His serum creatinine peaked at 247µmol/L on day 6 post methotrexate (CTCAE toxicity grade 2). Phosphate dropped to 0.72 mmol/L on day 3 post methotrexate (CTCAE toxicity grade 2). ALT remained elevated at maximum of 260 IU/L on day 5 post methotrexate (CTCAE toxicity grade 3). His albumin dropped down

to 27 g/L on day 5 post methotrexate (CTCAE toxicity grade 2). No bone marrow suppression was noted following the second dose of methotrexate despite his delayed clearance.

Assessment on day 15 of cycle 1 took place on 21 August 2008, after two doses of methotrexate. SAPH did not clear his second methotrexate dose until 27 August 2008; 14 days post his second dose. SAPH had oral candidiasis and a small ulcer on his lower lip (CTCAE for mucositis clinical and functional, toxicity grade 2). He also had diarrhoea (CTCAE toxicity grade 1). He had right iliac fossa pain and generalised abdominal tenderness. An abdominal X-ray revealed dilated small bowel loops and possibly oedematous bowel wall (enteritis CTCAE toxicity grade 1) which were managed conservatively. His creatinine was elevated at 238 µmol/L (CTCAE toxicity grade 1). His calculated GFR was low at 45.5 ml/min/1.73 m^2. ALT was elevated at 126 IU/L (CTCAE toxicity grade 2). His serum phosphate was elevated at 2.11mmol/L and his calcium was low at 1.66 mmol/L (CTCAE toxicity grade 3). His albumin was also low at 27 g/L (CTCAE toxicity grade 2). No significant bone marrow suppression was noted throughout the first cycle of treatment. In view of delayed methotrexate excretion, renal deterioration and mucositis on day 15 of cycle 1, **"day 15" criteria were not met.**

In view of renal deterioration it was decided not to receive further treatment with methotrexate.

Antiglucarpidase antibodies were negative on days 1, 8, 15 and 30 of cycle 1.

TRGO-13	
Date of birth	09 April 1978 (30 years), female
Date of diagnosis	December 2005 chondroblastic osteosarcoma of proximal tibia February 2008 pulmonary metastases
Diagnosis	December 2005: chondroblastic osteosarcoma of proximal tibia, treated with chemotherapy (?agents), excision and reconstruction, all treatment given in Nantes, France February 2008: pulmonary embolism and pulmonary metastases, inoperable, decided to be managed with methotrexate followed by ifosfamide and etoposide
Other medical problems	Suicidal symptoms
Date of consent	2 September 2008
Concomitant medication at trial entry	Duloxetine 60 mg, nocte, antidepressant (serotonin–norepinephrine reuptake inhibitor) Aripiprazole 15 mg, nocte, antipsychotic, antidepressant Asthma inhalers Had been on warfarin until 18 August 2009, stopped in view of pending portacath insertion
Examination at trial entry	Unremarkable apart from previous right tibial replacement
GFR prior to trial entry	87 ml/min/1.73 m^2 on 20th May 2008

TRGO started treatment on the GLU 1 clinical trial on 02 September 2008. She was randomised to arm B and received glucarpidase in cycle 1.

Her clinical and laboratory assessments on day 1 of cycle 1 were unremarkable.

MTX dose 1: TRGO had the first dose of methotrexate on 02 September 2008 with glucarpidase rescue on 03 September 2008. Folinic acid rescue was started on 03 September 2008. Methotrexate plasma levels at 24 hours were 17.47 µmol/L. Methotrexate was cleared at 72 hours. Creatinine prior to the first methotrexate dose was 74 µmol/L but peaked at 114 µmol/L, on day 5 post first dose of methotrexate (CTCAE toxicity grade 1). Phosphate dropped to 0.67 mmol/L on day 3 post methotrexate (CTCAE toxicity grade 2). ALT peaked at 506 IU/L on day 8 post methotrexate (CTCAE toxicity grade 3). No bone marrow suppression was noted following the first dose of methotrexate. There were no adverse events related to glucarpidase.

Assessment on day 8 of cycle 1 revealed one oral ulcer and erythema in the oral cavity. The patient needed analgesia (Difflam mouthwash). There was also eruptive facial rash which was attributed to steroids used as part of antisickness regimen. Creatinine was elevated at 107 µmol/L (CTCAE toxicity grade 1) although calculated GFR was acceptable at 78.6 ml/min/1.73 m^2. ALT was also elevated at 506 IU/L (CTCAE toxicity grade 3). In view of the elevated creatinine and mucositis thesecond dose of methotrexate was delayed by one week.

MTX dose 2: TRGO had the second dose of methotrexate on 15 September 2008 with glucarpidase rescue on 16 September 2008. Folinic acid rescue was started on 16 September 2008. Methotrexate plasma levels at 24 hours were 43.86 µmol/L. Methotrexate was cleared at 120 hours. Calculated GFR prior to second dose of methotrexate was 106 ml/min/1.73 m^2. Creatinine prior to the second methotrexate dose was 83µmol/L but peaked at 141 µmol/L, on day 5 post methotrexate (CTCAE toxicity grade 1). Phosphate dropped to 0.59 mmol/L on day 3 post methotrexate (CTCAE toxicity grade 3). ALT peaked at 1017 IU/L on day 8 post methotrexate (CTCAE toxicity grade 4). No bone marrow suppression was noted following the second dose of methotrexate. There were no adverse events related to glucarpidase.

Assessment on day 15 of cycle 1 took place on 15 September 2008, prior to the second of methotrexate. It revealed one small recovering ulcer on the left side of her tongue. The patient was still requiring analgesia (Difflam mouthwash). Calculated GFR and creatinine were within normal range at 106 ml/min/1.73 m^2 and 83 µmol/L respectively. ALT was elevated at 319 IU/L (CTCAE toxicity grade 3). No bone marrow suppression was noted throughout the first cycle of treatment. In view of the one week delay for the second dose of methotrexate **"day 15" criteria were not met.**

In view of delayed methotrexate excretion and creatinine elevation post second dose of methotrexate, patient's GFR (radioisotope) was examined on 24 September 2008 and was found to be decreased at 51 ml/min/1.73 m^2. Therefore it was decided not to proceed with further doses of methotrexate.

Antiglucarpidase antibodies were negative on days 1, 8 and 30 of cycle 1.

ABBS-14	
Date of birth	27 November 1992 (14 years), male
Date of diagnosis	June 2008
Diagnosis	Localised chondroblastic osteosarcoma of right distal femur, intermediate grade
Other medical problems	Already received 2 courseS of cisplatin 120 mg/m^2, 2 courses of doxorubicin 75 mg/ m^2 and 4 courses of methotrexate 12 gr m^2 Excision of tumour and distal femoral replacement in the UK on 03 November 2008, pathology: high grade periosteal osteosarcoma with chondroblastic and osteoblastic features, poor response to pre-operative chemotherapy with <90% necrosis
Date of consent	17 November 2008
Concomitant medication at trial entry	Lactulose 15mls, BD, PO, Paracetamol 1 gr, QDS, PO, Fragmin, 2500 units, OD, SC
Examination at trial entry	Unremarkable apart from distal femoral replacement
GFR prior to trial entry	106 ml/min/1.73 m^2 on 14 November 2008

ABBS started treatment on the GLU 1 clinical trial on 17 November 2008. He was randomised to arm A and was planned to receive glucarpidase in cycle 2.

CYCLE 1

His clinical and laboratory assessments on day 1 of cycle 1 were unremarkable apart from haemoglobin of 9.7 gr/dl (CTCAE toxicity grade 1).

MTX dose 1: ABBS had his first dose of methotrexate on 17 November 2008. Folinic acid rescue was started on 18 November 2008. Methotrexate plasma levels at 24 hours were 30.04 µmol/L. Methotrexate was cleared within 96 hours post dose. His serum creatinine prior to first methotrexate dose was 66 µmol/L and remained within normal range post methotrexate (CTCAE toxicity grade 0). Phosphate dropped to 0.64 mmol/L on day 4 post methotrexate (CTCAE toxicity grade 2). ALT peaked at 333 IU/L on day 4 post methotrexate (CTCAE toxicity grade 3). No further bone marrow suppression was noted following the first dose of methotrexate.

Assessment on day 8 of cycle 1 revealed no oral mucositis. However, he had oral thrush. There was some mild central abdominal discomfort but bowel sounds were present and he was not constipated. His right knee was swollen, hot and tender and ABBS was reluctant to move his right knee due to pain. He remained on the same

medication as prior to starting GLU 1 trial treatment. Creatinine was 71 µmol/L (CTCAE toxicity grade 0) and calculated GFR was 136 ml/min/1.73 m^2. ALT was elevated at 223 IU/L (CTCAE toxicity grade 2). His phosphate and bilirubin were within normal range. In view of his effusion he was started on IV teicoplanin. Blood cultures from 14 November 2008 grew acinobacter calcoaceticus and IV ciprofloxacin was added to his antibiotic treatment. His right knee effusion was tapped on 26 November 2008. In view of his knee effusion, GLU 1 clinical trial treatment was discontinued and **"day 15 of cycle 1" criteria were not met.**

Blood sample for antiglucarpidase antibodies was collected on day 1 of cycle 1 but not processed as patient off treatment by day 8 of cycle 1, prior to receiving any glucarpidase.

SETI-15	
Date of birth	14 May 1971 (37 years), male
Date of diagnosis	15 December 2008
Diagnosis	Localised high-grade osteosarcoma of maxilla
Other medical problems	Nil of note
Date of consent	25 January 2009
Concomitant medication at trial entry	nil
Examination at trial entry	Unremarkable apart from ulcerated area in the oral cavity at the site of his primary disease (palate and maxilla)
GFR prior to trial entry	88 ml/min/1.73 m^2 on 14 January 2009

SETI started treatment on the GLU 1 clinical trial on 26 January 2009. He was randomised to arm B and received glucarpidase in cycle 1.

CYCLE 1

His clinical and laboratory assessments on day 1 of cycle 1 were unremarkable apart from ulcerated area in the oral cavity at the site of his primary disease.

MTX dose 1: SETI had his first dose of methotrexate on 26 January 2009. He had glucarpidase on 27 January 2009. Folinic acid rescue was started on the same day. Methotrexate plasma levels at 24 hours were 12.78 µmol/L. Methotrexate was cleared within 72 hours post dose. His serum creatinine prior to first methotrexate dose was 79µmol/L and remained within normal range post methotrexate (CTCAE toxicity grade 0). Phosphate dropped to 0.5 mmol/L on day 3 post methotrexate (CTCAE toxicity grade 3). ALT peaked at 130 IU/L on day 2 post methotrexate (CTCAE toxicity grade 2). No further bone marrow suppression was noted following the first dose of methotrexate. There were no adverse events related to glucarpidase.

Assessment on day 8 of cycle 1 revealed no mucositis. There was an ulcerated area in his oral cavity at the site of his primary disease as before. In view of his hypophosphataemia he was started on Phosphate Sandoz 2 tablets, BD, PO. He was also on Lanzoprazole 30mg, OD, orally. Creatinine was 72 µmol/L (CTCAE toxicity grade 0) and calculated GFR was 188 ml/min/1.73 m^2. ALT was slightly elevated at 64

IU/L (CTCAE toxicity grade 1). His phosphate and bilirubin were within the normal range. He was fit to proceed to his second dose of methotrexate on time.

MTX dose 2: SETI had his second dose of methotrexate on 02 February 2009. He had glucarpidase on 03 February 2009. Folinic acid rescue was started on the same day. Methotrexate plasma levels at 24 hours were 16.89 µmol/L. Methotrexate was cleared within 72 hours post dose. His serum creatinine remained within normal range post methotrexate. Phosphate dropped to 0.66 mmol/L on day 4 post methotrexate (CTCAE toxicity grade 2). ALT peaked at 107 IU/L on day 2 post methotrexate (CTCAE toxicity grade 1). No further bone marrow suppression was noted following the second dose of methotrexate. There were no adverse events related to glucarpidase.

Assessment on day 15 of cycle 1 took place on 09 February 2009, after two doses of methotrexate. SETI had confluent ulcers at the back of his mouth and his lower lips. The rest of his oral mucosa was erythematous (CTCAE mucositis clinical toxicity grade 3, CTCAE mucositis functional toxicity grade 2). He remained on Lanzoprazole 30mg, OD, PO. Calculated GFR and creatinine were within normal range at 190 ml/min/1.73 m^2 and 71µmol/L respectively. ALT was elevated at 70 IU/L (CTCAE toxicity grade 1). His serum phosphate and bilirubin were within normal range. No further bone marrow suppression was noted throughout the first cycle of treatment. In view of his mucositis on day 15 of cycle 1, “day 15” criteria were not met.

CYCLE 2

On clinical assessment on day 1 of cycle 2 on 16 February 2009 his oral ulcers were almost healed (CTCAE mucositis clinical toxicity grade 1, CTCAE mucositis functional toxicity grade 0). He was on Lanzoprazole 30mg, OD, PO, DIfflam mouthwash, Aspirin mouthwash and Phosphate Sandoz 2 tablets, BD, PO. Creatinine was 83µmol/L (CTCAE toxicity grade 0) and calculated GFR was within normal range at 154 ml/min/1.73 m^2. His ALT was only slightly elevated at 59 IU/L (CTCAE toxicity grade 1). His serum phosphate and bilirubin were within normal range. His Hb was 12.1gr/dl (CTCAE toxicity grade 1). He was fit to proceed to his third dose of methotrexate on time.

MTX dose 3: SETI had the third dose of methotrexate on 16 February 2009. Folinic acid rescue was started on 17 February 2009. Methotrexate plasma levels at 24 hours were 26.12 µmol/L. Methotrexate was cleared within 96 hours post dose. His serum creatinine remained within normal range. His phosphate dropped to 0.82 mmol/L (CTCAE toxicity grade 1) on day 3 post methotrexate. His ALT peaked at 162 IU/L on day 2 post methotrexate (CTCAE toxicity grade 2). No further bone marrow suppression was noted following the third dose of methotrexate.

Clinical assessment on day 8 of cycle 2 was unremarkable apart from folliculitis at the inner thighs for which he was started on Canesten cream topically. There was no mucositis. Serum creatinine, phosphate and bilirubin were within normal range. His calculated GFR was 137ml/min/1.73 m^2. ALT was elevated at 110 IU/L (CTCAE toxicity grade 1). He was fit to receive his fourth dose of methothrexate on time.

MTX dose 4: SETI received his fourth dose of methotrexate on 23 February 2009. Folinic acid rescue was started on 24 February 2009. Methotrexate plasma levels at 24 hours were 23.19 µmol/L. Methotrexate was cleared within 96 hours post dose. His serum creatinine peaked at 136 µmol/L on day 4 post methotrexate (CTCAE toxicity grade 1). His ALT peaked at 185 IU/L (CTCAE toxicity grade 2) on day 2 post methotrexate. His phosphate dropped to 0.48 mmol/L (CTCAE toxicity grade 3) on day 3 post methotrexate. His bilirubin remained within normal range. His haemoglobin remained stable but his platelets dropped to 132 x 10^9/L (CTCAE toxicity grade 1) on day 5 post methotrexate.

Assessment on day 15 of cycle 2 took place on 02 March 2009, after four doses of methotrexate. His inner thigh folliculitis was healing. He had an ulcer on his lower lip and one on his uvula. The rest of his oral mucosa was erythematous (CTCAE mucositis clinical toxicity grade 2, CTCAE mucositis functional toxicity grade 1). Otherwise his clinical examination was unremarkable. He remained on Lansoprazole 30mg, OD, PO, Canesten cream topically on inner thighs and domperidone 20 mg, QDS, PO. His calculated GFR was 118 ml/min/1.73 m^2. His serum creatinine, bilirubin and phosphate were within normal range. His ALT was slightly elevated at 75 IU/L (CTCAE toxicity grade 1). No further bone marrow suppression was noted following the fourth dose of methotrexate. “Day 15” criteria were not met in view of his mucositis.

Antiglucarpidase antibodies were negative on days 1, 8 and 15 of cycle and 1 and 8 of cycle 2. They were present on days 15 and 30 of cycle 2.

DAOA-16	
Date of birth	07 January 1990 (19 years), male
Date of diagnosis	11 December 2008
Diagnosis	High grade spindle cell sarcoma of right distal tibia
Other medical problems	Already received 1 course of cisplatin 120 mg/m^2 and doxorubicin 75 mg/ m^2
Date of consent	26 January 2009
Concomitant medication at trial entry	MTS 20mg BD,PO Sodium Docusate 200mg TDS,PO
Examination at trial entry	Unremarkable apart from pain and swelling at his right ankle at the site of his primary disease
GFR prior to trial entry	94 ml/min/1.73 m^2 on 21 January 2009

DAOA started treatment on the GLU 1 clinical trial on 27 January 2009. He was randomised to arm A and received glucarpidase in cycle 2.

CYCLE 1

His clinical and laboratory assessments on day 1 of cycle 1 were unremarkable apart from pain and swelling at his right ankle at the site of his primary disease and haemoglobin of 12.6 gr/dl (CTCAE toxicity grade 1).

MTX dose 1: DAOA had his first dose of methotrexate on 27 January 2009. Folinic acid rescue was started on 28 January 2009. Methotrexate plasma levels at 24 hours were 18.38µmol/L. Methotrexate was cleared within 72 hours post dose. His serum creatinine prior to first methotrexate dose was 81 µmol/L and remained within normal range post methotrexate (CTCAE toxicity grade 0). Phosphate dropped to 0.49 mmol/L on day 3 post methotrexate (CTCAE toxicity grade 3). ALT peaked at 499 IU/L on day 2 post methotrexate (CTCAE toxicity grade 3). No further bone marrow suppression was noted following the first dose of methotrexate.

Assessment on day 8 of cycle 1 revealed one healing ulcer and two tiny almost healed lesions on the left side of his buccal mucosa (CTCAE mucositis clinical toxicity grade 1, CTCAE mucositis functional toxicity grade 0). The patient remained on the same medication that he was at the beginning of the trial treatment, i.e. MTS 20mg BD, PO and Sodium Docusate 200mg TDS, PO as well as Senna 2 tablets nocte and Difflam

mouthwash. Creatinine was 62 µmol/L (CTCAE toxicity grade 0) and calculated GFR was 168 ml/min/1.73 m^2. ALT was elevated at 551 IU/L (CTCAE toxicity grade 3). His phosphate was normal at 1.24 mmol/L. He was fit to proceed with his second dose of methotrexate on time.

MTX dose 2: DAOA had his second dose of methotrexate on 4th February 2009, delayed by 1 day due to weather conditions. Folinic acid rescue was started on 5th February 2009. Methotrexate plasma levels at 24 hours were 20 µmol/L. Methotrexate was cleared within 72 hours post dose. His serum creatinine remained within normal range post methotrexate. Phosphate dropped to 0.58 mmol/L on day 4 post methotrexate (CTCAE toxicity grade 3). ALT peaked at 803 IU/L on day 7 post methotrexate (CTCAE toxicity grade 3). No further bone marrow suppression was noted following the second dose of methotrexate.

Assessment on day 15 of cycle 1 took place on 10 February 2009, after two doses of methotrexate. DAOA had mucositis on the left side of his buccal mucosa (CTCAE mucositis clinical toxicity grade 1, CTCAE mucositis functional toxicity grade 2). He was able to eat and drink as long as he used Difflam mouth spray. Calculated GFR and creatinine were within normal range at 134 ml/min/1.73 m^2 and 84 µmol/L respectively. ALT was elevated at 803 IU/L (CTCAE toxicity grade 3). His serum phosphate and bilirubin were within normal range. No further bone marrow suppression was noted throughout the first cycle of treatment. In view of his mucositis on day 15 of cycle 1, “day 15” criteria were not met.

CYCLE 2

Clinical assessment on day 1 of cycle 2 on 16 February 2009 was unremarkable and his mucositis had settled completely. He remained on MST, Senna and Sodium Docusate. Creatinine was 75 µmol/L (CTCAE toxicity grade 0) and calculated GFR was within normal range at 145 ml/min/1.73 m^2. His ALT remained elevated at 276 IU/L (CTCAE toxicity grade 3). His serum phosphate was within normal range. His haemoglobin was stable at 12 gr/dl (CTCAE toxicity grade 1) but his neutrophils dropped to 1.9 x 10^9/L (CTCAE toxicity grade 1). He was fit to proceed to his third dose of methotrexate on time.

MTX dose 3: DAOA had the third dose of methotrexate on 17 February 2009. He had glucarpidase on 18 February 2009. Folinic acid rescue was started on the same day. Methotrexate plasma levels at 24 hours were 26 µmol/L. Methotrexate was cleared within 72 hours post dose. His serum creatinine remained within normal range. His phosphate dropped to 0.49 mmol/L (CTCAE toxicity grade 3) on day 3 post methotrexate. ALT was not measured until 23 February 2009 when it was 290 IU/L (CTCAE toxicity grade 3). No further bone marrow suppression was noted following the third dose of methotrexate. There were no adverse events related to glucarpidase.

Clinical assessment on day 8 of cycle 2 was unremarkable. In particular, there was no mucositis. Serum creatinine, phosphate and bilirubin were within normal range. His calculated GFR was 136ml/min/1.73 m^2. ALT was elevated at 290 IU/L (CTCAE toxicity grade 3). He was fit to receive his fourth dose of methothrexate on time.

MTX dose 4: DAOA received his fourth dose of methotrexate on 24 February 2009. He had glucarpidase on 25 February 2009. Folinic acid rescue was started on the same day. Methotrexate plasma levels at 24 hours were 16.97 µmol/L. Methotrexate was cleared within 72 hours post dose. His serum creatinine and bilirubin remained within normal range. His phosphate dropped to 0.82 mmol/L (CTCAE toxicity grade 1) on day 3 post methotrexate. ALT was not measured until 03 March 2009 when it was 426 IU/L (CTCAE toxicity grade 3). No further bone marrow suppression was noted following the forth dose of methotrexate. A mouth swab taken on 27 February 2009 revealed a moderate growth of candida (CTCAE toxicity grade 2 for infection with normal ANC). There were no adverse events related to glucarpidase.

Assessment on day 15 of cycle 2 took place on 02 March 2009, after four doses of methotrexate. He had a small ulcer on his lower lip. His right ankle, site of primary disease, remained swollen. Otherwise his clinical examination was unremarkable. He remained on MST 15mg BD, PO, Senna and Sodium Docusate.. His calculated GFR was 136 ml/min/1.73 m^2. His serum creatinine was within normal range. His serum bilirubin and phosphate were within normal range. His ALT remained elevated at 426 IU/L (CTCAE toxicity grade 3). No further bone marrow suppression was noted following the forth dose of methotrexate. “Day 15” criteria were met.

Antiglucarpidase antibodies were negative on days 1, 8 and 15 of cycle 2 and day 1 of cycle 2. They were present on days 8 and 15 of cycle 2.

APPENDIX 13. ANTIGLUCARPIDASE ANTIBODY RESPONSE, INTERIM ANALYTICAL REPORT BY MILLIPORE BIOPHARMA (NOVEMBER 2011)

Millipore Interim Analytical Report Study Number 10/056-010	
Study Title:	Determination of Anti-Glucarpidase Antibodies by ELISA in Human Serum Samples From 'A Randomised, Cross-Over, Phase II Study, To Investigate The Efficacy And Safety Of Glucarpidase For Routine Use After High Dose Methotrexate In Patients With Bone Sarcoma'.
Sponsor:	Joint UCLH/UCL Biomedical Research Unit (JBRU) University College London 1st Floor Maple House 149 Tottenham Court Road London W1T 7NF
Clinical Study Reference:	BRD/06/085
EudraCT Number:	2006-003203-40
Analysis Sponsor:	Protherics Medicines Development Ltd, A BTG Company 5 Fleet Place London, EC4M 7RD
Analysis Sponsor Study Reference:	PR001-CLN-009
Sponsor Document Reference:	PR001-CLN-rptBAX050
Analytical Laboratory:	Millipore BioPharma Services 91 Milton Park Abingdon Oxon OX14 4RY UK

ANALYTICAL PROJECT MANAGERS STATEMENT

I, the undersigned, hereby declare that the work was performed by me or under my supervision and that the findings provide a true and accurate record of the results obtained.

The study was performed in accordance with the agreed analytical plan, the analytical procedure and Millipore BioPharma Services Standard Operating Procedures, unless otherwise stated, and the study objectives were achieved.

D Shaw
Analytical Project Manager
Millipore BioPharma Services

18 Nov 2011
Date

REVIEWING SCIENTIST STATEMENT

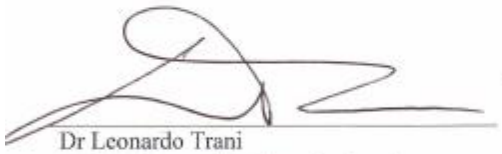
I, the undersigned, hereby declare that I have reviewed this report in conjunction with the Analytical Project Manager and that the interpretation and presentation of the data in the report are consistent with the results obtained.

M Bentley
Bioanalytics Service Manager
Millipore BioPharma Services

18 Nov 2011
Date

SPONSOR REPORT ACCEPTANCE

The signatory below confirms that this report has been reviewed for compliance with the analytical plan and the final report is acceptable to the study Sponsor.



22/11/11

Dr Leonardo Trani
Clinical Research Fellow in Oncology
University College Hospital, Department of Oncology

Date

STUDY PERSONNEL

The following Millipore BioPharma Services personnel were involved in the study:

Name	Role in Study
T Evans	Analyst
C Langley	Analyst
D Langley	Analyst
P Martindale	Data Checker
H Woodcock	Analyst
S Wyatt	Analyst

STUDY SCHEDULE

The study schedule, for the contents of this report, was as follows:

Study initiation: (Date Analytical Project Manager signed analytical plan)	02 Mar 2011
Experimental start date: (Date of recording first study specific data)	12 Mar 2011
Experimental completion date: (Date of capture of final reported data)	21 Sep 2011
Study completion:	Date final report is signed by the Analytical Project Manager

DATA ARCHIVE AND MATERIAL RETENTION STATEMENT

All study specific data generated will be archived for a period of 3 years from the date of issue of the final report.

Materials (samples, reference standards and Sponsor supplied assay materials) resulting from this study will be retained by Millipore BioPharma Services, at the appropriate storage temperature, for a period of one month from the date of issue of the final report, unless they are to be used in subsequent studies.

After these time periods, the Sponsor will be contacted to determine the fate of the study data and materials. Should the data archive and material retention periods require extension; further charges will be made based on the quantity of data/materials and the period of extension.

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LIST OF ABBREVIATIONS

BALAP	Analytical Procedure
CD	Compact disc
%CV	Coefficient of variation (precision)
ELISA	Enzyme linked immunosorbent assay
Hi QC	High quality control
LLOQ	Lower limit of quantification
Lo QC	Low quality control
Me QC	Medium QC
NCS	Negative control serum
OD	Optical density
PMDL	Protherics Medicines Development Ltd
pdf	Portable document format
%RE	Percent relative error
QC	Quality control
SOP	Standard operating procedure
SB	Sample batch
SV	Software validation
TB	Test batch
UCL	University College London

SUMMARY

Ninety two serum samples from 14 patients on Sponsor study reference BRD/06/085 (Analytical Sponsor's reference PR001-CLN-009) were analysed for the presence of anti-glucarpidase (Voraxaze™) antibodies.

The observed assay responses at the positive/negative cut-point and the data for the anti-glucarpidase positive control calibration standards and QC samples indicated that the method performed reliably during the reported study sample analysis. All reported batches and samples complied with the assay acceptance criteria.

A total of 23 samples from eight of the subjects were screened as being potentially anti-glucarpidase positive. Of these, ten samples from six subjects were confirmed as being true anti-glucarpidase positive. Of the ten confirmed positive samples, six had low measured concentrations that were below the limit of quantification of the assay (<62.5 ng/mL). The day 180 cycle 2 sample for subject 2 was confirmed with a concentration of anti-glucarpidase antibodies 2526.2 ng/mL. The three confirmed samples for subject 7 at days 1, 15 and 23 for cycle 2 had concentrations of 526.7 ng/mL, 172.4 ng/mL and 123.9 ng/mL, respectively.

1 OBJECTIVE

The objective of this study was to determine the presence of anti-glucarpidase (Voraxase™) antibodies, using ELISA techniques, in serum samples generated in the Osteosarcoma Clinical Trial study EudraCT reference 2006-003203-40 (BTG reference PR001-CLN-009). All samples were subject to a screen for the presence of anti-glucarpidase antibodies. This assay included a calibration curve for semi-quantification of responses that were confirmed as true positives. All positive samples from the screening assay were analysed in a confirmatory assay. The concentrations measured in the screening assay were reported only for samples subsequently confirmed as true positives.

Samples confirmed as true positive will be sent to second contract laboratory for analysis in a neutralising antibody assay as the contractor is yet to be confirmed, these results will be reported independently.

2 MATERIALS AND METHODS

2.1 Positive Control Antibody (Analyte)

Affinity purified rabbit anti-glucarpidase antibodies from Covance Research Products (Denver PA, USA Study No. 2006-0074) (lot number PR070522A), used in the preparation of calibration standards and QC samples, was received from the Sponsor on 17 Apr 2008. On receipt it was assigned the Millipore BioPharma Services reference standard number RS08-113. Thirty 1 mL aliquots at a concentration of 1.26 mg/mL were received. No expiry date was specified. On receipt at Millipore BioPharma Services it was stored at -70°C (nominal). At first use of each aliquot they were dispensed as single sub-aliquots and re-frozen. Aliquots from two stock vials were used in the current study; RS08-113e and RS08-113f.

2.2 Critical Reagents

Twelve vials of lyophilised glucarpidase (Voraxaze™) (lot number 2090601) used to coat the assay plates and in the immunodepletion of positive samples, were received from the Sponsor on 12 Oct 2010 and stored refrigerated (+4°C nominal) until reconstitution. These were assigned Millipore BioPharma Services reference standard number RS10-218. The content of each vial was

1000U at a specific activity of 455 U/mg (2.2 mg protein content). The end of the clinical shelf life was specified as 31 March 2011[1].

For analytical use the Voraxaze was reconstituted with 1 mL of sterile 0.9% saline; once reconstituted a frozen (-70°C nominal). The different reconstitution dates were used in the following study batches.

Lot	Reconstitution Date	Expiry Date[a]	Batch Use
RS10-218f	03 May 11	10 May 11	TB06, SB01, SB02
RS10-218g	11 May 11	18 May 11	TB07, SB03-SB07

Footnote:
Initial 7 day expiry extended to 6 months based on stability study results [2]

The provisional frozen expiry date given in the Analytical Procedure was 7 days; the stability of this critical reagent under these specific storage conditions is the subject of an ongoing investigation [2]. At the time the current study was performed a stability period of 6 months had been demonstrated, and so all reconstituted Voraxaze™ was considered to be in date when used in the study.

Biotinylated Voraxaze™ (RS08-149, 40:1 labelling) used as the detection reagent in the assay was prepared in study number 08/056-002 [3]. This was prepared at a concentration of 440 µg/mL stored frozen (-70°C nominal) since preparation on 06 Jun 2008. The stability of this critical reagent is the subject of investigation [2].

All other reagents used are specified in the Analytical Procedure BALAP 08/021 version (07) presented in the Annex. Unless otherwise stated in the Deviations section (see Section 3), all reagents were within specification at the time of use.

2.3 Control Matrices

Pooled control human serum used as a blank and in the preparation of calibration standards and QC samples was prepared from individual sera previously screened during validation [4].

[1] The continued use of theVoraxaze past is clinical shelf life is supported by the results from the ongoing stability study 08/056-004 [2].

Pooled sera 10HPS243 and 11HPS185 were prepared as an equal mix of the following seven individuals:

10HIS051, 10HIS053, 10HIS056, 10HIS059, 10HIS068, 10HIS069 and 10HIS070

An expiry date of 05 Feb 2015 was assigned for these pools.

The following individual normal sera were used in the screening batches SB01 to SB04 to supplement the number of baseline samples for establishing the assay cut-point.

10HIS052, 10HIS054, 10HIS055, 10HIS058, 10HIS062, 10HIS063, 10HIS064, 10HIS065, 10HIS066, 10HIS067.

An expiry date of 05 Feb 2015 was assigned for these.

Individual sera were obtained from SeraLabs International (Haywards Heath, UK) and stored at -70°C (nominal) each was assigned an expiry date of 5 years from receipt at Millipore BioPharma Services. All sera were in date when used in this study.

2.4 Calibration Standards and QC Samples

Positive control antibody calibration standards were prepared fresh on the day of analysis over the whole serum equivalent range 15.6 ng/mL to 1000 ng/mL.

Positive control QC samples of 100, 400 and 750 ng/mL were bulked spiked and stored at -70°C (nominal) as aliquots in polypropylene tubes as follows:

Preparation Date	Prepared in Study	Tested in Batch	Expiry Date	Batch Use
05 May 2011	10/056-010	TB06	05 Jun 2011[x1]	SB01-SB04
15 Sep 2011	10/056-010	TB07	16 Mar 2011[x2]	SB05-SB08

Footnotes:
x1 = Initial expiry set as per BALAP 08/021 (07), subsequently resed to 05 Nov 2011 based on extended stability data obtained in study 08/056-004 [2].
x2 = Expiry set based on extended stability data obtained in study [2].

Two sets of QCs (TB06 and TB07) were prepared during the study; the results for the testing of these are shown in appendix 1.

2.5 Analytical Procedure

The analysis of clinical samples for the presence of antibodies to glucarpidase was performed in accordance with the methodology described in the Analytical Procedure:

BALAP 08/021: Detection of Antibodies to Voraxaze™ in Human Serum by Bridging ELISA

Briefly, the principle of the method was as follows:

Screening/semi-quantitative assay:
Voraxaze™ was adsorbed onto microtitre plates at a concentration of 0.5 µg/mL in PBS (100 µL/well) overnight at 4°C (nominal). Following blocking (2 hours, 2% w/v BSA in PBS, 200 µL/well) the plates were washed (four times, 0.05% v/v Tween 20 in PBS, 300 µL/well). Human serum samples, anti-glucarpidase standards and blanks were diluted 1 in 2 with 2% w/v BSA in PBS prior to adding to the plates in duplicate adjacent wells (100 µL/well). The plates were incubated at 25°C (nominal) for 2 hours with gentle shaking. The plates were washed (four times, 0.05% v/v Tween 20 in PBS, 300 µL/well) and biotin-labelled Voraxaze is added (0.5 µg/mL, 100 µL/well). The plates were incubated for 2 hours at 25°C (nominal) with gentle shaking and then washed (four times, 0.05% v/v Tween 20 in PBS, 300 µL/well). Extravidin peroxidase reagent (1:10,000 dilution, 100 µL/well) was added and the plates incubated for 1 hour at 25°C (nominal). Following washing (four times, 0.05% v/v Tween 20 in PBS, 300 µL/well), TMB substrate was added (100 µL/well) and the plates incubated for 15 minutes at 25°C (nominal). The reaction was stopped by the addition of sulphuric acid (0.5 M, 100 µL/well) and the absorbance read at 450 nm with background correction at 540 nm.

Confirmatory assay:
Six replicates of samples with positive Screen assay results were re-analysed as described above following a 30 minute pre-incubation at 25°C (nominal) with and without Voraxaze™ added to a final concentration of 0.19 µg/mL. The responses were compared by t-test.

The version (07) used in the reported analysis of all clinical samples is included as an annex to this report.

2.6 Screening & Semi-Quantitative Assay

Positive/negative cut-point determination:

The pre-dose serum samples from the clinical study were used in conjunction with individual normal human sera and together used for the determination of a negative cut-off value within each plate. A Grubbs test for statistical outliers was performed on the ten sera according to the method described in SOP BAL-157; one statistical outlier was removed from SB01.

The negative cut-off was derived from the mean absorbance (OD) values of the 10 individual sera (pre-dose and individual negative controls) + 1.645 × their standard deviation, according to the validated method. Any samples greater than or equal to the negative cut-off value were considered to have screened positive and a relative mass unit score was derived from the positive control standard curve if positivity was subsequently confirmed. Samples which gave a value below the negative cut-off value were scored as negative.

In addition to the above, and as part of the ongoing in-study validation of the cut-point determination, a further control sample was included in the screening assay. Two replicates (n = 4 wells) of a pooled normal control serum (NCS, batch 10HPS077) were analysed on each assay plate. This NCS sample was analysed during the pre-study validation study and a normalisation factor obtained for it by dividing the plate cut-points (derived from n = 50 individual control sera) by the mean value observed for the NCS on individual assay plates. The normalisation factor was calculated in the pre-study validation as 1.51 [4]. Normalised cut-points for each plate within this clinical study were therefore calculated by multiplying the mean NCS assay response by the normalisation factor shown above. A comparison with the cut-points used for reporting sample results was made for information purposes only (see Appendix 3).

2.6.1 Batch Acceptance Criteria

Each batch included a positive control antibody calibration curve, 2 × Lo QC, 2 × Me QC, 2 × Hi QC samples and 2 × NCS. All of the QC samples must be positive when assessed against the cut-point value for the screening assay. Additionally, where antibody concentrations were to be reported 4 of the 6 QC

replicates should be within 30% of their nominal concentration. Two QC samples may be outside of 30%, but these must be at different concentrations

The precision between duplicate OD values of the Lo QC samples should be ≤ 30%.

The precision between duplicate OD values of the Me QC and Hi QC samples should be ≤ 20%.

2.6.2 Sample Acceptance Criteria

The precision between duplicate ODs of positive samples with a mean measured OD of ≤ 0.250 should be ≤ 30%.

The precision between duplicate ODs of positive samples with a mean measured OD of > 0.250 should be ≤ 20%.

For the calibration curve to be acceptable, the calibration standards should be within 25% of the nominal concentration (i.e. percent relative error (%RE) ≤ 25%). The accuracy of the calibration standards around the limit of quantification (i.e. 62.5 ng/mL calibration standards) should be ≤ 30%.

The curve may be edited by removing points which do not meet the above acceptance criteria. A maximum of 2 individual replicates or one complete calibration point should be removed. There was no requirement to remove points which do not meet the calibration curve acceptance criteria if the overall curve fit was considered acceptable.

Any sample with a percentage difference between duplicates of greater than 30% and with one replicate below and one above the assay negative cut-off value should be treated as a potential positive sample and analysed in the confirmatory assay.

Negative samples have no precision acceptance criteria so long as both replicate OD values for that sample are less than the assay negative cut-off value.

2.7 Confirmatory Assay

The confirmatory batch acceptance criteria as specified in the analytical plan (see Annex) were as follows:

2.7.1 Batch Acceptance Criteria

Each confirmatory assay plate included six replicate wells of a Lo QC sample analysed with and without Voraxaze at 0.19 µg/mL as described in plan Section 7.2. The batch was considered acceptable if the Lo QC sample was confirmed as a true positive as described below in Section 2.7.2.

2.7.2 Sample Acceptance Criteria

The precision between the six replicate OD values of the spiked and un-spiked samples should be $\leq 30\%$.

If the drug-spiked response was significantly ($p < 0.05$) less than the un-spiked response and the difference is greater than 20% (of the un-spiked) it was designated as confirmed antibody positive. Mass unit equivalent concentrations derived from the positive control calibration curves in the screening assay (section 2.6) were reported for these confirmed samples.

In cases where the pre-dose sample is negative and the post-dose sample is confirmed as positive according to the above critera, the response is defined as treatment emergent.

If the post-treatment sample is positive in the screening assay but is found not to be drug specific in the confirmatory assay, the sample is reported as 'negative' and no statement regarding the treatment emergent status is made since both results are negative.

Where both the pre- and post-dose samples are confirmed as drug-specific, a comparison between six replicates of pre-dose and post-dose samples will be analysed. The responses will be compared by t-test. If the post-treatment response is significantly greater ($p < 0.05$) than the pre-treatment response and the difference is greater than 20 % (of the pre-treatment), it will be designated treatment-emergent.

2.8 Sample Receipt and Storage

Two shipments of study samples were received and were assigned Millipore sample receipt references S11-088 and S11-291.

S11-088 was received on 17 Mar 2011 and contained 166 aliquots, comprising 79 unique samples from 12 patients.
S11-291 was received on 09 Sep 2011 and contained 26 aliquots comprising 13 unique samples from two patients.

For each patient samples were received for up to 8 nominal time points as specified in the clinical protocol and Analytical Plan:

> Baseline, day 8 cycle 1, day 15 cycle 1, day 1 cycle 2, day 8 cycle 2, day 15 cycle 2, day 30 cycle 2, day 90 cycle 2, day 180 cycle 2.

The samples were received frozen on dry-ice on receipt all samples were stored at -70°C (nominal) prior to analysis. No deviations from the sample storage temperatures from the date of receipt to the date of analysis of the last sample were recorded. Samples for patient 5 and 14 were not available for analysis (patient 5 receievd emergency treatment of Voraxaze™ and patient 14 never received Voraxaze™).

3 DEVIATIONS

The following deviations from the Analytical Plan, Analytical Procedure, and SOPs were noted during the course of the sample analysis. The risk of all deviations on the integrity of the validation data is discussed.

3.1 Analytical Plan

None recorded

3.2 Analytical Procedure BALAP 08/021 version (07)

None recorded

3.3 Millipore BioPharma Services SOPs

- **Failure to print Instrument Method File before commencing analysis: BAL-019 Organisation and Storage of Study Data [E] Section 4.2.9.**
  The analyst performing the first analysis batch neglected to prepare and print a study-specific instrument method file having been working on another study

utilising the same analytical procedure. All analyses were performed using the correct methodology and a method file was produced retrospectively, and so there considered to be no risk to the integrity of the study.

- **Failure to prepare Reference Standard Documentation at time of sub-aliquoting: BAL-015 [K] Reference Standards, Section 6.1.3.**
  At the time that the positive control antibody, RS08-113e was thawed and sub-aliquoted the necessary reference standard paperwork was not completed. The details of the paperwork were reconstructed from the ItemTracker records and so this deviation from procedure is not considered to have had an impact on the integrity of the study data.

- **Use of Voraxaze beyond stated Clinical Shelf Life: BAL-045 (06) Section 4**
  The clinical shelf life expiry date for Lot 2090601 of Glucarpidase (Voraxaze) used in the current study was set as 31 March 2011, 5 years from manufacture. The Sponsor ended the GMP stability study at that time point (i.e. no further stability data was collected). For consistency with the previous rounds of sample analysis, the current lot of Voraxaze was used for the ongoing sample analysis. An ongoing stability study designed to assess the performance of lot 2090601 of Voraxaze with the intention of generating data to support its continued use in the clinical sample analysis studies is ongoing. At the time of issue of this memo (6 month time point in study 08/056-004) the performance of the assay on plates coated with lot 2090601 (both freshly reconstituted and stored frozen at -70°C nominal) was considered to be comparable the performance at the start of the stability period; as such there considered to be no risk to the integrity of the study.

- **Use of reconstituted Voraxaze beyond stated expiry date: BAL-045 (06) Section 4**
  As for the lyophilised Voraxaze stocks, the reconstituted, frozen (-70°C nominal) Voraxaze was used beyond its 1 week expiry date based on the results of the ongoing stability study, and so there considered to be no risk to the integrity of the study.

- **Use of positive control samples beyond stated expiry date: BAL-045 (06) Section 4**
  The quality control samples used in the current study were prepared on 05 May 2011 and used over a period of four months, rather than the 1 month stated in BALAP 08/021 (07). This extension to the usage period for the QCs

is based the acceptable performance of the QC sample in the ongoing stability study, and so there considered to be no risk to the integrity of the study.

4 RESULTS

The analysis of samples from Sponsor study PR001-CLN-009 was performed using a method previously developed and validated at Millipore BioPharma Services [5, 4].

Due to rounding differences between spreadsheet applications and calculators some data may contain minor differences when checked using an alternative method. These differences are so small that they are considered acceptable as they do not affect the integrity of the data generated.

4.1 Summary of Analysis

Eight reported analytical batches were performed; four screening and four confirmatory. Additionally, eight test batches were performed, six for training purposes and two for qualification of positive control samples. A summary of all batches performed is shown in Appendix 1.

A total of 92 screening and 23 confirmatory results were reported from 14 patients.

4.2 Repeat Analysis

A summary of all repeat analysis is included in Appendix 2.

4.3 Screen Assay Cut-Point

Samples were analysed in four screening batches (SB01 - SB04).

The mean absorbance for all blank sera (pre-treatment and normal) used in the calculation of the cut-point are shown in Table 1.

The range of blank responses in the two screening batches was 0.012 to 0.040 OD, one statistical outlier (2 tailed Grubb's $p = 0.10$) was identified in batch SB01. The data were normally distributed (Shapiro-Wilk test). The calculated cut-points for the four batches were between 0.023 and 0.041 OD.

Table 2 shows an assessment of the mean results for the NCS blank serum relative to the calculated cut-points in all screening batches. Five of the eight replicates of NCS screened negative in the four screening batches.
Also shown in Table 2 are the individual replicate ODs of the lowest calibration standard (15.6 ng/mL). Both replicates screened positive relative to the calculated plate cut-point in all screening batches; OD ratios of between 1.1 and 1.8. These results at very low positive control antibody concentrations thus confirm good control of the cut-point in the screening batches.
Additional data in Table 3 shows all QC samples were positive when assessed against the plate cut-points. The screening assay criteria were therefore met.

4.4 Calibration Standard Data

The raw absorbance data for the positive control calibrators are shown in Table 4. The corresponding back-calculated concentrations are shown in Table 5. The data show that the calibration standard assay batch acceptance criteria were met.

4.5 QC Data

Quantitative QC Data

Table 6 shows the inter-assay precision and accuracy of the Lo, Me and Hi QC sample concentrations for all batches from which semi-quantitative data were reported. All batches except SB01 met the acceptance criteria that at least 4 out of 6 QC samples should be within ± 30% of their nominal value. In batch SB01 both Lo QC samples over-recovered, therefore no semi-quantitative data is reported from this batch.

From an in-study validation perspective, the inter-assay precision of the QC samples from batches did not exceed 22.7% (Lo QC). The overall inter-assay accuracies were between 7.8% and 16.3%. This data is presented for information purposes as there are no acceptance criteria associated with it.

Confirmatory QC Data

The data in Table 7 show the assessment of the glucarpidase immunodepletion of the Lo QC sample in all confirmatory assay batches. These data show that all QC samples successfully immunodepleted by at least 83.3%. The differences between the spiked and un-spiked LoQC samples was statistically significant by t-test ($p < 0.05$). The assay acceptance criteria were therefore met.

4.6 Sample Data

The overall screening and confirmatory results for all subjects are presented in Table 8. The patients are presented in ascending numerical order based on the patient number and time point. The statistical assessment of the immunodepletion of the samples screened as positive is shown in Table 9.

Of the 92 samples tested from 14 subjects, 23 (25%) screened as potential positives for anti-glucarpidase antibodies. Of these, ten samples (10.9%) from six subjects were confirmed positive.

The Day 180 cycle 2 sample for subject 2 (ANME) was confirmed as a treatment emergent, anti-glucarpidase specific response at a concentration of 2526.2 ng/mL. The semi-quantitative result was obtained following analysis at a 1 in 5 dilution.

Three of the post treatment samples from subject 7 (IFOK) were confirmed as treatment emergent, anti-glucarpidase specific responses. The measured antibody equivalent concentrations were 526.7 ng/mL, 172.4 ng/mL and 123.9 ng/mL at Cycle 2 timepoints of Day 1, Day 15 and Day 23, respectively.

The remaining six confirmed positive samples from subjects 8, 9, 15 and 16 were all from Cycle 2 timepoints and gave low measured antibody equivalent concentrations that were below the limit of quantitation of the assay (<62.5 ng/mL).

5 DISCUSSION

The acceptable performance of the cut-point, the rabbit anti-glucarpidase calibration standards and quality control samples indicated that the method performed reliably during the study sample analysis. All reported batches complied with the calibration and quality control acceptance criteria.

In addition to the established low QC sample of 100 ng/mL, the lowest calibration standard (15.6 ng/mL) was also assessed against the plate cut-point. The sample was found to be positive in both screening batches having an absorbance that was 1.1 to 1.8 times greater than the cut-point. Thus, confirmation that recovery of very low

positive anti-Voraxaze antibody was possible in both screening batches further ensured against the possibility of false negatives being recorded in the test samples.

Twenty three of the 92 clinical samples were screened as being potential anti-glucarpidase antibody positives. Of these, 10 samples (10.9%) were subsequently confirmed as being true anti-glucarpidase antibody positive.

6 REFERENCES

[1] Clinical protocol: A Randomised, Cross-Over, Phase II Study, to Investigate the Efficacy and Safety of Glucarpidase for Routine use After High Dose Methotrexate in Patients with Bone Sarcoma. BRD/06/085 (PR001-CLN-pro009).

[2] BioAnaLab Study 08/056-004 (Sponsor Ref No. PR001-CLN-proBA041): Assessment of the Stability of Critical Assay Reagents and Anti-Glucarpidase Antibodies in Human and Cynomolgus Monkey Sera at -70°C.

[3] BioAnaLab Study 08/056-002 (Sponsor Ref No. PR001-CLN-proSP001): Preparation and Quality Control of Biotin-labelled Voraxaze™

[4] BioAnaLab Study 08/056-003 (Sponsor Ref No. PR001-CLN-proBA028): Pre-Study Validation of an Analytical Procedure to Detect Antibodies to Voraxaze™ in Human Serum by Bridging ELISA.

[5] BioAnaLab Study 08/056-001 (Sponsor Ref No. PR001-CLN-pro BA025): Development of a Screening ELISA to Detect Antibodies against Voraxaze™ in Human Serum.

DATA TABLES

Table 1

Determination of Anti-Glucarpidase Antibodies by ELISA in Human Serum Samples From 'A Phase II Study, To Investigate The Efficacy And Safety Of Glucarpidase For Routine Use After High Dose Methotrexate In Patients With Bone Sarcoma' BRD/06/085 (PR001-CLN-009)

Cut-Point and Outlier Determination of Screening Batches SB01 to SB04

Batch Number							
SB01		SB02		SB03		SB04	
Individual IDs 1-10	Mean OD	Individual IDs 1-10	Mean OD	Individual IDs 1-10	Mean OD	Individual IDs 1-10	Mean OD
JASH	0.022	SAWI	0.017	NIFI	0.017	015 D1C1	0.021
CHWC	0.027	IFOK	0.035	JACA	0.015	016 D1C1	0.020
PEFR	0.022	PAHE	0.012	ANME	0.015	10HIS052	0.020
SAPH	0.021	10HIS054	0.024	HO	0.018	10HIS054	0.019
10HIS054	0.031	10HIS055	0.034	TRGO	0.013	10HIS055	0.020
10HIS063	0.039	10HIS062	0.040	10HIS052	0.024	10HIS058	0.022
10HIS064	0.023	10HIS063	0.027	10HIS054	0.028	10HIS062	0.016
10HIS065	0.031	10HIS065	0.027	10HIS063	0.022	10HIS063	0.015
10HIS066	0.070[x1]	10HIS066	0.032	10HIS065	0.026	10HIS064	0.014
10HIS067	0.033	10HIS067	0.023	10HIS067	0.029	10HIS065	0.015
Mean	0.028		0.027		0.021		0.018
Std Dev	0.0062		0.0085		0.0059		0.0029
n	9		10		10		10
Cut-point	0.038		0.041		0.031		0.023

Footnotes:

x1 = Value excluded as a statistical outlier by Grubbs two-tailed test (p = 0.10)

{Click to return to Section 4.3}

Table 2

Determination of Anti-Glucarpidase Antibodies by ELISA in Human Serum Samples From 'A Phase II Study, To Investigate The Efficacy And Safety Of Glucarpidase For Routine Use After High Dose Methotrexate In Patients With Bone Sarcoma' BRD/06/085 (PR001-CLN-009)

Assessment of NCS and Lowest Calibrator Samples Against Plate Cut Point

Batch	Plate Cut Point	Replicate	Screening Results (Positive / Negative)				
			NCS		15.6 ng/mL Calibrator		
			Mean OD	Result	OD	Result	Ratio
SB01	0.038	1	0.040	Positive	0.056	Positive	
		2	0.037	Negative	0.051	Positive	1.4
SB02	0.041	1	0.025	Negative	0.043	Positive	
		2	0.026	Negative	0.046	Positive	1.1
SB03	0.031	1	0.034	Positive	0.056	Positive	
		2	0.033	Positive	0.058	Positive	1.8
SB04	0.023	1	0.017	Negative	0.038	Positive	
		2	0.018	Negative	0.038	Positive	1.7

{Click to return to Section 4.3}

Table 3

Determination of Anti-Glucarpidase Antibodies by ELISA in Human Serum Samples From 'A Phase II Study, To Investigate The Efficacy And Safety Of Glucarpidase For Routine Use After High Dose Methotrexate In Patients With Bone Sarcoma' BRD/06/085 (PR001-CLN-009)

Assessment of QC Samples Against Plate Cut point

Batch	Plate cutpoint	Replicate	QC Screening Results					
			LoQC		MeQC		HiQC	
			Mean OD	Result	Mean OD	Result	Mean OD	Result
SB01	0.038	1	0.165	Positive	0.675	Positive	1.423	Positive
		2	0.184	Positive	0.819	Positive	1.699	Positive
SB02	0.041	1	0.209	Positive	0.931	Positive	1.819	Positive
		2	0.192	Positive	0.909	Positive	2.022	Positive
SB03	0.031	1	0.226	Positive	1.016	Positive	2.030	Positive
		2	0.204	Positive	0.892	Positive	1.743	Positive
SB04	0.023	1	0.134	Positive	0.680	Positive	1.617	Positive
		2	0.149	Positive	0.719	Positive	1.497	Positive
Mean OD			0.183		0.830		1.731	
Standard deviation (n-1)			0.0315		0.1275		0.2222	
Precision (%)			17.2		15.4		12.8	
n			8		8		8	

{Click to return to Section 4.3}

Table 4

Determination of Anti-Glucarpidase Antibodies by ELISA in Human Serum Samples From 'A Phase II Study, To Investigate The Efficacy And Safety Of Glucarpidase For Routine Use After High Dose Methotrexate In Patients With Bone Sarcoma' BRD/06/085 (PR001-CLN-pro009)

Precision of Calibration Standards (Raw Absorbance Values)

Batch	Nominal Calibrator concentration (ng/mL)						
	15.6	31.3	62.5	125	250	500	1000
SB01	0.056	0.072	0.093	0.147	0.334	0.878	1.621
	0.051	0.066	0.086	0.134	0.287	0.785	1.727
SB02	0.043	0.064	0.111	0.217	0.465	1.099	2.403
	0.046	0.064	0.105	0.203	0.427	0.987	2.469
SB03	0.056	0.084	0.132	0.246	0.516	1.277	2.433
	0.058	0.081	0.124	0.249	0.497	1.155	2.418
SB04	0.038	0.061	0.093	0.182	0.401	0.978	2.175
	0.038	0.066	0.100	0.187	0.423	0.982	2.159
Mean	0.048	0.070	0.106	0.196	0.419	1.018	2.176
SD	0.0082	0.0085	0.0160	0.0420	0.0781	0.1559	0.3321
Precision (%)	16.9	12.2	15.2	21.4	18.6	15.3	15.3
n	8	8	8	8	8	8	8

{Click to return to Section 4.4}

Table 5

Determination of Anti-Glucarpidase Antibodies by ELISA in Human Serum Samples From 'A Phase II Study, To Investigate The Efficacy And Safety Of Glucarpidase For Routine Use After High Dose Methotrexate In Patients With Bone Sarcoma' BRD/06/085 (PR001-CLN-009)

Precision and Accuracy of Calibration Standards ($1/y^2$ Weighting)

Batch	Back-calculated Concentrations (ng/mL) Nominal Calibrator concentration (ng/mL)						
	15.6	31.3	62.5	125.0	250.0	500.0	1000.0
SB01	11.3	40.0	68.3	119.8	242.1	527.1	982.6
SB02	15.1	32.1	63.3	124.7	245.0	502.0	1007
SB03	14.6	33.4	62.2	125.0	241.4	522.4	987.7
SB04	13.8	36.6	61.9	119.7	247.3	516.0	995.6
Mean (ng/mL)	13.7	35.5	63.9	122.3	244.0	516.9	993.2
SD	1.69	3.53	2.98	2.95	2.72	10.91	10.58
Precision (%)	12.3	9.9	4.7	2.4	1.1	2.1	1.1
RE (%)	-12.2	13.7	2.3	-2.2	-2.4	3.4	-0.7
n	4	4	4	4	4	4	4

{Click to return to Section 4.4}

Table 6

Determination of Anti-Glucarpidase Antibodies by ELISA in Human Serum Samples From 'A Phase II Study, To Investigate The Efficacy And Safety Of Glucarpidase For Routine Use After High Dose Methotrexate In Patients With Bone Sarcoma' BRD/06/085 (PR001-CLN-009)

Inter-assay Precision and Accuracy of Quality Control Samples

Batch	Replicate	LoQC		MeQC		HiQC	
		100.0	%RE	400.0	%RE	750.0	%RE
SB01	1	140.4 x1	40.4	445.9	11.5	838.6	11.8
	2	155.3 x1	55.3	520.6 x1	30.2	998.8 x1	33.2
SB02	1	124.0	24.0	457.0	14.3	793.4	5.8
	2	114.3	14.3	448.0	12.0	864.4	15.3
SB03	1	114.5	14.5	445.7	11.4	833.7	11.2
	2	103.0	3.0	397.1	-0.7	723.3	-3.6
SB04	1	87.7	-12.3	379.8	-5.0	781.9	4.3
	2	97.0	-3.1	398.1	-0.5	734.0	-2.1
Mean (ng/mL)		116.3	-	431.2	-	818.4	-
Standard deviation (n-1)		26.35	-	51.61	-	100.68	-
Precision (%)		22.7	-	12.0	-	12.3	-
RE (%)		16.3	-	7.8	-	9.1	-
n		6	-	6	-	6	-

Footnotes:
x1 = Accuracy (%RE) >30%

{Click to return to Section 4.5}

Table 7

Determination of Anti-Glucarpidase Antibodies by ELISA in Human Serum Samples From 'A Phase II Study, To Investigate The Efficacy And Safety Of Glucarpidase For Routine Use After High Dose Methotrexate In Patients With Bone Sarcoma' BRD/06/085 (PR001-CLN-009)

Assessment of Immunodepletion of LoQC Samples

Batch	Well Replicate	Unspiked LoQC	Voraxaze Spiked LoQC	Results		
		OD Value	OD Value	% Inhibition	t-test	Final Result
SB05	1	0.179	0.018	89.8	0.0000004	Anti-drug pos
	2	0.194	0.019			
	3	0.188	0.021			
	4	0.175	0.018			
	5	0.210	0.020			
	6	0.200	0.021			
SB06	1	0.181	0.019	88.7	0.00000001	Anti-drug pos
	2	0.185	0.021			
	3	0.183	0.020			
	4	0.174	0.021			
	5	0.188	0.022			
	6	0.191	0.021			
SB07	1	0.190	0.030	83.3	0.00000008	Anti-drug pos
	2	0.196	0.029			
	3	0.178	0.031			
	4	0.180	0.034			
	5	0.183	0.031			
	6	0.187	0.031			
SB08	1	0.180	0.024	85.6	0.00000002	Anti-drug pos
	2	0.166	0.024			
	3	0.173	0.026			
	4	0.171	0.025			
	5	0.174	0.026			
	6	0.181	0.025			

{Click to return to Section 4.5}

Table 8

Determination of Anti-Glucarpidase Antibodies by ELISA in Human Serum Samples From "A Phase II Study, To Investigate The Efficacy And Safety Of Glucarpidase For Routine Use After High Dose Methotrexate In Patients With Bone Sarcoma" BRDA060085 (PR001-CLN-009)
Screening and Confirmatory Results: Batches SB01 to SB07, Subjects 1 to 16
Millipore BioPharma Services Study 10/056-010

Subject Number	Subject Initials	Nominal Time Point	Sample Date	Plate Cut Point	Sample OD	Screening Result	Confirmatory Result	Concentration (ng/mL)	Comments
1	HEOL	baseline	08 Jul 2007	0.081	0.018	Negative	-	-	-
1	HEOL	day 8 cycle 1	10 Jul 2007	0.081	0.016	Negative	-	-	-
1	HEOL	day 15 cycle 1	17 Jul 2007	0.081	0.018	Negative	-	-	-
1	HEOL	day 1 cycle 2	24 Jul 2007	0.081	0.015	Negative	-	-	-
1	HEOL	day 8 cycle 2	31 Jul 2007	0.081	0.013	Negative	-	-	-
1	HEOL	day 30 cycle 2	28 Sep 2007	0.081	0.016	Negative	-	-	-
1	HEOL	day 90 cycle 2	25 Nov 2007	0.081	0.016	Negative	-	-	-
2	ANME	baseline	03 Sep 2007	0.081	0.015	Negative	-	-	-
2	ANME	day 8 cycle 1	10 Sep 2007	0.081	0.014	Negative	-	-	-
2	ANME	day 15 cycle 1	17 Sep 2007	0.081	0.034	Positive	Not Anti-Glucarpidase Positive	-	-
2	ANME	day 1 cycle 2	01 Oct 2007	0.081	0.013	Negative	-	-	-
2	ANME	day 8 cycle 2	08 Oct 2007	0.081	0.015	Negative	-	-	-
2	ANME	day 15 cycle 2	15 Oct 2007	0.081	0.025	Negative	-	-	-
2	ANME	day 180 cycle 2	11 Mar 2008	0.081	3.623	Positive	Treatment emergent, Anti-Glucarpidase Positive	25262	Screening result from SB01, Semi-quantitative result from SB04 at 1 in 5 dilution
3	JACA	baseline	25 Sep 2007	0.081	0.013	Negative	-	-	-
3	JACA	day 8 cycle 1	02 Oct 2007	0.081	0.021	Negative	-	-	-
3	JACA	day 15 cycle 1	09 Oct 2007	0.081	0.019	Negative	-	-	-
3	JACA	day 1 cycle 2	16 Oct 2007	0.081	0.015	Negative	-	-	-
3	JACA	day 15 cycle 2	29 Oct 2007	0.081	0.017	Negative	-	-	-
3	JACA	day 90 cycle 2	18 Feb 2008	0.081	0.015	Negative	-	-	-

Table continues

{Click to return to Section 4.6}

Table 8 continued

Subject Number	Subject Initials	Nominal Time Point	Sample Date	Plate Cut Point	Sample OD	Screening Result	Confirmatory Result	Concentration (ng/mL)	Comments
4	NFI	baseline	01 Oct 2007	0.031	0.017	Negative	-	-	-
4	NFI	day 8 cycle 1	08 Oct 2007	0.031	0.019	Negative	-	-	-
4	NFI	day 15 cycle 1	15 Oct 2007	0.031	0.021	Negative	-	-	-
4	NFI	day 30 cycle 1	25 Oct 2007	0.031	0.087	Positive	Not Anti-Glucapidase Positive	-	-
4	NFI	day 60 cycle 1	25 Nov 2007	0.031	0.022	Negative	-	-	-
6	SA WI	baseline	28 Jun 2008	0.041	0.017	Negative	-	-	-
6	SA WI	day 8 cycle 1	04 Feb 2008	0.041	0.013	Negative	-	-	-
6	SA WI	day 15 cycle 1	11 Feb 2008	0.041	0.013	Negative	-	-	-
6	SA WI	day 1 cycle 2	25 Feb 2008	0.041	0.017	Negative	-	-	-
6	SA WI	day 8 cycle 2	04 Mar 2008	0.041	0.023	Negative	-	-	-
6	SA WI	day 15 cycle 2	11 Mar 2008	0.041	0.040	Negative	-	-	-
6	SA WI	day 30 cycle 2	01 Apr 2008	0.041	0.015	Negative	-	-	-
6	SA WI	day 90 cycle 2	24 Jun 2008	0.041	0.011	Negative	-	-	-
7	IF OK	baseline	18 Feb 2008	0.041	0.035	Negative	-	-	-
7	IF OK	day 8 cycle 1	25 Feb 2008	0.041	0.062	Positive	Not Anti-Glucapidase Positive	-	-
7	IF OK	day 15 cycle 1	03 Mar 2008	0.041	0.041	Positive	Not Anti-Glucapidase Positive	-	-
7	IF OK	day 1 cycle 2	17 Mar 2008	0.041	1.105	Positive	Treatment emergent; Anti-Glucapidase Positive	526.7	-
7	IF OK	day 8 cycle 2	27 Mar 2008	0.041	0.419	Positive	Not Anti-Glucapidase Positive	-	-
7	IF OK	day 15 cycle 2	31 Mar 2008	0.041	0.299	Positive	Treatment emergent; Anti-Glucapidase Positive	172.4	-
7	IF OK	day 28 cycle 2	08 Apr 2008	0.041	0.209	Positive	Treatment emergent; Anti-Glucapidase Positive	123.9	-
7	IF OK	day 180 cycle 2	15 Sep 2008	0.041	0.040	Negative	-	-	-

Table continues

{Click to return to Section 4.6}

Table 8 continued

Subject Number	Subject Initials	Nominal Time Point	Sample Date	Plate Cut Point	Sample OD	Screening Result	Confirmatory Result	Concentration (ng/mL)	Comments
8	PA HE	baseline	18 Feb 2008	0.041	0.012	Negative	-	-	-
8	PA HE	day 8 cycle 1	26 Feb 2008	0.041	0.024	Negative	-	-	-
8	PA HE	day 15 cycle 1	03 Mar 2008	0.041	0.019	Negative	-	-	-
8	PA HE	day 1 cycle 2	13 Mar 2008	0.041	0.054	Positive	Treatment emergent, Anti-Glucarpidase Positive	BLQ	< 62.5 ng/mL Treatment delayed by 1 week; cycle 2 begun on 19 Mar 2008
8	PA HE	day 1 cycle 2	19 Mar 2008	0.041	0.036	Negative	-	-	-
8	PA HE	day 8 cycle 2	27 Mar 2008	0.041	0.021	Negative	-	-	-
8	PA HE	day 15 cycle 2	06 Apr 2008	0.041	0.017	Negative	-	-	-
8	PA HE	day 90 cycle 2	27 Jun 2008	0.041	0.015	Negative	-	-	-
8	PA HE	day 180 cycle 2	10 Oct 2008	0.041	0.018	Negative	-	-	-
9	PE FR	baseline	12 May 2008	0.038	0.022	Negative	-	-	-
9	PE FR	day 8 cycle 1	19 May 2008	0.038	0.021	Negative	-	-	-
9	PE FR	day 15 cycle 1	27 May 2008	0.038	0.024	Negative	-	-	-
9	PE FR	day 8 cycle 2	09 Jun 2008	0.038	0.058	Positive	Not Anti-Glucarpidase Positive	-	-
9	PE FR	day 15 cycle 2	18 Jun 2008	0.038	0.087	Positive	Treatment emergent, Anti-Glucarpidase Positive	BLQ	< 62.5 ng/mL (Screening result from SB01, Semi-quantitative result from SB04)
9	PE FR	day 30 cycle 2	09 Jul 2008	0.038	0.033	Negative	-	-	-
9	PE FR	day 90 cycle 2	14 Oct 2008	0.038	0.020	Negative	-	-	-
10	CH WC	baseline	26 May 2008	0.038	0.027	Negative	-	-	-
10	CH WC	day 8 cycle 1	02 Jun 2008	0.038	0.026	Negative	-	-	-
10	CH WC	day 15 cycle 1	09 Jun 2008	0.038	0.026	Negative	-	-	-
10	CH WC	day 1 cycle 2	26 Jun 2008	0.038	0.031	Negative	-	-	-
10	CH WC	day 8 cycle 2	03 Jul 2008	0.038	0.029	Negative	-	-	-
10	CH WC	day 15 cycle 2	10 Jul 2008	0.038	0.025	Negative	-	-	-
10	CH WC	day 90 cycle 2	14 Oct 2008	0.038	0.027	Negative	-	-	-
11	JA SH	baseline	27 May 2008	0.038	0.022	Negative	-	-	-
11	JA SH	day 8 cycle 1	03 Jun 2008	0.038	0.021	Negative	-	-	-
11	JA SH	day 15 cycle 1	10 Jun 2008	0.038	0.020	Negative	-	-	-
11	JA SH	day 1 cycle 2	16 Jun 2008	0.038	0.024	Negative	-	-	-
11	JA SH	day 8 cycle 2	23 Jun 2008	0.038	0.022	Negative	-	-	-
11	JA SH	day 15 cycle 2	30 Jun 2008	0.038	0.018	Negative	-	-	-
11	JA SH	day 30 cycle 2	21 Jul 2008	0.038	0.020	Negative	-	-	-
11	JA SH	day 90 cycle 2	23 Sep 2008	0.038	0.020	Negative	-	-	-

Table continues

{Click to return to Section 4.6}

Table 8 continued

Subject	Subject	Nominal	Sample Date	Plate Cut	Sample	Screening	Confirmatory	Concentration	Comments
12	SA PH	baseline	07 Aug 2008	0.038	0.021	Negative	-	-	-
12	SA PH	day 8 cycle 1	14 Aug 2008	0.038	0.128	Positive	Not Anti-Glucarpidase Positive	-	-
12	SA PH	day 15 cycle 1	21 Aug 2008	0.038	0.431	Positive	Not Anti-Glucarpidase Positive	-	-
12	SA PH	day 30 cycle 2	17 Sep 2008	0.038	0.036	Negative	-	-	-
13	TR GO	baseline	02 Sep 2008	0.031	0.013	Negative	-	-	-
13	TR GO	baseline	02 Sep 2008	0.031	0.017	Negative	-	-	-
13	TR GO	baseline	02 Sep 2008	0.031	0.015	Negative	-	-	-
15	SE TI	day 1 cycle 1	26/01/2009	0.023	0.021	Negative	-	-	-
15	SE TI	day 1 cycle 2	16/02/2009	0.023	0.058	Positive	Not Anti-Glucarpidase Positive	-	-
15	SE TI	day 8 cycle 1	02/02/2009	0.023	0.046	Positive	Not Anti-Glucarpidase Positive	-	-
15	SE TI	day 8 cycle 2	23/02/2009	0.023	0.052	Positive	Not Anti-Glucarpidase Positive	-	-
15	SE TI	day 15 cycle 1	09/02/2009	0.023	0.050	Positive	Not Anti-Glucarpidase Positive	-	-
15	SE TI	day 15 cycle 2	03/03/2009	0.023	0.029	Positive	Treatment emergent, Anti-Glucarpidase Positive	BLQ	< 62.5 ng/mL
15	SE TI	Follow up	25/03/2009	0.023	0.027	Positive	Treatment emergent, Anti-Glucarpidase Positive	BLQ	< 62.5 ng/mL
16	DA OA	day 1 cycle 1	27/01/2009	0.023	0.020	Negative	-	-	-
16	DA OA	day 1 cycle 2	17/02/2009	0.023	0.017	Negative	-	-	-
16	DA OA	day 8 cycle 1	04/02/2009	0.023	0.020	Negative	-	-	-
16	DA OA	day 8 cycle 2	24/02/2009	0.023	0.027	Positive	Treatment emergent, Anti-Glucarpidase Positive	BLQ	< 62.5 ng/mL
16	DA OA	day 15 cycle 1	10/02/2009	0.023	0.034	Positive	Not Anti-Glucarpidase Positive	-	-
16	DA OA	day 15 cycle 2	23/03/2009	0.023	0.049	Positive	Treatment emergent, Anti-Glucarpidase Positive	BLQ	< 62.5 ng/mL

{Click to return to Section 4.6}

Table 9

Determination of Anti-Glucarpidase Antibodies by ELISA in Human Serum Samples From 'A Phase II Study, To Investigate The Efficacy And Safety Of Glucarpidase For Routine Use After High Dose Methotrexate In Patients With Bone Sarcoma' BRD/06/085 (PR001-CLN-009)

Assessment of Immunodepletion of Positive Samples

Batch	Sample ID	Well Replicate	Unspiked Sample	Voemaze Spiked Sample	Results		
			OD Value	OD Value	% Inhibition	t-test	Final Result
SB05	IFOR 28/02/08	1	0.036	0.030	14.7	0.946	Not Anti-Glucarpidase Positive
		2	0.038	0.023			
		3	0.037	0.039			
		4	0.032	0.022			
		5	0.036	0.069 x1			
		6	0.031	0.024			
SB05	IFOR 03/03/08	1	0.026	0.018	25.5	0.245	Not Anti-Glucarpidase Positive
		2	0.029	0.020			
		3	0.023	0.024			
		4	0.024	0.021			
		5	0.147 x1	0.018			
		6	0.040	0.026			
SB05	IFOR 17/03/08	1	0.452	0.419	28.5	0.0488	Anti-Glucarpidase Positive
		2	0.363	0.418			
		3	0.485	0.331			
		4	0.452	0.294			
		5	0.614	0.352			
		6	0.616	0.319			
SB05	IFOR 27/03/08	1	0.128	0.143	6.0	0.344	Not Anti-Glucarpidase Positive
		2	0.148	0.113			
		3	0.139	0.117			
		4	0.142	0.123			
		5	0.142	0.156			
		6	0.161	0.156			
SB05	IFOR 31/03/08	1	0.129	0.093	32.6	0.001	Anti-Glucarpidase Positive
		2	0.134	0.102			
		3	0.151	0.077			
		4	0.129	0.090			
		5	0.145	0.095			
		6	0.113	0.083			
SB05	IFOR 08/04/08	1	0.106	0.085	32.1	0.041	Anti-Glucarpidase Positive
		2	0.082	0.073			
		3	0.099	0.080			
		4	0.154	0.079			
		5	0.139	0.132 x1			
		6	0.127	0.083			

Table continues

{Click to return to Section 4.6}

Table 9 Continued

Batch	Sample ID	Well Replicate	Unspiked Sample	Veterinary Spiked Sample	Results		
			OD Value	OD Value	% Inhibition	t-test	Final Result
SB06	PEFR 09/06/08	1	0.029	0.025	6.0	0.197	Not Anti-Glucarpidase Positive
		2	0.026	0.023			
		3	0.029	0.026			
		4	0.026	0.024			
		5	0.047	0.026			
		6	0.023	0.026			
SB06	PEFR 18/06/08	1	0.034	0.026	27.2	0.010	Anti-Glucarpidase Positive
		2	0.036	0.029			
		3	0.030	0.024			
		4	0.033	0.028			
		5	0.046	0.025			
		6	0.034	0.023			
SB06	SAPH 14/08/08	1	0.044	0.044	28.9	0.052	Not Anti-Glucarpidase Positive
		2	0.046	0.042			
		3	0.047	0.035			
		4	0.045	0.034			
		5	0.071	0.031			
		6	0.062	0.038			
SB06	SAPH 21/08/08	1	0.159	0.137	11.9	0.192	Not Anti-Glucarpidase Positive
		2	0.150	0.125			
		3	0.121	0.102			
		4	0.163	0.112			
		5	0.105	0.138			
		6	0.151	0.134			
SB06	NIFI 25/10/07	1	0.039	0.038	-1.8	0.632	Not Anti-Glucarpidase Positive
		2	0.035	0.036			
		3	0.038	0.037			
		4	0.034	0.041			
		5	0.039	0.038			
		6	0.037	0.036			
SB06	ANME 17/09/07	1	0.024	0.026	-6.0	0.107	Not Anti-Glucarpidase Positive
		2	0.022	0.025			
		3	0.025	0.027			
		4	0.024	0.027			
		5	0.029	0.027			
		6	0.026	0.027			

Table continues

{Click to return to Section 4.6}

Table 9 Continued

Batch	Sample ID	Well Replicate	Unspiked Sample	Veonuate Spiked Sample	Results		
			OD Value	OD Value	% Inhibition	t-test	Final Result
SB07	PAHE 13/03/08	1	0.045	0.035	20.9	0.003	Anti-Glucarpidase Positive
		2	0.037	0.032			
		3	0.047	0.036			
		4	0.041	0.030			
		5	0.047	0.034			
		6	0.037	0.034			
SB07	ANME 11/03/08 1 in 5	1	1.334	0.037	96.9	0.0000002	Anti-Glucarpidase Positive
		2	1.349	0.049			
		3	1.238	0.036			
		4	1.200	0.050			
		5	1.398	0.034			
		6	1.365	0.040			
SB07	Subject 015 Day 1 Cycle 2	1	0.040	0.063	-15.5	0.269	Not Anti-Glucarpidase Positive
		2	0.044	0.061			
		3	0.040	0.056			
		4	0.042	0.056			
		5	0.070	0.062			
		6	0.067	0.052			
SB07	Subject 015 Day 8 Cycle 1	1	0.069	0.044	21.0	0.070	Not Anti-Glucarpidase Positive
		2	0.043	0.042			
		3	0.053	0.030			
		4	0.046	0.037			
		5	0.047	0.040			
		6	0.042	0.044			
SB07	Subject 015 Day 8 Cycle 2	1	0.045	0.049	6.2	0.342	Not Anti-Glucarpidase Positive
		2	0.040	0.040			
		3	0.048	0.037			
		4	0.040	0.043			
		5	0.043	0.034			
		6	0.044	0.041			
SB07	Subject 015 Day 15 Cycle 1	1	0.044	0.040	7.7	0.440	Not Anti-Glucarpidase Positive
		2	0.060 x1	0.039			
		3	0.040	0.039			
		4	0.039	0.053 x1			
		5	0.042	0.037			
		6	0.043	0.037			

Table continues

{Click to return to Section 4.6}

Table 9 Continued

Batch	Sample ID	Well Replicate	Unspiked Sample	Veronate Spiked Sample	Results		
			OD Value	OD Value	% Inhibition	t-test	Final Result
SB08	Subject 015 Day 15 Cycle2	1	0.087	0.061	23.9	0.003	Anti-Glucarpidase Positive
		2	0.069	0.046			
		3	0.071	0.066			
		4	0.066	0.053			
		5	0.073	0.055			
		6	0.060	0.043			
SB08	Subject 015 Follow Up	1	0.033	0.026	31.3	0.019	Anti-Glucarpidase Positive
		2	0.035	0.024			
		3	0.031	0.025			
		4	0.030	0.025			
		5	0.052	0.036			
		6	0.052	0.024			
SB08	Subject 016 Day 8 Cycle2	1	0.016	0.015	22.3	0.020	Anti-Glucarpidase Positive
		2	0.015	0.014			
		3	0.016	0.012			
		4	0.016	0.013			
		5	0.020	0.012			
		6	0.020	0.014			
SB08	Subject 016 Day 15 Cycle1	1	0.013	0.015	-4.1	0.435	Not Anti-Glucarpidase Positive
		2	0.014	0.015			
		3	0.013	0.013			
		4	0.014	0.014			
		5	0.014	0.015			
		6	0.015	-0.016 xl			
SB08	Subject 016 Day 15 Cycle 2	1	0.046	0.022	43.4	0.000	Anti-Glucarpidase Positive
		2	0.039	0.023			
		3	0.038	0.024			
		4	0.041	0.024			
		5	0.053	0.028			
		6	0.041	0.025			

Footnotes:
xl = result removed by Dixon's Q test at 95% confidence limit

{Click to return to Section 4.6}

APPENDIX 1

Batch Summary

Table A1.1

Determination of Anti-Glucarpidase Antibodies by ELISA in Human Serum Samples From ' A Phase II Study, To Investigate The Efficacy And Safety Of Glucarpidase For Routine Use After High Dose Methotrexate In Patients With Bone Sarcoma ' BRD.06-085 (PR001-CLN-pro009)

Batch Summary

Preparation Date (dd mmm yyyy)	Batch Number	Analyst(s)	Batch Contents	Pass/Fail	Reason for Failure/Comments
11 Apr 2011	TB01	HW	Training batch	Pass	NA
12 Apr 2011	TB02	TME	Training batch	Pass	NA
09 Aug 2011	TB03	TME	Training batch	Pass	NA
25 Aug 2011	TB04	HW	Training batch	Pass	NA
03 May 2011	TB05	TME	Preparation and Test of QCs	Fail	Over recovery of QCs
05 May 2011	TB06	CL / HW	Preparation and Test of QCs	Pass	NA
09 May 2011	SB01	TME	Screening of S11-088 subjects 11, 10, 9, 12	Pass / fail	Screening passes, Semi-quantitative fails due to QCs over recovery failed
10 May 2011	SB05	DL	Screening of S11-088 subjects 6, 7, 8	Pass	NA

Table continues

Table A1.1 Continued

Preparation Date (dd mmm yyyy)	Batch Number	Analyst(s)	Batch Contents	Pass/Fail	Reason for Failure/Comments
12 May 2011	SB03	TME	Screening of S11-088 subjects 4, 3, 2, 1, 13	Pass	NA
14 Sep 2011	SB04	DL	Screening of S11-291 subjects 15 & 16	Pass	NA
15 Sep 2011	TB07	DL	Preparation and Test of QCs	Pass	NA
16 Sep 2011	TB08	SW	Training batch	Pass	NA
19 Sep 2011	SB05	DL	Confirmatory samples	Pass	NA
20 Sep 2011	TB09	SW	Training batch	Pass	NA
20 Sep 2011	SB06	DL	Confirmatory samples	Fail	Cals + QCs failed
21 Sep 2011	TB10	SW	Training batch	Pass	NA
21 Sep 2011	TB11	DL	Training batch	Pass	NA
21 Sep 2011	SB07	DL	Confirmatory samples	Pass	NA
26 Sep 2011	SB08	TME	Confirmatory samples	Pass	NA

APPENDIX 2

Sample Re-Analysis Summary

Table A2.1
Determination of Anti-Glucarpidase Antibodies by ELISA in Human Serum Samples From 'A Phase II Study, To Investigate The Efficacy And Safety Of Glucarpidase For Routine Use After High Dose Methotrexate In Patients With Bone Sarcoma' BRD/06/085 (PR001-CLN-pro009)

Sample Re-Analysis Summary

Sample ID	Batch Number	Reason for Repeat	Re-analysis Batch Number	Result on Repeat (Pass/Fail)
Subject 9, day 15 cycle 2	SB01	Quantitative QCs failed	SB04	Pass
Subject 2, day 180 cycle 2	SB03	Dilution Repeat	SB04	Pass

APPENDIX 3

Qualification of QC Samples

Table A3.1

Determination of Anti-Glucarpidase Antibodies by ELISA in Human Serum Samples From 'A Phase II Study, To Investigate The Efficacy And Safety Of Glucarpidase For Routine Use After High Dose Methotrexate In Patients With Bone Sarcoma' BRD/06-085 (PR001-CLN-pro009)

Inter-assay Precision and Accuracy of Quality Control Samples
QCs prepared and tested in 10/056-010 TB06

Batch	Replicate	LoQC		MeQC		HiQC	
		100.0	%RE	400.0	%RE	750.0	%RE
TB06	1	105.9	5.9	453.3	13.3	893.6	19.1
	2	113.8	13.8	451.8	12.9	980.0 x1	30.7
	3	121.9	21.9	478.3	19.6	924.2	23.2
Mean (ng/mL)		109.9	-	452.6	-	936.8	-
Standard deviation (n-1)		5.59	-	1.06	-	61.09	-
Precision (%)		5.1	-	0.2	-	6.5	-
RE (%)		9.9	-	13.1	-	24.9	-
n		2	-	2	-	2	-

Footnotes:
x1 = Accuracy (%RE) >30%

Table A3.2

Determination of Anti-Glucarpidase Antibodies by ELISA in Human Serum Samples From 'A Phase II Study, To Investigate The Efficacy And Safety Of Glucarpidase For Routine Use After High Dose Methotrexate In Patients With Bone Sarcoma' BRD/06-085 (PR001-CLN-pro009)

Inter-assay Precision and Accuracy of Quality Control Samples
QCs prepared and tested in 10/056-010 TB07

Batch	Replicate	LoQC		MeQC		HiQC	
		100.0	%RE	400.0	%RE	750.0	%RE
TB07	1	92.0	-8.0	355.2	-11.2	649.7	-13.4
	2	93.8	-6.2	359.8	-10.1	683.5	-8.9
	3	93.5	-6.5	351.8	-12.0	632.2	-15.7
	4	101.3	1.3	365.5	-8.6	644.0	-14.1
	5	95.5	-4.5	351.5	-12.1	811.6 [x1]	8.2
	6	99.7	-0.3	357.6	-10.6	632.3	-15.7
Mean (ng/mL)		96.0	-	356.9	-	675.6	-
Standard deviation (n-1)		3.72	-	5.31	-	69.26	-
Precision (%)		3.9	-	1.5	-	10.3	-
RE (%)		-4.0	-	-10.8	-	-9.9	-
n		6	-	6	-	6	-

Footnotes:
x1 = Imprecision between replicates (%CV) >20%

APPENDIX 4

Cut-Point Normalisation

During validation of the assay a cut-point normalisation factor was generated from three independent repeat analyses of a group of 50 normal control sera (NCS). The normalisation factor was calculated by dividing the individual plate cut-points by the mean value for all replicates of the NCS analysed on that plate. The average of these individual normalisation factors was calculated as 1.51 [4].

In the current study a minimum of 2 replicates (4 wells) of the same pooled normal control serum were analysed on each assay plate. A normalised cut-point was then calculated for each plate by multiplying the mean NCS result by the normalisation factor, the results are shown in Table A4.1, and summarised below.

The value of the normalised cut-point was compared to the individual plate cut-points derived from the pre-treatment or control sera (where applicable).

Batch	Plate Cut-Point (Positives)	Normalised Cut-Point (Positives)
SB01	0.038 (4)	0.058 (4)
SB02	0.041 (7)	0.039 (9)
SB03	0.031 (3)	0.039 (2)
SB04	0.023 (9)	0.050 (3)

In the screening and confirmatory assay, all replicates of the Lo QC samples were above the plate and normalised cut-points. Relative to the calibration standards in the screening batches the normalised cut-points were comparable with the values obtained for the lowest calibrator (15.6 ng/mL) meaning that both replicates in SB01 and SB04 fell below the normalised cut point, whilst in SB02 and SB03 both were above.

The results for the calculation of a normalised assay cut-point demonstrate the suitability of using this to set the assay sensitivity.

There are several advantages of applying a normalised cut-point to future analyses. There is an increased level of consistency in setting the threshold for determining which samples are positive as the determination is based on the performance of the

same NCS sample in all batches and it stems from a larger body of data; 50 samples rather than 10, and takes into account assay variability; 3 replicate analyses rather than a single analysis. Basing the assay cut-point on the performance of the NCS also allows for the calculation of a cut-point where ten individuals are not analysed, such as confirmatory assays.

Table A4.1

Determination of Anti-Glucarpidase Antibodies by ELISA in Human Serum Samples From 'A Phase II Study, To Investigate The Efficacy And Safety Of Glucarpidase For Routine Use After High Dose Methotrexate In Patients With Bone Sarcoma' BRD/06-085 (PR001-CLN-pro009)

Assessment of Plate Cut-Point vs Normalised Cut-Point

Batch (Study)	Replicate	Well OD	Mean NCS OD	%CV	Cut Point	
					Plate	Normalised[x]
SB01	1	0.041	0.040	3.5		
		0.039				
	2	0.038	0.037	3.8		
		0.036				
			0.039	**5.4**	0.038	**0.058**
SB02	1	0.025	0.026	2.8		
		0.026				
	2	0.026	0.026	0.0		
		0.026				
			0.026	**1.9**	0.041	**0.039**
SB03	1	0.034	0.034	2.1		
		0.033				
	2	0.033	0.033	0.0		
		0.033				
			0.033	**1.5**	0.031	**0.050**
SB04	1	0.018	0.018	4.0		
		0.017				
	2	0.018	0.018	0.0		
		0.018				
			0.018	**2.8**	0.023	**0.027**
SB05	1	0.038	0.039	1.8		
		0.039				
	2	0.040	0.042	6.7		
		0.044				
			0.040	**6.5**	**NA**	**0.061**
SB06	1	0.038	0.039	1.8		
		0.039				
	2	0.039	0.039	0.0		
		0.039				
			0.039	**1.3**	**NA**	**0.059**
SB07	1	0.038	0.038	0.0		
		0.038				
	2	0.060	0.052	23.3		

		0.043				
			0.045	**23.3**	**NA**	**0.068**
SB08	1	0.043	0.041	8.7		
		0.038				
	2	0.051	0.045	20.7		
		0.038				
			0.043	**14.4**	**NA**	**0.064**

Mean		0.035				0.049
Stdev		0.0096				0.0135
Precision (%CV)		27.2				27.6
n		32				8

Footnote:
x1 = Normalised cut point calculated as 1.51 x mean NCS OD
NA = Not applicable

ANNEX

The annex consists of 45 pages, including this one, and contains:

- Analytical Plan (23 pages)
- Analytical Procedure BALAP 08/021 version (07) (21 pages)

Analytical Plan
Study Number: 10/056-010

Millipore BioPharma Services Study Title:	Determination of Anti-Glucarpidase Antibodies by ELISA in Human Serum Samples From 'A Randomised, Cross-Over, Phase II Study, To Investigate The Efficacy And Safety Of Glucarpidase For Routine Use After High Dose Methotrexate In Patients With Bone Sarcoma'.
Sponsor:	Joint UCLH/UCL Biomedical Research Unit (JBRU) University College London 1st Floor Maple House 149 Tottenham Court Road London W1T 7NF
Clinical Study Reference:	BRD/06/085
EudraCT Number:	2006-003203-40
Analysis Sponsor:	Protherics Medicines Development Ltd, A BTG Company
Analysis Sponsor Study Reference:	PR001-CLN-009

ANALYTICAL PLAN APPROVAL

Dr Jeremy Whelan Chief Investigator University College Hospital, Department of Oncology	17/03/2011 Date
D Shaw Analytical Project Manager Millipore BioPharma Services	02 Mar 2011. Date

ANALYTICAL PLAN QA REVIEW

P Harrison
Quality Manager
Millipore BioPharma Services

02 Mar 2011
Date

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Study Schedule:

Laboratory phase start date:	Feb 2011
Laboratory phase completion date:	Feb 2012
Final Draft report date:	30 Mar 2012

Dates quoted are for internal scheduling purposes only. No deviation procedure required if dates are not adhered to.

LIST OF ABBREVIATIONS

BAL-	Millipore equipment or SOP ID number
BALAP	Analytical procedure
CNS	Central nervous system
CTC	Cancer Trials Centre
%CV	Coefficient of variation (precision)
DVD	Digital versatile disc
ELISA	Enzyme-linked immunosorbent assay
GCP	Good clinical practice
Hi QC	High quality control
ICH	International Conference on Harmonisation
ID	Identification
Lo QC	Low quality control
Me QC	Medium quality control
MHRA	Medicines and Healthcare Products Regulatory Agency
OD	Optical density
PDF	Portable document format
PMDL	Protherics Medicines Development Ltd.
QA	Quality assurance
QC	Quality control
%RE	Percent relative error
SOP	Standard operating procedure
SB	Sample batch
SV	Software validation
TB	Test batch
UCL	University College London

1. OBJECTIVE

The objective of this study is to determine the presence of anti-glucarpidase (Voraxase™) antibodies, using ELISA techniques, in serum samples generated in the Osteosarcoma Clinical Trial study EudraCT reference 2006-003203-40 (BTG reference PR001-CLN-009). All samples will be subject to a screen for the presence of anti-glucarpidase antibodies. This assay will include a calibration curve for quantification of any responses that are confirmed as true positives. All positive samples from the screening assay will be analysed in a confirmatory assay. The concentrations measured in the screening assay will be reported only for samples subsequently confirmed as true positives. As samples will be delivered and analysed on a patient by patient basis the screening and confirmatory assays may be combined in a single analysis.

Samples confirmed as true positive will also be sent to second contract laboratory for analysis in a neutralising antibody assay (contractor to be confirmed). Details of the identity of the contractor, and the procedure for transfer of the samples will be added by amendment.

Upon completion of the study a copy of the finalised neutralising antibody assay report will be supplied to Millipore BioPharma services for inclusion as an Annex to the study report.

2. SPONSOR RESPONSIBILITIES

In order to ensure that the requirements of GCP [1, 2, 3] are adhered to, it is the responsibility of the Sponsor to ensure that the clinical study protocol contains the following information:

- The name and address of Millipore BioPharma Services
- The identity of the testing being performed by Millipore BioPharma Services
- Patient consent for the testing required
- The identity of the study groups or cohorts and the nominal time points which require analysis

In order to ensure that any changes to the clinical protocol which could potentially affect the sample analysis detailed in the analytical plan are communicated to Millipore BioPharma Services, it is the responsibility of the Sponsor to provide Millipore BioPharma Services with copies of all amendments. Signed copies of the

clinical protocol and all amendments must be provided to Millipore BioPharma Services as soon as possible after issue.
For changes to the clinical protocol that impact on the analytical work performed by Millipore BioPharma Services, draft copies of the clinical protocol should be provided for review and comment prior to issue.

The Sponsor is responsible for informing Millipore BioPharma Services if patient consent is withdrawn.

The Sponsor is responsible for reporting all serious breaches of Good Clinical Practice regulations and guidelines to the MHRA. If a serious breach of GCP is discovered during testing, Millipore BioPharma Services will report the serious breach to the Sponsor within 48 hours of confirmation according to our procedure BAL-209. Millipore BioPharma Services will initiate an investigation into the serious breach and will update the Sponsor with the findings of the investigation and any corrective or preventive actions.

PMDL, a BTG company is responsible for selecting and approving the sub-contractor to perform the neutralising antibody assay. Millipore BioPharma Services has the responsibility to include a copy of neutralising antibody assay report as an appendix to the final study report, with no QC or QA of this report being performed by Millipore BioPharma Services.

3. REFERENCE STANDARD

Affinity purified rabbit anti-glucarpidase antibodies (the analyte), prepared by Covance Research Products (Denver PA, USA Study No. 2006-0074), will be provided by PMDL, a BTG company If available, certificates of analysis quoting the batch number, concentration and expiry date (where appropriate) will be obtained for each batch of anti-glucarpidase antibody supplied.

If the batch number of this antibody changes from that used in the validation of the assay (lot number PR070522A, RS08-113) [4], the new batch numbers will require qualification prior to use in the analysis of study samples.

4. REAGENTS

Voraxaze™ will be provided by PMDL, a BTG company. Documentation, if available, will be provided by the sponsor quoting the batch number, concentration, excipients and expiry/re-test date as appropriate.

Biotin-labelled Voraxaze™ was prepared by Millipore BioPharma Services as described in study 08/056-002 (RS08-149) [5]. Since the validation was limited to a single lot of biotinylated Voraxaze™, if the lot number of this reagent changes qualification of the new lot number must be performed to demonstrate equivalence in assay performance with the old lot prior to the use of the new lot in the analysis of clinical samples.

All other assay reagents will be supplied by Millipore BioPharma Services. These reagents are not considered critical for the performance of the assay and there is no requirement to assess change of lot numbers on the performance of the assay. The identity, specification and preferred source of all reagents are specified in the analytical procedure (see Section 7).

5. CONTROL MATRIX

Control normal human serum used in the preparation of calibration standards and quality control (QC) samples will be supplied by the Millipore BioPharma Services. The source and storage conditions at Millipore BioPharma Services will be documented in the raw data and the report.

6. STUDY SAMPLES

In accordance with the clinical protocol, the following nine nominal time points for samples from up to 38 patients will be analysed for all subjects:

Pre-Treatment
Baseline
Treatment Cycle 1
Day 8 & Day 15
Treatment Cycle 2
Day 1, Day 8 & Day 15
Follow Up (from Day 1 of Treatment Cycle 2)
Day 30, Day 90 (3 months) & Day 180 (6 months)

Patients are randomised to one of two arms in equal numbers. Arm A receive high dose Methotrexate with standard Folinic acid rescue in cycle 1, and then Folinic acid plus glucarpidase in cycle 2. Treatment order is reversed in Arm B.

The study was initiated in 2007 with the first patient being recruited in July of that year. The protocol has been amended several times; this analytical plan represents the

analytical requirements in the current version of the protocol (Version 5, issued 05 October 2010).

If any samples for additional time points or additional patients are received for analysis these should not be analysed until confirmation that the clinical protocol has been amended to include the additional time points and/or patients has been issued. Any additional samples requiring analysis should be documented in an amendment to this analytical plan.

If unscheduled analysis or evaluation is required for urgent clinical reasons, for example, as a result of adverse events, the Sponsor should notify Millipore BioPharma Services that such analysis or evaluation is required. Millipore BioPharma Services will document this as a study plan amendment, and will schedule the extra work as a priority.

Samples should be labeled with the following information as a minimum:

- Clinical Study Reference
- Patient ID code
- Nominal sample collection time point

The actual sample collection dates and times can be provided separately (e.g. copy of CRF or Excel spreadsheet).

On no account should details of confidential patient information such as name, date of birth, address, etc... be supplied to Millipore BioPharma Services. In the event that the samples, or associated documents, are supplied with this information, the samples will be coded and the information obliterated. A copy of the sample coding will be supplied to the study monitor. The study monitor will be notified and instructed that this information must not be provided in future sample shipments.

The Sponsor will be responsible for the samples until they are delivered to Millipore BioPharma Services, and will make appropriate arrangements with the clinical site(s) for sample shipment and temperature logging (if required). Within 48 to 24 hours prior to shipment of each set of samples the Sponsor will notify Millipore BioPharma Services of the shipment date, the courier, waybill number and expected arrival date. Samples should not be sent to arrive at a weekend except by prior agreement between the Sponsor and Millipore BioPharma Services.

Millipore BioPharma Services will inform the Sponsor within 24 hours of any sample shipments which are not received as expected.

Each sample shipment will include a statement of identity (or equivalent) specifying the number and identity of the individual samples. On arrival, Millipore BioPharma Services will check the condition of the samples and notify the Sponsor within 48 hours of delivery and of any damage or discrepancies between the sample labels and the inventory. Such discrepancies should be resolved before the analysis of the samples. If the samples are accompanied by a temperature logger, Millipore BioPharma Services will stop the logger and return it to the Sponsor by first class mail within 4 days of sample receipt unless arrangement has been made with the courier to stop and return the logger.

Upon receipt at Millipore BioPharma Services the samples will be stored at -70°C (nominal).

The exact number and identity of samples received, analysed and sent for analysis of neutralising antibodies will be documented in the final report.

Upon first analysis, the remaining volume of each sample will be divided into sub-aliquots. An aliquot of 0.5 ml volume will be prepared for use in confirmation of any positive responses; a second aliquot of 0.20 to 0.25 mL will be made for use in the neutralising antibody assay. The sub-aliquots will be stored at -70°C (nominal) together with any remaining volume of the original sample.

Upon confirmation of a positive antibody response the smaller aliquot together with any remaining sample volume will be sent for analysis of neutralising antibodies (details to be added by amendment).

7. SAMPLE ANALYSIS

The assay was previously validated at Millipore BioPharma Services in study number 08/056-003 [3]. The methodology for the assay will be documented in the following Analytical Procedure:

> BALAP 08/021 version: Detection of Antibodies to Voraxaze in Human Serum by Bridging ELISA

7.1 Screening & Quantitative Assay

Samples from the same subject (pre- and post-dose) will be analysed on the same plate. Once a sample has reached 3 freeze-thaws, the second aliquot should be used. If no second aliquot is available and the samples have exceeded 3 freeze-thaws, additional validation of the assay may be required.

For the purposes of quantification of antibody responses, a calibration curve will be included in the screening assay. Concentrations will be reported only for samples subsequently confirmed as true responses.

Pre-treatment "false" positive samples will be treated the same as other post treatment positive results and subjected to analysis in the confirmatory assay.

In the screening assay study samples will be divided into analysis batches consisting of a maximum of 33 samples exclusive of blanks and QC samples.

Clinical study samples will be analysed following a set of rabbit anti-glucarpidase antibody calibration standards and between two sets of a control human serum blank, Lo Me and Hi QC samples. All samples and QC samples will be analysed in duplicate.

The pre-dose serum samples from the clinical study, supplemented by commercially obtained normal human sera, will be used for the determination of a negative cut point value within each plate. The cut point will be calculated from up to 10 samples as the mean OD values+ 1.645 × their standard deviation. If any cut-off sample is identified as an outlier (by Grubbs test) it will be excluded from the cut point calculation. Any samples which are greater than or equal to the negative cut-off value will be scored as positive and will be subject to analysis in the confirmatory assay. Samples which give a value below the negative cut-off value will be scored as negative.

The results (+/-) will be determined relative to the plate cut point calculated from the pre-treatment samples. As part of the in-study validation of the method, the validity of the cut point normalisation factor generated during the validation study [4] will be assessed during sample analysis. The mean result for the 4 replicate negative control serum wells will be multiplied by the normalisation factor; results will be compared to the individual plate cut points. A cut point normalisation factor derived from the repeated analysis of

50 individual sera during validation will be used to calculate a normalised cut point for each assay plate. The value of the normalisation factor will be specified by study memo.

The validity of these different approaches to assessing the cut-off will be determined based on the results obtained. Depending on the results, alternative approaches to calculating the cut point may be investigated, any such approaches will be discussed and agreed with the Analytical Sponsor (PMDL) and documented by amendment to this plan.

7.2 Confirmatory Assay

Any sample which gives a positive result in the screening assay (i.e. above the cut-point) will be analysed in the confirmatory assay. Screening assay positive samples will be tested as follows:

Screening result	Confirmatory assay
Pre-dose -ve, Post-dose -ve	not required
Pre-dose +ve, Post-dose -ve	6 replicates pre-dose (unspiked) 6 replicates pre-dose (spiked*)
Pre-dose -ve, Post-dose +ve	6 replicates post-dose (unspiked) 6 replicates post-dose (spiked*)
Pre-dose +ve, Post-dose +ve	6 replicates pre-dose (unspiked) 6 replicates pre-dose (spiked*) 6 replicates post-dose (unspiked) 6 replicates post-dose (spiked*)

*Samples spiked with Voraxaze at 0.19 µg/mL (final concentration).

The principle of the confirmatory assay is as follows:

a) Determination of drug inhibition

Four replicates of the positive samples will be analysed with and without the addition of Voraxaze to a final concentration of 0.19 µg/mL. The responses will be compared by t-test. If the drug-spiked response is significantly ($p<0.05$) less than the unspiked response and the difference is reduced by greater than 20% (of the unspiked) it will be designated drug-specific (indicative of anti-glucarpidase antibodies).

b) Determination of treatment-emergent response
In cases where the pre-dose sample is negative and the post-dose sample is confirmed as drug-specific, the response will be defined as treatment emergent.

If the post-dose sample is positive in the screening assay but is found to be non-drug specific in the confirmatory assay, the sample will be reported as 'negative' and no statement regarding the treatment emergent status will be made since both results are negative.

Where both the pre- and post-dose samples are confirmed as drug-specific, a comparison between six replicates of pre-dose and post-dose samples will be analysed. The responses will be compared by t-test. If the post-treatment response is significantly greater ($p < 0.05$) than the pre-treatment response and the difference is greater than 20 % (of the pre-treatment), it will be designated treatment-emergent.

Samples with OD readings greater than 2 during screening should be diluted to approximately this level in the confirmatory assay.

7.3 Semi-Quantitative Assay

The semi-quantitative assay will be performed as part of the initial screening assay, rather than as a separate analysis. Samples which are subsequently confirmed as true positive will have their measured concentration (see section 7.1) reported as equivalent antibody mass. The apparent concentration in samples that are not confirmed will not be reported. Samples that are confirmed as true positives, but fall below the limit of quantification will be reported as "Anti-Glucarpidase Positive, concentration not determined".

8. CALIBRATION STANDARDS

The rabbit anti-glucarpidase antibody (see Section 3) will be the reference standard used to produce the calibration standards. Calibration standards will be spiked in control human serum on the day of analysis. The volume of the rabbit anti-glucarpidase antibody stock solutions used for the preparation of the serum calibration standards will not exceed 5% of the final working volume and will be constant in all calibration standards.

The calibration standards used will be as follows:

15.6 (1), 31.3 (2), 62.5 (3), 125 (4), 250 (5), 500 (6) and 1000 (7) ng/mL

The calibration standards at 15.6 ng/mL and 31.3 ng/mL are considered to be anchor points.

The curve fit used will be a weighted ($1/y^2$) 4-parameter logistic model. The curve fit used will be the same for all batches analysed.

9. QUALITY CONTROL SAMPLES

The rabbit anti-glucarpidase antibody (see Section 3) will be the reference standard used to produce the QC samples. The QC samples will be prepared in bulk in pooled control human serum (10HPS077, or equivalent pools) at the start of the sample analysis and stored at -70°C (nominal) for up to 34 days[1]. Should the opportunity arise during the course of the study, this period of stability will be extended by the re-analysis of expired QCs using spare plate capacity.

The volume of the rabbit anti-glucarpidase antibody stock solutions used for the preparation of the serum QC samples will not exceed 5% of the final working volume and will be constant in all QC samples.

The QC concentrations will be as follows:

- Lo QC: 100 ng/mL
- Me QC: 400 ng/mL
- Hi QC: 750 ng/mL

10. DATA COLLECTION AND PROCESSING

The data will be collected and processed using Softmax Pro software version 5.2. This software has been subject to formal validation at Millipore BioPharma Services Limited as documented in software validation report SV-019, issue date 16 May 2008. Controls and samples will be compared to the assay cut-point value for the plate that they were analysed on and assigned negative or positive status depending on whether

[1] This may be extended as appropriate, based on the results of an ongoing stability study (08/056-004) [6]

the mean measured OD was less than the cut-point or greater than or equal to the cut-point respectively.

The concentration of anti-glucarpidase antibody in calibration standards, QC samples, and confirmed positive samples will be determined using the parameters derived from a weighted ($1/y^2$) 4-parameter logistic curve fit of the mean (n = 2) absorbance values of the anti-glucarpidase antibody calibration standards (y-axis) plotted against the log anti-glucarpidase antibody concentration (x-axis).

11. ACCEPTANCE CRITERIA

11.1 Screening & Semi-Quantitative Assay Batch Acceptance Criteria

Each batch will include a calibration curve, 2 Lo QC, 2 Me QC, 2 Hi QC samples and 2 negative control sera. All of the QC samples must be positive when assessed against the cut-point value for the screening assay. Additionally, where antibody concentrations are to be reported 4 of the 6 QC replicates should be within 30% of their nominal concentration. Two QC samples may be outside of 30%, but these must be at different concentrations

The precision between duplicate OD values of the Lo QC samples should be ≤30%.

The precision between duplicate OD values of the Me QC and Hi QC samples should be ≤20%.

11.2 Screening & Semi-Quantitative Assay Sample Acceptance Criteria

The precision between duplicate ODs of positive samples with a mean measured OD of ≤0.250 should be ≤30%.

The precision between duplicate ODs of positive samples with a mean measured OD of >0.250 should be ≤20%.

For the calibration curve to be acceptable, the calibration standards should be within 25% of the nominal concentration (i.e. percent relative error (%RE) ≤ 25%). The accuracy of the calibration standards around the limit of quantification (i.e. 62.5 ng/mL calibration standards) should be ≤30%.

The curve may be edited by removing points which do not meet the above acceptance criteria. A maximum of 2 individual replicates or one complete calibration point could be removed. There is no requirement to remove points which do not meet the calibration curve acceptance criteria if the overall curve fit is considered acceptable.

Any sample with a percentage difference between duplicates of greater than 30% and with one replicate below and one above the assay negative cut-off value should be treated as a potential positive sample and analysed in the confirmatory assay.

Negative samples have no precision acceptance criteria so long as both replicate OD values for that sample are less than the assay negative cut-off value.

11.2 Confirmatory Assay Batch Acceptance Criteria

Each confirmatory assay plate will include a Lo QC sample which will be analysed with and without Voraxaze™ at 0.19 µg/mL as described in Section 7.2. The batch will be considered acceptable if the Lo QC sample is confirmed as a true positive.

11.2.2 Confirmatory Assay Sample Acceptance Criteria

The precision between the six replicates OD values of the spiked and un-spiked samples should be ≤30%.

12. REPEAT ANALYSIS

Samples may only be re-analysed in each assay for analytical reasons; there is no requirement for the analysis of incurred samples in this study. A summary of all samples which are re-analysed will be maintained in the raw data and will be summarised in the final report.

Examples of analytical reasons for the repeat analysis of samples are instrument malfunction, unacceptable replicates, and batch rejected due to failure to meet acceptance criteria.

For all of the above reasons the samples will initially be re-analysed once. The original value will be ignored and the raw data annotated to state why the sample has been selected for re-analysis.

In the confirmatory assay in samples which give a low absorbance signals (as expected for false positive samples) samples may fail to meet the acceptance criteria on precision (percentage difference) between replicates. Samples which fail to meet this acceptance criteria will be analysed a maximum of three times in the confirmatory assay. If no acceptable result is determined after three attempts, the sample will be reported as false positive.

Samples that are confirmed as treatment emergent, true positive responses shall be returned to Covance Laboratories on dry ice, to be analysed for the presence of neutralising antibodies. As described in section 6, the remaining volume in the original aliquot (~225 µL) plus both sub-aliquots used in the confirmation and quantification assays shall be returned.

13. DATA REPORTING

13.1 Expedited Reporting

Since the study samples were collected over a period of time spanning the duration of treatment and the analysis is being performed retrospectively, the results obtained are not expected to indicate any issues which could be indicative of an immediate risk to patient safety and thus there is no requirement for expedited reporting.

13.3 Interim Data Reporting

Interim 100% QC checked results for each subject in Excel Spreadsheet format will be sent electronically via email to the Study Monitor before the subject's scheduled 3 month follow up clinic visit.

The interim data will be as indicated in section 13.4.

13.3 Sample Analysis Report

A study report will be prepared to include, but is not to, the following information:

- The name and address of the analytical laboratory

- The signature of the Analytical Project Manager involved in the study as authentication of the report
- The signature of the QA auditor as verification of compliance with the analytical plan, analytical procedure and SOP requirements
- The signature of the scientific reviewer as confirmation of accuracy of the study data and any conclusions drawn
- The signature(s) of the Sponsor as confirmation of acceptance of the final report
- The dates on which the study was initiated and completed
- The names and roles of all Millipore BioPharma Services staff involved in the study
- A summary of the sample analysis
- The objective as stated in this Analytical Plan
- Any deviations from the analytical plan, the Analytical Procedure and Millipore BioPharma Services SOPs
- The identity of the reference materials used in the preparation of calibration standards and QC samples. Information on batch number, concentration, expiry date and storage conditions will be included as appropriate.
- The identity of the control matrices used. Information on batch number, source, expiry date and storage conditions will be included as appropriate.
- The identity of the critical assay reagents. Information on batch number, concentration, expiry date and storage conditions will be included as appropriate.

- The data listed in data presentation (see Section 13.4)
- A discussion of the study data and any anomalous results
- A summary of all sample analysis and test batches performed as an appendix
- This analytical plan, and any amendments, as an annex
- A copy of the Analytical Procedure(s) used in the analysis of the samples as an annex
- A copy of the bioanalytical report for the neutralising antibody assay supplied by Covance Laboratories.

Prior to issuing the final report, the Sponsor and Analysis Sponsor will each be issued with a draft report (1 copy) for approval. If either the Sponsor or Analysis Sponsor has any comments for inclusion in the final report, a single round of collated comments from all reviewers (at each site) should be supplied to Millipore BioPharma Services within one month of issue of the draft report. Additional rounds of review may incur further financial charges. If no comments are received within one month the report will be issued as final. Changes made after issue of the report will be addressed as an amendment to the final report, and will incur further financial charges.

The Sponsor and Analysis Sponsor will each be issued with 1 bound copy of the final report. Each will also be provided with an electronic copy of the report in pdf format on writable DVD, as well as the corresponding Excel datasets. The pdf copy of the report will be hyperlinked and not password protected.

One unbound copy and one electronic copy (in pdf format on writable DVD) of the final report will be archived with the study data. One bound copy of the final report will be retained at Millipore BioPharma Services indefinitely. All electronic data, including the final report, will be archived with the study data and will also be retained at Millipore BioPharma Services indefinitely.

13.4 Data Presentation

The reported data will be presented as follows:

- Measured mean OD values to three decimal places
- Negative cut-off value for each plate to three decimal places
- Back calculated concentrations (ng/mL rabbit anti-glucarpidase antibody equivalent) of each calibration standard to 1 decimal place
- Screening assay results of study samples and QC sample as 'Positive' or 'Negative'.
- Sample results from confirmatory assay as 'Treatment Emergent' or 'Not Treatment Emergent'
- Sample results from confirmatory assay as 'Anti-Glucarpidase Positive' or 'Not Anti-Glucarpidase'
- Confirmatory assay t-statistics to three decimal places
- Back calculated concentrations (ng/mL rabbit anti-glucarpidase antibody equivalent) of each calibration standard to 1 decimal place
- Measured concentrations of QC samples analysed in the semi-quantitative assay to one decimal place
- Measured concentrations of confirmed positive study sample analysed in the quantitative assay to one decimal place
- Where the measured concentrations of confirmed positive study sample fall below the range of the assay, results will be reported as "Anti-Glucarpidase Positive, Concentration Not Determined"

Where appropriate, sample dates will be reported as dd mmm yyyy and sample times will be reported in 24 hour clock format (i.e. hh:mm).

14. REGULATORY REQUIREMENTS

14.1 Regulatory Guidelines

The study will be conducted in accordance with the principles of Good Clinical Practice [1, 2, 3] and Good Clinical Laboratory Practice [7].

The procedures will be performed in accordance with principles of Good Laboratory Practice [8, 9].

14.2 Standard Operating Procedures (SOPs)

All procedures directly and indirectly involved in the analysis of the study samples are the subject of detailed Millipore BioPharma Services SOPs.

14.3 Amendments and Deviations

Any planned changes to the Analytical Plan will be detailed in an Analytical Plan Amendment issued by Millipore BioPharma Services and approved by the Sponsor.

Any unplanned deviations from the Analytical Plan will be documented by Study Memo which will be approved by the Analytical Project Manager. Any deviations from Millipore BioPharma Services SOPs and the Analytical Procedure will also be documented by Study Memo. All major or critical deviations as defined in Millipore BioPharma Services SOP BAL-026, will be reported within 3 working days of confirmation.

The risk of all deviations on the integrity of the study data will be documented in the Study Memos.

14.4 Quality Assurance (QA) Evaluation

The raw data, supporting data and the report will be subject to QA evaluation (audit) as verification for compliance with the Analytical Plan, Analytical Procedure and SOPs.

The neutralising antibody bioanalytical report will not be subject to QA audit at Millipore BioPharma Services. It is the responsibility of nominated contract laboratory to ensure QA audit of this report.

14.5 Inspection by Regulatory Authorities

In the event of inspection by a regulatory authority, the Sponsor will be consulted before the authority is granted access to any of the study data unless required by law or regulation.

15. DATA ARCHIVE AND SAMPLE RETENTION

All study specific data generated will be archived for a period of 3 years from the date of issue of the final report at Millipore BioPharma Services. If longer term storage is required after the initial archive period, this may be sub-contracted to a Millipore BioPharma Services approved supplier. No movement of data will be performed without consent of the Sponsor.

Samples and materials resulting from this study will be retained by Millipore BioPharma Services, at the appropriate storage temperature, for a period of one month from the date of issue of the final report.

After this time the Sponsor will be contacted to determine the fate of the study data and samples. Sponsor will arrange for transfer of samples to a long-term storage facility. Should the archive periods require extension further charges will be made based on the quantity of data and samples and the period of extension.

16. REFERENCES

[1] Statutory Instrument No. 2004/1031. The Medicines for Human Use (Clinical Trials) 2004 as amended by Statutory Instruments No.1928 and 2984, 2006, and No. 941, 2008 and No.1164, 2009.

[2] ICH Topic E6: Guideline for Good Clinical Practice. Note for Guidance on Good Clinical Practice (CPMP/ICH/135/95). January 1997.

[3] MHRA, Good Clinical Practice: Guidance on the maintenance of regulatory compliance in laboratories that perform the analysis or evaluation of clinical trial samples. Issue 1, July 2009

[4] BioAnaLab Study 08/056-003: Pre-Study Validation of an Analytical Procedure to Detect Antibodies to Voraxaze™ in Human Serum by Bridging ELISA

[5] BioAnaLab study 08/056-002: Preparation and Quality Control of Biotin-labelled Voraxaze™

[6] Millipore BioPharma Services study 08/056-004 (PMDL ref No PR001-CLN-pro BA041): Assessment of the Stability of Critical Assay Reagents and Anti-Glucarpidase Antibodies in Human and Cynomolgus Monkey Sera at -70°C.

[7] BARQA (2003). Good Clinical Laboratory Practice (GCLP): A Quality System for Laboratories that Undertake the Analyses of Samples from Clinical Trials. ISBN 1-904610-00-5.

[8] Statutory Instrument No.3106 1999. The Good Laboratory Practice Regulations 1999

[9] Statutory Instrument 2004 No.994. The Good Laboratory Practice Regulations (Codification Amendments Etc) 2004

ANALYTICAL PROCEDURE

TITLE: Detection of Antibodies to Voraxaze in Human Serum by Bridging ELISA	
ANALYTICAL PROCEDURE NUMBER: BALAP 08/021	VERSION NUMBER: (07)

AUTHOR APPROVAL:	DATE:
REVIEWER APPROVAL:	DATE:
QA REVIEW:	DATE:

COPY APPROVAL	
Study Number: 10/056-010	
Approved by: David Shaw	Date: 25 Nov 2011.

1. OBJECTIVE

The objective of this analytical procedure is to detect anti-glucarpidase (Voraxaze) antibodies in human serum samples using a bridging ELISA.

2. PRINCIPLE

Voraxaze is adsorbed onto microtitre plates at a concentration of 0.5 µg/mL in PBS (100 µL/well) overnight at 4°C (nominal). Following blocking (2 hours, 2% w/v BSA in PBS, 200 µL/well) the plates are washed (four times, 0.05% v/v Tween 20 in PBS, 300 µL/well). Human serum samples, anti-glucarpidase standards and blanks are diluted 1 in 2 with 2% w/v BSA in PBS prior to adding to the plates (100 µL/well). The plates are incubated at 25°C (nominal) for 2 hours with gentle shaking. The plates are washed (four times, 0.05% v/v Tween 20 in PBS, 300 µL/well) and biotin-labelled Voraxaze is added (0.5 µg/mL, 100 µL/well). The plates are incubated for 2 hours at 25°C (nominal) with gentle shaking and are then washed (four times, 0.05% v/v Tween 20 in PBS, 300 µL/well). Extravidin peroxidase reagent (1:10,000 dilution, 100 µL/well) is added and the plates are incubated for 1 hour at 25°C (nominal). Following washing (four times, 0.05% v/v Tween 20 in PBS, 300 µL/well), TMB substrate is added (100 µL/well) and the plates incubated for 15 minutes at 25°C (nominal) with gentle shaking. The reaction is stopped by the addition of sulphuric acid (0.5 M, 100 µL/well) and the absorbance is read at 450 nm with background correction at 540 nm.

3. REFERENCES

Cobbold, S.P., Rebello, P.R.U.B., Davies, H.F.S., Friend, P.J., & Clark, M.R. (1990). A simple method for measuring patient anti-globulin responses against isotypic or idiotypic determinants. J. Immunol. Meth., **127**, 19-24.

BioAnaLab study 08/056-001: Development of a Screening ELISA to Detect Antibodies against Voraxaze™ in Human Serum

4. MATERIALS AND EQUIPMENT

The names and addresses of the suppliers are listed in Appendix 2. If the specified materials or equipment are not available at the time of analysis, equivalent materials and equipment can be used. In the case where alternatives to the materials and equipment specified are used, the details **MUST** be recorded in the batch record and the risk of using alternatives acknowledged by Study Memo.

4.1 Chemicals

Name	Grade/Specification and Catalogue Number	Supplier
Anti-glucarpidase affinity purified antibody	Rabbit, affinity purified on Protein G followed by Voraxaze	Sponsor
Biotin-labelled Voraxaze	Study number 08/056-002, RS08-149 [x1]	Millipore BioPharma Services
Bovine Serum Albumin (BSA)	Cat No. A-9647	Sigma
Elix water	Millipore, Elix	Millipore BioPharma Services
Extravidin-peroxidase	Cat No. E-2886	Sigma
Human Serum	Individual Samples	SLI

[x1] Any other lots of chemical maybe used and will be documented in the raw data.

Name	Grade/Specification and Catalogue Number	Supplier
Phosphate buffered saline tablets	Cat No. 18912-014	Invitrogen
Sodium Chloride	Cat No. S-7653	Sigma
Sulphuric Acid (H_2SO_4)	Cat No. J/8430/15 0.5 M	Thermo Scientific
Tween 20	Cat No. P-1379	Sigma
Voraxaze (Glucarpidase)	NA	Sponsor
3,3',5,5' Tetramethylbenzidine (TMB)	Cat No. M0701C	Europa

4.2 Consumables

Name	Grade/Specification and Catalogue Number	Supplier
1.2 mL Cluster Tubes	Cat No. CC751	Appleton Woods
Duran bottles	Various volumes	Thermo Scientific
Disposable reagent reservoirs	50 mL	Thermo Scientific
Greiner V bottom 96 well plates	Cat No. MSCPNPP00/ Cat No. 651201	Millipore or Greiner Bio One
0.22 µm Filters	Sterile	Various Suppliers
Nunc Maxisorb microtitre plates	Cat No 439454	Thermo Scientific

Name	Grade/Specification and Catalogue Number	Supplier
2.0 mL micro tubes	Cat No. FBS6055	Thermo Scientific
Pipette tips	Various sizes	Thermo Scientific
Plate sealers	Cat No. LW2770	Alpha Labs
Universals	Various sizes	Thermo Scientific
Weighing boats	Various sizes	Thermo Scientific

4.3 Equipment and Software

Name	Make and Model	Supplier
Analytical Balance	Adam	Thermo Fisher Scientific
BioControl Pipettors	Various	Thermo Fisher Scientific
Class 2 Microbiological Safety Cabinet	BS.5726.1992	RS Biotech
Magnetic Fleas	Various	Thermo Fisher Scientific
Magnetic stirrer	Various	Thermo Fisher Scientific
Microtitre Plate Reader	VERSAmax or SpectraMax	Molecular Devices
Multichannel Pipettor	Various	Various

Name	Make and Model	Supplier
Plate Reader Software	Softmax Pro 5.2	Molecular Devices
Pipettors	Various Volumes	Various
Vortex mixer	Various	Thermo Fisher Scientific

5. REAGENT PREPARATION

5.1 Sterile 0.9% (w/v) NaCl

Weigh 0.9 g NaCl and dissolve in 100 mL Elix water. Filter sterilise through a 0.22 µm filter into a sterile polypropylene tube in a biological safety cabinet.

Storage condition:	Refrigerate
Expiry:	1 month from preparation date
Container material:	Polypropylene or Glass

5.2 Voraxaze Stock Solution

Re-constitute 1 vial of lyophilised Voraxaze with sterile 0.9% (w/v) NaCl in a biological safety cabinet. The volume required will be specified on the certificate of analysis provided for the lot number of Voraxaze. Dissolve by swirling for at least 1 minute.

For each new vial reconstituted label as RSXX-XXXa/b/c etc and dispense into 250 µL aliquots in sterile polypropylene tubes and store at -70ºC (nominal).

Storage condition:	Frozen
Expiry:	7 days from preparation date
Container material:	Polypropylene

5.3 Phosphate Buffered Saline

To 2000 mL Elix water add 4 PBS tablets. Stir until dissolved.

Storage condition:	Refrigerate
Expiry:	1 month from preparation date
Container material:	Glass

5.4 Voraxaze Intermediate Stock (100 µg/mL)

Prepare an intermediate stock at 100 µg/mL by adding the appropriate amount of Voraxaze stock solution to PBS.

Expiry:	Use on day of preparation
Container material:	Polypropylene

5.5 Voraxaze Coating Solution (0.5 µg/mL)

Add 60 µL of the 100 µg/mL Voraxaze intermediate stock to 11.94 mL PBS.

Expiry:	Use on day of preparation
Container material:	Polypropylene

5.6 Assay Buffer (2% BSA (w/v) in PBS)

To 400 mL PBS add 8 g BSA. Mix by stirring until BSA is dissolved.

Storage condition:	Refrigerate
Expiry:	1 week from preparation date
Container material:	Glass

5.7 Wash Buffer (0.05% (v/v) Tween 20 in PBS)

To 1.6 L of PBS add 0.8 mL of Tween 20. Mix by inversion.

Storage condition:	Room Temperature
Expiry:	1 week from preparation date
Container material:	Glass

5.8 Biotin-labelled Voraxaze Working Solution (0.5 µg/mL)

Add 14 µL of the 440 µg/mL biotin-labelled Voraxaze (20:1 labelling ratio) stock solution to 11.986 mL assay buffer. Note: Any other lots of reagent preparation will be calculated accordingly.

Expiry:	End of day of preparation
Container material:	Polypropylene

5.9 Extravidin Peroxidase (1:10,000 Dilution)

To 40 mL assay buffer add 4 µL of extravidin peroxidise. Vortex gently to mix.

Expiry:	End of day of preparation
Container material:	Polypropylene

5.10 TMB substrate solution

Remove approximately 12 mL TMB solution per plate and place in incubator at 25°C (nominal) for approx 30 minutes prior to use in the assay

Expiry:	End of day of preparation
Container material:	Polypropylene

5.11 Stop solution (0.5 M H_2SO_4)

No preparation required – supplied as 0.5M stock solution

Storage condition:	Room Temperature
Expiry:	Manufacturers Expiry
Container material:	Manufacturers Container

6. PREPARATION OF CALIBRATION STANDARDS

6.1 Anti-Glucarpidase Antibody Stock (Stock A)

On first thaw of each vial, the contents should be dispensed into 20 µL aliquots in polypropylene tubes in order to avoid freeze/thawing.

Each time a vial is thawed and sub-aliquotted it should labelled as the same reference standard number as the original but with the suffix "a","b", "c" etc after sub-aliquoting.

Storage condition:	-70°C (nominal)
Expiry:	TBD
Container material:	Polypropylene

6.2 Anti-Glucarpidase Antibody Intermediate Stocks

Prepare intermediate stock solutions in assay buffer as follows:

Stock Solution ID	Stock Solution Concentration (µg/mL)	Volume of stock solution (µL)/stock solution ID	Volume of Assay Buffer (µL)	Final Volume (µL)
A	1260	NA	NA	NA
B	20	8 A	496	504
C	10	150 B	150	300
D	5	75 B	225	300
E	2.5	37.5 B	262.5	300
F	1.25	20 B	300	320
G	0.625	20 B	620	640
H	0.312	20 B	1260	1280

Expiry:	Use on day of preparation
Container material:	Polypropylene

6.3 Anti-Glucarpidase Antibody Human Serum Calibration Standards

Prepare human serum calibration standards as follows:

Calibration Standard Concentration (ng/mL)	Intermediate Stock Volume/ID (µL)	Human Serum Volume (µL)	Final Volume (µL)
15.63	20 H	380	400
31.25	20 G	380	400
62.5	20 F	380	400
125	20 E	380	400
250	20 D	380	400
500	20 C	380	400
1000	20 B	380	400
Expiry:	Use on day of preparation		
Container material:	Polypropylene		

7. PREPARATION OF QUALITY CONTROL SAMPLES

7.1 Anti-Glucarpidase Antibody Intermediate QC Stocks

Prepare intermediate QC stock solutions in assay buffer as follows:

Stock Solution ID	Stock Solution Concentration (µg/mL)	Volume of stock solution (µL)/stock solution ID	Volume of Assay Buffer (µL)	Final Volume (µL)
A	1260.00	NA	NA	NA
B	15.00	8 A	664	672
C	8.00	160 B	140	300
D	2.00	40 B	260	300

Expiry:	Use on day of preparation
Container material:	Polypropylene

7.2 Anti-Glucarpidase Antibody Human Serum QC Samples

Prepare human serum QC samples as follows:

Volumes are sufficient for 10 quantitative / screening assay plates with one replicate at the start and end of the plate.

QC Sample ID	QC Sample Concentration (ng/mL)	Intermediate Stock Volume/ID (µL)	Human Serum Volume(µL)	Final Volume (µL)
Lo QC	100.00	140 D	2660	2800
MeQC	400.00	140 C	2660	2800
Hi QC	750.00	140 B	2660	2800

Storage condition:	-70°C (nominal)
Expiry:	1 month from preparation date
Container material:	Polypropylene
Aliquot volume:	140 µL

7.3 Preparation of Voraxaze Spiked Assay Buffer

Specificity samples should be prepared in assay buffer at 0.38 µg/mL.
Stock A is the Voraxaze reference standard the concentration of each lot is stated in the reference standard log.

Stock Solution ID	Voraxaze Stock Concentration (µg/mL)	Volume of stock solution (µL)/stock solution ID	Volume of Assay Buffer (µL)	Final Volume (µL)
B	380.00	NA	NA	NA
C	0.38	10A	9990	10,000

Expiry:	Use on day of preparation
Container material:	Polypropylene

This calculation MUST be checked by a second analyst.

LoQC Samples should be diluted 1 in 2 with the desired spiked assay buffer, as described above, and incubated for approximately 30 minutes before use at room temperature. Samples do not require further dilution prior to addition to the assay plate.

8. SAMPLE PREPARATION

8.1 Sample Thawing

Step	Procedure
1	Remove the required serum samples, control serum (blank) and QC samples from the freezer.
2	Place samples in an incubator at 25°C (nominal) until thawed for a maximum of 15 minutes. Remove immediately once thawed.
3	Remove samples from incubator and leave at room temperature for no more than 4 hours. Record whether any samples appear to be clotted, haemolysed, lipaemic or icteric.
4	Re-freeze samples as soon as practicable after use.

8.2 Sample Dilution

Samples to be diluted into the assay range, or for determination of titre values, should be diluted by the appropriate factor using control matrix in polypropylene micro-tubes.

All matrix blanks, calibrators, QC samples, samples and diluted samples should be diluted 1 in 2 with assay buffer prior to addition to the assay plate by addition of 125 µL of sample to 125 µL assay buffer. This should be done in the Greiner (Millipore) polystyrene V-bottom well plates stated in section 4.2.

If larger volumes are required (i.e. for multiple plates during troubleshooting) then dilution in 500 µL polypropylene "cluster tubes" is acceptable.

9. METHODOLOGY

Step	Procedure
1	Add 100µL/well of the 0.5 µg/mL Voraxaze coating solution to each well of the 96 well plate.
2	Incubate overnight at 4°C (nominal) for 16 – 20 hours, flick out the coating solution then blot the plates dry on an absorbent tissue
3	Add 200 µL per well of assay buffer to all wells
4	Incubate plates for 2 hours (± 10 minutes) at 25 °C (nominal) with gentle shaking.
5	Flick out the assay buffer then blot the plates dry on an absorbent tissue
6	Wash the plate four times with 300 µL per well of wash buffer, by hand washing using a multi channel pipette with a 30 second soak. Blot plate and rotate plate 180° between washes and repeat until 4 washes completed. Blot plates dry after the final wash.
7	Add 100 µL/well of the blanks, calibration standards, QC samples and study samples according to the plate map
8	Seal plates and incubate for 2 hour (± 5 min) at 25°C (nominal) with gentle shaking
9	Flick out the samples and blot plates on absorbent tissues
10	Wash the plate four times with 300 µL per well of wash buffer, by hand washing using a multi channel pipette with a 30 second soak. Blot plate and rotate plate 180° between washes and repeat until 4 washes completed. Blot plates dry after the final wash.
11	Add 100 µL/well of the 0.5 µg/mL biotin-labelled Voraxaze working solution to the appropriate wells according to the plate maps

12	Seal plates and incubate for 2 hours (± 5 min) at 25°C (nominal) with gentle shaking
13	Flick out the plates and blot on absorbent tissue
14	Wash the plate four times with 300 µL per well of wash buffer, by hand washing using a multi channel pipette with a 30 second soak. Blot plate and rotate plate 180° between washes and repeat until 4 washes completed. Blot plates dry after the final wash.
15	Add 100 µL per well of the 1:10,000 extravidin peroxidise reagent to the appropriate wells
16	Seal plates and incubate for 1 hour (± 5 min) at 25°C (nominal) with gentle shaking
17	Flick out the plates and blot on absorbent tissue
18	Wash the plate four times with 300 µL per well of wash buffer, by hand washing using a multi channel pipette with a 30 second soak. Blot plate and rotate plate 180° between washes and repeat until 4 washes completed. Blot plates dry after the final wash.
19	Add 100 µL of TMB substrate solution to all wells
20	Incubate plates at 25°C (nominal) with gentle shaking and monitor colour development (should be approximately 15 minutes)
21	After 15 minutes (± 1 minute), or when adequate colour has developed, add 100 µL 0.5 M H_2SO_4 to each well to stop the reaction.
22	Read absorbance values at 450 nm with 540 nm background correction within 15 minutes of stopping the reaction

9. INSTRUMENT PARAMETERS

Parameter	Setting
Reading type	Dual wavelength endpoint at 450nm with 540nm background correction
Processing type	Normal
Mixing	5 Seconds
Curve-fitting options	Weighted ($1/y^2$) 4-parameter logistic
X-axis	Log

10. DATA ANALYSIS

The calibration function is fitted using a weighted, $1/y^2$, 4-parameter logistic curve fit of the mean background corrected absorbance values (y-axis) plotted against the log anti-glucarpidase antibody concentration (x-axis). The concentrations of anti-glucarpidase antibody in calibration standards, QC samples and the equivalent level of anti-glucarpidase antibody in study samples is determined from the background corrected absorbance values and the curve fit model parameters.

APPENDIX 1

Revision History

Analytical Procedure

DCC No and Date	Original Author	Version Number	Description of Revision
DCC-0701 Nov 2008	G Hubbard	01	New analytical procedure
DCC-0803 Feb 2009	G Hubbard	02	Section 4.1 Details of Carboxypeptidase B, Leucovorin and Methotrexate add to list of chemicals Section 7 Added sections 7.3 to 7.5.5 for preparation of selectivity and specificity samples Section 8.2 Added text to describe how samples should be diluted to obtain titre values.
DCC-0808 Feb 2009	C Cox	03	Section 4.1 Details of Carboxypeptidase B, Leucovorin and methotrexate removed from list of chemicals. Sections 7.1 and 7.2 Lo QC changed to 100 ng/mL. Details of preparation of LLOQ, and Me QCs, and their corresponding stocks, removed. Expiry date of human serum QC samples set to 1 month from date of preparation. Sections 7.3 and 7.4 Sections removed. Subsequent sections re-numbered Section 7.5.1 Section amended as only one concentration of Voraxaze required. Sections 7.5.2, 7.5.3, 7.5.4 and 7.5.5 Sections removed. Section 8.1 Sample thawing changed from waterbath to incubator.

DCC-0992	G Hubbard	04	Minor pagination changes throughout Section 7: Added provision for making MeQC and intermediate stocks
DCC-1044 26 Feb 2010	G Hubbard	05	Updated version numbers throughout Section 2 – Updated to reflect new extravidin dilution Section 4.2 – Added supplier and catalogue number for offline dilution plate/tubes. Section 5.9 – Changed extravidin dilution to 1 in 10,000 for revised procedure Section 6.2 – Updated concentrations and volumes to reflect revised calibrator concentrations. Section 6.3 – Updated concentrations to revised levels. Section 7.1 – Updated with new intermediate QC concentrations in line with revised final Qc concentrations. Section 7.2 – Updated to new Qc concentration and scaled volumes up sufficient for 10 plates and specified this in the text. Specified aliquot volume. Section 7.3 – Changed Stock IDs and added text to clarify how stocks should be prepared. Section 8.2 – Added text to specify tube and plate types to be used for dilutions. Section 9 – step 15 changed to state correct extravidin dilution Section 9 – step 21 clarified colour development to be 15 min ± 1 min
DCC-1101 26 April 2010	C Langley	06	1. Addition of alternative catalogue number for Greiner V bottom 96 well plates. As the plates are sourced through Millipore but manufactured by Greiner and have different catalogue numbers. 2. Section 4.3 removal of reference to plate washer. 3. Section 9 Removal of plate washing and replacement with hand washing with a 30 second soak throughout this section due to drift seen during the assay.

			4. Millipore added to list of suppliers.
DCC-1215 Oct 2010	C Langley	07	1. Copy approval section page 1 removal of BioAnaLab 2. In all headers company logo amended to Millipore one. 3. Section 2 addition of 'with gentle shaking' added to TMB sentence. 4. Section 4.1 amended details of BioAnaLab to Millipore and addition of other lots of reagent. 5. Section 4.3 removal of a blank row. 6. Section 5.8 addition of sentence specifying other lots maybe used. 7. Section 6.2 changed preparation details of calibration standards. 8. Section 9.20 added 'with gentle shaking'

Batch Record

DCC No and Date	Original Author	Version Number	Description of Revision
DCC-0701 November 2008	G Hubbard	01	New batch record
DCC-0803 February 09	G Hubbard	02	No changes.
DCC-0808 February 2009	C Cox	03	No changes.
DCC-0992	G Hubbard	04	Section 4: Made provision for recording Me QC Level preparation details Section 6: Added table to record incubations for plates 2 and 3 if used and to match number of plate plans in section 8
DCC-1044 26 Feb 2010	G Hubbard	05	Updated version numbers throughout Section 2 – Updated order of reagent prep to better reflect that used when preparing assay. Section 2.9 - Added for preparation of Preparation of

			Voraxaze Spiked Assay Buffer previously omitted. Section 2.11 – Changed to read 1 in 10,000 dilution Sections 2.12 - Removed instance of duplicate data recording boxes for expiry date and added box for recording time TMB placed at 25°C Sections 2.12 - Removed instances of duplicate data recording boxes for expiry date. Section 3.1 – Updated with new concentrations and specified matrix expiry date to be recorded. Section 4 – Updated with new QC concentrations and specified to record matrix expiry date and aliquot size. Section 5.1 – Updated to better reflect procedure in AP. Section 5.2 – Updated to allow recording of specified tube/plate catalogue and lot numbers. Section 6.1 – Add text to box so only BAL number need be recorded. Section 6.2 – Add text to box so only BAL number need be recorded. Section 8 – Updated with revised Calibrator concentrations.
DCC-1101 26 April 2010	C Langley	06	1) Section 6.2 Removal of reference to plate washer.
DCC-1215 Oct 2010	C Langley	07	1. Copy approval section page 1 removal of BioAnaLab. 2. In all headers company logo amended to Millipore one. 3. Status on completion addition of 'reason for failure box' 4. Section 1 addition of 'pipette database completed by/Date' 5. Section 2.2 added details for records NaCl usage. 6. Section 2.10 added space to record calculation checked by. 7. Section 2.14 addition of space to document offline plate details. 8. Section 3.1 amended preparation details. 9. Section 6.2 added details for recording plate shaker details

APPENDIX 2

Names and Addresses of Suppliers

Name	Address
Alpha Labs	40 Parham Drive, Eastleigh, Hampshire, SO50 4NU, UK
Greiner Bio-One	Brunel Way, Stroudwater Business Park, Stonehouse, Glos. GL10 3SX, UK
Europa	Europa House, 15-17 North Street, Wicken, Ely, Cambridge, CB7 5XW, UK
Invitrogen Ltd	Inchinnan Business Park, 3 Fountain Drive, Paisley, PA4 9RF, UK
Millipore	Bioscience Division, Units 3 & 5, The Court Yard, Hatters Lane, Watford, Herts. WD18 8YH
Molecular Devices Ltd	660 - 665 Eskdale Road, Winnersh Triangle, Wokingham, Berkshire, RG41 5TS, UK
RS Biotech	Tower Works, Well St., Finedon, Northants NN9 5JP, UK
Sigma	Fancy Road, Poole, Dorset, BH12 4QH, UK
SLI	492 Richmond Road, East Meadow, NY 11554, USA
Thermo Scientific UK Ltd	Bishop Meadow Road, Loughborough, Leics, LE11 ORG, UK

APPENDIX 3

Health and Safety

Normal laboratory precautions as specified in the most current version of BioAnaLab SOP BAL-024 must be adhered to at all times when performing this procedure.

In addition, this Analytical Procedure poses the following risks:

A3.1 The Stop Solution used in this procedure is Sulphuric Acid, which is corrosive, toxic and hygroscopic and cause severe burns. Care should be taken when handling this solution.

A3.2 The development reagent 3,3',5,5'-Tetramethylbenzidine (TMB) although not carcinogenic (Ames test negative), is an irritant (contact and respiratory) and identified by some sources as a potential mutagen (unconfirmed).

A3.3 Human Serum samples, although sourced from suppliers with stringent control procedures, could be a potential source of adventitious infection. As such these should be handled with caution.

APPENDIX 14. NEUTRALISING ANTIGLUCARPIDASE ANTIBODIES, DRAFT REPORT BY COVANCE (MARCH 2012)

Draft Report

Study Title	Analysis of Human Serum Samples from Clinical Study PR001-CLN-pro009 using a Spectrophotometric Analytical Procedure to Detect Neutralising Anti-glucarpidase Antibodies
GMO Status	Non GMO
Author	Name: S Little Phone: +44 (0)1423 848298 Email: samantha.little@covance.com Fax: +44 (0) 1423 569595
Test Facility	Covance Laboratories Ltd Otley Road, Harrogate North Yorkshire, HG3 1PY United Kingdom
Study Monitor	Name: L Trani Email: Leonardo.Trani@uclh.nhs.uk Phone: +44 (0) 845 155 5000 ext 77583
Sponsor	University College Hospital 250 Euston Road 1st Floor East NW1 2PG UK
Financial Client	Protherics Medicines Development Ltd. (Part of BTG International Ltd.) 5 Fleet Place, London, EC4M 7RD, UK
Covance Client Identifier	1000484
Covance Study Number	8257561
Client Reference Number	XXXXXX
Report Issued	Draft Report (March 2012)
Page Number	1 of 20 (Excluding Annex)

STUDY MANAGER AUTHENTICATION STATEMENT

Analysis of Human Serum Samples from Clinical Study PR001-CLN-pro009 using a Spectrophotometric Analytical Procedure to Detect Neutralising Anti-glucarpidase Antibodies

I, the undersigned, hereby declare that the work was performed under my supervision and that the findings provide a true and accurate record of the results obtained.

The study was performed in accordance with the agreed Protocol and Protocol Amendments and with Covance Laboratories Limited Standard Operating Procedures, unless otherwise stated, and the study objectives were achieved. The data generated was scientifically acceptable and valid. This Report provides a true and accurate record of the results obtained.

This study was not required to be subject to Good Laboratory Practice Regulation and, consequently, a claim of GLP compliance has not been made. However, the laboratory procedures satisfied the current requirements of the UK and OECD GLP regulations. The study was conducted in accordance with the following regulations and standards:

- UK Statutory Instrument 2004 No.1031 the Medicines for Human Clinical Use (Clinical Trials) Regulations 2004 plus subsequent amendments

- Good Clinical Practice: Consolidated Guideline ICH Topic E6, adopted by CPMP, July 1996, issued as CPMP/ICH/135/95.

S Little
Study Manager
Covance Laboratories Limited

Date

QUALITY ASSURANCE STATEMENT

Analysis of Human Serum Samples from Clinical Study PR001-CLN-pro009 using a Spectrophotometric Analytical Procedure to Detect Neutralising Anti-glucarpidase Antibodies

This study has been reviewed by the GLP Quality Assurance Unit of Covance and the Report accurately reflects the raw data. The following inspections were conducted and findings reported to the Study Manager (SM) and associated Management.

Critical procedures, which are performed routinely in an operational area, may be audited as part of a "process" inspection programme. This can be in addition to phases scheduled on an individual study basis. Selected process inspections conducted and considered applicable to this study are included below.

In addition to the inspection programmes detailed below, a facility inspection programme is also operated. Details of this programme, which covers all areas of the facility annually (at a minimum), are set out in standard operating procedures.

Inspection Dates		Phase	Date Reported to SM and SM Management
From	To		

Process

Inspection Dates		Phase	Date Reported to SM and SM Management
From	To		

Representative
Quality Assurance Unit

Date

RESPONSIBLE PERSONNEL

Analysis of Human Serum Samples from Clinical Study PR001-CLN-pro009 using a Spectrophotometric Analytical Procedure to Detect Neutralising Anti-glucarpidase Antibodies

In addition, the following staff was responsible for key elements of the study:

Study Manager:	S Little
Study Supervisor:	A Michalak

STUDY SCHEDULE

The study schedule was as follows:

Study Initiation:	13th January 2012 (Date Study Manager signed Protocol)
Experimental Start:	19th January 2012 (Date of recording first study specific data)
Experimental Completion:	19th January 2012 (Date of final data capture for Report)
Study Completion:	Date the Final Report was signed by the Study Manager

ARCHIVE STATEMENT

Analysis of Human Serum Samples from Clinical Study PR001-CLN-pro009 using a Spectrophotometric Analytical Procedure to Detect Neutralising Anti-glucarpidase Antibodies

All primary data, or authenticated copies thereof, specimens or samples will be retained in the Covance Laboratories Limited archives for one year after issue of the Final Report. At this time, the Client will be contacted to determine whether the data should be returned, retained or destroyed on their behalf. The Client will be notified of the financial implications of each of these options at that time. One copy of the Definitive Protocol and Final Report respectively will be held indefinitely in the Covance Laboratories Limited archives.

Samples must only be retained for as long as they may be needed for the clinical trial and should be discarded in a respectful manner as soon as they are no longer required. Covance deem an acceptable time period being 6 weeks after either QA audited data is provided or 6 weeks after data has been locked in the clinical data base. The Client will be contacted before samples are fully disposed.

CONTENTS

OBJECTIVE

The objective of the study was to detect the presence of neutralising anti-glucarpidase antibodies (NAb) in Human serum samples using a UV spectrophotometric procedure.

INTRODUCTION

The method had previously been subjected to a validation exercise (CLEH Study Number 8255048) at Covance Laboratories Limited, Harrogate (CLEH).

Samples were generated from the following clinical study:

"A randomized, cross-over, phase II study, to investigate the efficacy and safety of glucarpidase for use after high-dose methotrexate in patients with bone sarcoma" (Clinical Protocol Number PR001-CLN-pro009).

For the duration of the study all items constituting the test system were identified and appropriately labelled.

This analytical investigation was conducted in the Immunochemistry Department, Covance Laboratories Limited, Otley Road, Harrogate, North Yorkshire, United Kingdom.

MATERIALS AND METHODS

Full details of the analytical method, the reagents and equipment used are presented in the Definitive Protocol and Analytical Procedure found in the Annex.

ASSAY SPECIFIC INFORMATION

The analytical programme was conducted in accordance with the Definitive Protocol and Protocol Amendment 1 along with the current version of Analytical Procedure IA-08-050, which was used during this study (see Annex).

VALIDATED PARAMETERS

- Estimated sensitivity, as defined by the cut point, is confirmed as 7.5µg/mL relative to a rabbit anti-glucarpidase positive control antiserum.
- Assay Cut Point (95th percentile) target patient population: -0.0940 (abs/min).
- Negative Control Serum (NCS) normalisation factor (95th percentile): 0.7315.
- Assay Cut Point (99th percentile) target patient population: -0.0933 (abs/min).
- Negative Control Serum (NCS) normalisation factor (99th percentile): 0.7261.
- Number of additional freeze-thaw cycles: 5 (confirmed within study 1845-161).
- Freezer stability: 20 days at -70°C nominal (confirmed within study 1845-161).

BATCH OVERVIEW

Two batches were undertaken under CLEH study number 8257561, SB01 and SB02. As per Protocol Amendment 1, the samples analysed under SB01, performed on the 16th January 2012, have not been reported. Batch SB02 was conducted on the 19th January 2012. The batch was considered acceptable and therefore the data produced from this batch is presented within this Report.

ANTIBODY REFERENCE STANDARD MATERIAL

Rabbit anti-glucarpidase affinity purified IgG antibody was supplied by Covance Research Products (CRP) (Lot Number PR070522A) on 4 June 2007, at a concentration of 1.26 mg/mL. Expiry was not known however, material was provided with an expiry date of 2 years from date of receipt (4 June 2009) when stored at <-50°C. The material had continued to be used past this date and a review of the control data supported activity of the material throughout the duration of the study.

A Product Specification for this material is presented in Appendix 1.

CRITICAL REAGENTS

Critical Reagent 1

Identity:	Glucarpidase (Voraxaze®)
Supplied by:	Client
Batch number:	2162-104
Date of manufacture:	04-Nov-2010
Storage:	2-8°C (as supplied)
Expiry date:	October 2013
Other information:	1085 Units/vial enzyme activity and 492 Units/mg protein

Critical Reagent 2

Identity:	Methotrexate hydrate (MTX)
Supplied by:	Sigma
Catalogue number:	A6770
Storage:	<-10°C
Retest date:	10th February 2012 (internally assigned by CLEH)

CONTROL MATRIX

Pooled and individual Human serum was sourced by CLEH. Details regarding supplier, lot number and expiry are stored in the departmental records.

A specific pool of human serum, CR11/1000484/677, was assigned for use as the negative control serum (NCS) in the study. This was a pool of individual matrix which was used throughout sample analysis. The NCS was also used in the preparation of the positive control standards.

PREPARATION OF STANDARDS AND POSITIVE CONTROL SAMPLES

Preparation of Methotrexate
MTX was prepared to a 28.4 µg/mL working solution in assay buffer. The working solution was stored at room temperature and used on the day of preparation.

Rabbit Anti-Glucarpidase Positive Control Standards
Rabbit anti-glucarpidase PC samples were prepared by dilution in negative control serum to the following concentrations: 2.5, 5.0, 7.5, 10 and 15 µg/mL.

The PC samples (180 µL) were incubated at 37°C nominal for 30 minutes with 20 µL glucarpidase (9 µg/mL). Upon completion of this incubation, all samples were stored on ice for a maximum of 2 hours. A 50 µL aliquot of each of the samples followed by 1 mL MTX was added to the assay cuvette and then analysed.

PREPARATION OF STUDY SAMPLES

Sample Storage
Serum samples were initially supplied to CLEH from multiple clinical sites. Aliquots were sent to Millipore Biopharma Services, UK, for the detection of anti-glucarpidase antibodies in a screening/confirmatory assay (Millipore study 09/056-006). Samples identified as positive were provided to CLEH from Millipore Biopharma Services, UK, where they were stored with any original unshipped aliquots at <-50°C until analysis. Samples must only be retained for as long as they are needed and should be discarded in a respectful manner as soon as they are no longer required. Actual details regarding the sample receipt at CLEH were contained within the raw data.

Sample Analysis

10 samples previously confirmed positive for anti-glucarpidase antibody, and 6 matched patient negative control samples were analysed.

The study samples were analysed within batch SB02 (analysis conducted under CLEH study number 8257561). All PC samples, NCS and study samples were analysed in triplicate. Each batch contained 2 sets of the PC samples (2.5, 5.0, 7.5, 10 and 15 µg/mL) and 4 sets of the NCS.

Study samples were prepared in the same manner as the PC samples (180 µL of sample to which 20 µL of a 9 µg/mL glucarpidase solution was added).

The reported data was the mean analytical slop x 60 (Abs/min) for 3 replicates reported for each sample analysed.

DATA COLLECTION

The data was collected on the Perkin Elmer lambda 35 UV Winlab software version no. 5.2.0.0646. This software has been validated for use at CLEH. The data was processed using Microsoft Excel.

ASSAY ACCEPTANCE CRITERIA

Full details of the acceptance criteria applied to the study can be found in the Definitive Protocol, associated Amendments and Analytical Procedure which are presented in the Annex.

In summary,

- The precision between triplicates for the PC samples should not have exceeded 25%. Where the precision was exceeded, the PCs in question have been highlighted.
- PC samples at 7.5, 10 and 15 µg/mL should have produced a positive result relative to the batch specific assay cut-point.
- Mean response of NCS < mean response of PC at 7.5 µg/mL < mean response of PC at 10 µg/mL < mean response of PC at 15 µg/mL.
- The precision of the NCS (mean slope) was not to exceed 25%. A single replicate of the NSB could be dropped if a clear outlier was identified. The mean slope should

have ideally fallen within -0.0826 and -0.1570 units (mean ± 3 SD limits based upon results from the 8255048 validation data).

- The precision between triplicates for each study sample was not to exceed 25%. Where the precision was exceeded, the sample in question has been highlighted.

- Where the mean slope value for the sample was ≥ batch specific cut-point, samples have been considered as neutralising reactive. To inform further upon the antibody specificity of the response however, and in the absence of any secondary confirmatory assay process, a comparison has been made (where feasible) between the antibody positive sample result and the result of the matched control sample previously shown to be negative for anti-glucarpidase antibody. Only where the control sample result was less than the cut-point (i.e. it is non-reactive) has the reactive sample been reported as positive for neutralising anti-glucarpidase antibody. Where the matched control sample was ≥ to the cut-point, the neutralising reactive sample has been reported as unconfirmed for neutralising anti-glucarpidase antibody.

- Where the mean slope value is < batch specific neutralising cut-point, the sample has been reported as negative for neutralising anti-glucarpidase antibody.

RESULTS AND DISCUSSION

Intra-Assay Precision of the Negative Control Serum

The NCS analysed within SB02 produced a mean slope of -0.1675 and had a precision of 11.1%. The performance of the NCS can be observed in Table 1.

The Definitive Protocol stated that the mean slope of the NCS should fall between -0.0826 and -0.1570 units. The mean slope obtained from the NCS analysed within SB02 was outside this range. It is appreciated however that the target range in which the NCS should fall was derived from a limited data set and was set for guidance purposes. Also, considering the results from the PC samples generated from batch SB02 in conjunction with the NCS results, the Study Manager considers the results obtained from the NCS as acceptable.

Inter-Assay Precision of the Positive Control Samples

As presented in Table 1, the mean response of the PCs at 2.5, 5, 7.5, 10 and 15 µg/mL was -0.1540, -0.1080, -0.0840, -0.0710 and -0.0480, respectively. The precision of the PCs at 2.5, 5, 7.5, 10 and 15 µg/mL was 9.2%, 7.9%, 3.4%, 17.9% and 5.9%, respectively.

When assessed against the batch specific cut points using both the 95th percentile normalisation factor (0.7315) and the 99th percentile normalisation factor (0.7261), all PCs at 5, 7.5, 10 and 15 µg/mL assessed produced a positive result (i.e. above the batch specific cut point).

Sample Results Summary

The results for the samples analysed are presented in Table 1 and Table 2. The results obtained were assessed against two batch specific cut points, one determined using a normalisation factor derived from the 95th percentile and one determined using a normalisation factor derived from the 99th percentile.

The results were firstly assessed to determine whether the samples could be considered as neutralising reactive. To determine whether the sample could be considered as neutralising reactive, the mean slope value for the sample was required to be ≥ batch specific cut-point. If the result was < batch specific cut-point, the sample was considered negative. Table 1 presents the data to indicate whether the sample was considered negative or neutralizing reactive.

After considering the sample's neutralising reactivity, a comparison was made (where feasible) between the neutralising reactive sample result and the result of the matched control sample previously shown to be negative for the anti-glucarpidase antibody. This comparison was undertaken in order to inform further upon the antibody specificity of the response (as there was no secondary confirmatory assay process). Where the control sample result was less than the batch specific cut-point (i.e. it was non-reactive), the reactive sample has been reported as positive for neutralising anti-glucarpidase antibody. Where the matched control sample was ≥ to the cut-point, the neutralising reactive sample has been reported as unconfirmed for neutralising anti-glucarpidase antibody. These results are presented within Table 2.

The CV% for 2 sample results (Subject 15, Follow Up and Day 15) exceeded 25%. The Study Manager considered this acceptable as high CVs are inevitable due to the low slope figures and especially when the response has mostly been neutralised and values were approaching zero. In addition, the result for the sample in question was obtained from two replicates, not three, as an analytical error occurred with the third replicate and thus, data from this third replicate has been omitted.

CONCLUSION

The analysis of the clinical samples was successfully completed and the performance of the assay was found to be acceptable. All data was reported from batches which had met the criteria outlined in the Definitive Protocol and subsequent Protocol Amendments.

TABLES

Table 1: Results Obtained from NCS, PCs and Sample Specimens (Undertaken Within Batch SB02, CLEH Study no. 8257561)

Sample ID	Time Point	Collection date	Concentration (µg/mL)	Replicate 1	Replicate 2	Replicate 3	Mean Slope	Mean Slope x 60 (Abs/min)	% CV	Mean Response (%CV)	Study Batch Specific Cut Point (-0.1225) NR/Negative	Study Batch Specific Cut Point (-0.1216) NR/Negative
NCS 1 start [X1]	-	-	-	-0.0022	-0.0024	-0.0025	-0.0024	-0.1420	6.5	-0.1675	-	-
NCS 2 start [X1]	-	-	-	-0.0023	-0.0030	-0.0031	-0.0028	-0.1680	15.6	(11.1)	-	-
NCS 3 end [X1]	-	-	-	-0.0029	-0.0032	-0.0032	-0.0031	-0.1860	5.6		-	-
NCS 4 end [X1]	-	-	-	-0.0030	-0.0031	-0.0026	-0.0029	-0.1740	9.1		-	-
Anti-Glucarpidase PC	-	-	15.00	-0.0009	-0.0007	-0.0007	-0.0008	-0.0460	15.1	-0.0480	NR	NR
Anti-Glucarpidase PC	-	-	15.00	-0.0009	-0.0007	-0.0009	-0.0008	-0.0500	13.9	(5.9)	NR	NR
Anti-Glucarpidase PC	-	-	10.00	-0.0011	-0.0012	-0.0008	-0.0010	-0.0620	20.1	-0.0710	NR	NR
Anti-Glucarpidase PC	-	-	10.00	-0.0015	-0.0014	-0.0011	-0.0013	-0.0800	15.6	(17.9)	NR	NR
Anti-Glucarpidase PC	-	-	7.50	-0.0013	-0.0015	-0.0013	-0.0014	-0.0820	8.4	-0.0840	NR	NR
Anti-Glucarpidase PC	-	-	7.50	-0.0014	-0.0017	-0.0012	-0.0014	-0.0860	17.6	(3.4)	NR	NR
Anti-Glucarpidase PC	-	-	5.00	-0.0020	-0.0018	-0.0019	-0.0019	-0.1140	5.3	-0.1080	NR	NR
Anti-Glucarpidase PC	-	-	5.00	-0.0016	-0.0014	-0.0021	-0.0017	-0.1020	21.2	(7.9)	NR	NR
Anti-Glucarpidase PC	-	-	2.50	-0.0021	-0.0026	-0.0025	-0.0024	-0.1440	11.0	-0.1540	Negative	Negative
Anti-Glucarpidase PC	-	-	2.50	-0.0028	-0.0028	-0.0026	-0.0027	-0.1640	4.2	(9.2)	Negative	Negative
Subject 15	Day 15	02/Mar/2009	-	-0.0045	-0.0046	-0.0017	-0.0036	-0.2160	**45.7**	-	Negative	Negative
Subject 15	Follow up	04/Sep/2009	-	-0.0051	-0.0043	-0.0021	-0.0038	-0.2300	**40.5**	-	Negative	Negative
Subject 15	Baseline	26/Jan/2009	-	-0.0030	-0.0028	-0.0026	-0.0028	-0.1680	7.1	-	Negative	Negative
Subject 16	Day 8	24/Feb/2009	-	-0.0031	-0.0033	-0.0031	-0.0032	-0.1900	3.6	-	Negative	Negative
Subject 16	Day 15	03/Mar/2009	-	-0.0033	-0.0029	-0.0031	-0.0031	-0.1860	6.5	-	Negative	Negative
Subject 16	Baseline	27 Jan 2009	-	-0.0035	-0.0031	-0.0032	-0.0033	-0.1960	6.4	-	Negative	Negative
Subject 2	Day 180	11/Mar/2008	-	-0.0014	-0.0021	-0.0017	-0.0017	-0.1040	20.3	-	NR	NR
Subject 2	Baseline	03/Sep/2007	-	-0.0025	-0.0035	-0.003	-0.0030	-0.1800	16.7	-	Negative	Negative
Subject 7	Day 1	17/Mar/2008	-	-0.0025	-0.0017	-0.0025	-0.0022	-0.1340	20.7	-	Negative	Negative
Subject 7	Day 15	31/Mar/2008	-	-0.0027	-0.0027	-0.003	-0.0028	-0.1680	6.2	-	Negative	Negative
Subject 7	Day 23	08/Apr/2008	-	-0.0027	-0.0029	-0.0027	-0.0028	-0.1660	4.2	-	Negative	Negative
Subject 7	Baseline	18/Feb/2008	-	-0.0028	-0.0035	-0.0033	-0.0032	-0.1920	11.3	-	Negative	Negative
Subject 8	Day 1	13/Mar/2008	-	-0.0028	-0.0025	-0.0027	-0.0027	-0.1600	5.7	-	Negative	Negative
Subject 8	Baseline	18/Feb/2008	-	-0.0032	-0.0034	-0.0031	-0.0032	-0.1940	4.7	-	Negative	Negative

Sample ID	Time Point	Collection date	Concentration (µg/mL)	Replicate 1	Replicate 2	Replicate 3	Mean Slope	Mean Slope x 60 (Abs/min)	% CV	Mean Response (%CV)	Study Batch Specific Cut Point (-0.1225) NR/Negative	Study Batch Specific Cut Point (-0.1216) NR/Negative
Subject 9	Day 15	18/Jun/2008	-	-0.0033	-0.0030	-0.0033	-0.0032	-0.1920	5.4	-	Negative	Negative
Subject 9	Baseline	12/May/2008	-	-0.0027	-0.0033	-0.0031	-0.0030	-0.1820	10.1	-	Negative	Negative

Run specific cut point-1 (95th percentile) =-0.1675 x 0.7315 = -0.1225
Run specific cut point-1 (99th percentile) =-0.1675x 0.7261 = -0.1216
X1= Negative Control Serum is CR11/1000484/677
NR = neutralising reactive
Bold = Co-efficient of variation > 25%

Table 2: Overall Summary of Sample Results

Subject Number	Sample Time Point	Sample Date	Mean Slope x 60 (Abs/min)	Assay Batch cut point (slope x 60) NF=0.7315, 95th %ile	Assay Batch cut point (slope x 60) NF=0.7261, 99th %ile	Sample status	Neutralising Antibody Result	Batch Sample Analysed
Subject 15	Baseline [X1]	26/Jan/2009	-0.1680	-0.1225	-0.1216	< Cut-point	NA	SB02
Subject 15	Day 15	02/Mar/2009	**-0.2160**	-0.1225	-0.1216	< Cut-point	Negative	SB02
Subject 15	Follow up	04/Sep/2009	**-0.2300**	-0.1225	-0.1216	< Cut-point	Negative	SB02
Subject 16	Baseline [X1]	27 Jan 2009	-0.1960	-0.1225	-0.1216	< Cut-point	NA	SB02
Subject 16	Day 8	24/Feb/2009	-0.1900	-0.1225	-0.1216	< Cut-point	Negative	SB02
Subject 16	Day 15	03/Mar/2009	-0.1860	-0.1225	-0.1216	< Cut-point	Negative	SB02
Subject 2	Baseline [X1]	03/Sep/2007	-0.1800	-0.1225	-0.1216	< Cut-point	NA	SB02
Subject 2	Day 180	11/Mar/2008	-0.1040	-0.1225	-0.1216	> Cut-point	Positive	SB02
Subject 7	Baseline [X1]	18/Feb/2008	-0.1920	-0.1225	-0.1216	< Cut-point	NA	SB02
Subject 7	Day 1	17/Mar/2008	-0.1340	-0.1225	-0.1216	< Cut-point	Negative	SB02
Subject 7	Day 15	31/Mar/2008	-0.1680	-0.1225	-0.1216	< Cut-point	Negative	SB02
Subject 7	Day 23	08/Apr/2008	-0.1660	-0.1225	-0.1216	< Cut-point	Negative	SB02
Subject 8	Baseline [X1]	18/Feb/2008	-0.1940	-0.1225	-0.1216	< Cut-point	NA	SB02
Subject 8	Day 1	13/Mar/2008	-0.1600	-0.1225	-0.1216	< Cut-point	Negative	SB02
Subject 9	Baseline [X1]	12/May/2008	-0.1820	-0.1225	-0.1216	< Cut-point	NA	SB02
Subject 9	Day 15	18/Jun/2008	-0.1920	-0.1225	-0.1216	< Cut-point	Negative	SB02

X1 = Matched control sample (negative for anti-glucarpidase antibody)
Bold = Co-efficient of variation > 25%
NA = Not applicable (sample negative for anti-glucarpidase antibody)

APPENDIX

Product Specification Sheet for Reference Material

COVANCE
THE DEVELOPMENT SERVICES COMPANY

Covance Research Products
P.O. Box 7200
Denver, PA 17517
Tel: 800/345-4114
717/336-4821
Fax: 717/336-3481

PRODUCT SPECIFICATIONS

Rabbit Immunoglobin (Ig)
Affinity Purified from Sera – CRPQ7177-02

Product Description:	Rabbit immunoglobin (Ig) affinity purified and buffer exchanged from 220 ml of sera.
Lot Number:	PR070522A

TECHNICAL DATA

Protein Concentration:	1.26 mg/ml (A_{280nm}, ε = 1.4 ml/mg cm)
Product Volume:	82.0 mL
Buffer:	Phosphate buffered saline, pH 7.25 (no preservative)
Purity:	Unknown
Sterility:	Not sterile
Shipping and Storage:	Product shipping on dry ice. **Store below -20°C upon receipt.** Avoid freeze-thaw cycles as product degradation may result. **CAUTION: This Product Is for Research and Laboratory Use Only.**

RECOVERY ASSAY SUMMARY

	50% Titer*	% Recovery
Starting Material	4513	n/a
Bound	525	12%
Unbound	2381	53%

1000484 TM Exc
8257561
11 NOV 11 A

* The 50% titer is not the same as an EIA 50% titer. This is just a reference that is used to determine if the purification has sufficiently exhausted the starting material.

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APPENDIX 15. GLU 1 INTERIM ANALYSIS: INDEPENDENT DATA MONITORING COMMITTEE (IDMC) REPORT

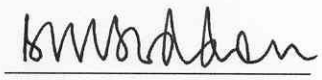
Title:	GLU 1 clinical trial
Sponsor Protocol Number:	**06/085**
Sponsor:	**UCL/UCLH**
Chief Investigator:	Dr Jeremy Whelan
EudraCT Number:	2006-003203-40
CTA number	23151/0002/001-0001
Date of MHRA approval:	10 October 2006
Name of Investigational Medicinal Product(s):	Voraxaze (glucarpidase)
Details of latest approved protocol (date and version number):	**Version 3.0, 4 December 2007**
Trial design:	Double-blind, randomised, cross-over, phase II study, to investigate the efficacy and safety of glucarpidase for routine use after high-dose methotrexate in patients with bone sarcoma.
Start of trial:	3 July 2007
Total number of subjects planned for the trial	28
Number of patients recruited so far	16
Number of patients with complete data set	11
Date of IDMC meeting to discuss interim analysis report	**26 May 2009**
IDMC members	**Dr Beatrice Seddon** **Dr Anna Cassoni** **Dr Rumana Omar**
Interim analysis of the primary outcome	**Analysis of the primary outcome has not shown a statistically significant benefit of glucarpidase with P<0.005** **Treatment with glucarpidase and folinic acid has not been not found to be significantly worse than standard treatment using an one-sided test with P<0.05**

Recruitment and drop-out rates	**Recruitment rate has been lower than expected**. Since 13 June 2007 16 patients have been recruited in over 22 months (while the expected accrual was 10-15 patients per year). **The drop-out rate has been significantly higher than expected with only 11 out of 16 participants contributing to complete data set. The expected drop-out rate was 5% while the actual drop-out rate has been 31%. The trial sample size needs to be re-calculated assuming that the drop-out rate will remain around 30%.** The competing EURAMOS 1 clinical trial will stop recruiting in June 2010. This will increase the number of eligible patients for the GLU 1 trial and boost recruitment rates.
SAEs and AEs reported between 10 October 2006 and 26 May 2009	**There were no AEs related to the IMP.** **Two SAEs were reported during this period, both unrelated to the IMP. The reported SAEs have not been considered to be a safety issue that might alter the current benefit-risk assessment.** **SAE 1** was reported on 18 October 07 regarding patient SHHI/GLU1/05; Short description of the SAE 1: acute renal impairment after treatment with high-dose methoterexate; reason for seriousness: prolongation of patient's hospitalization; causality assessment regarding the IMP: unrelated; causality assessment regarding protocol treatment apart from the IMP: related to MTX Resolution: 27 November 2007. **SAE 2** was reported on 27 March 08 regarding patient PAHE/GLU1/08; Short description of the SAE 2: right sided tension pneumothorax caused by patient's extensive pulmonary disease; reason for seriousness: life threatening required admission to Intensive Care Unit; causality assessment regarding protocol treatment apart from the IMP: unrelated; causality assessment regarding the IMP: unrelated; resolution: 09 April 2008
IMP shelf life extension	**The expiry date on the current Voraxaze trial stock (BN 2090601) was March 2009 (36 months post manufacture date). There is now stability data to support extension of the IMP shelf-life to 48 months.** **A substantial amendment to the protocol will be submitted in order to increase the IMP shelf-life to 48 months. Once the amendment is approved Protherics will over-label the study stock (38 vials at UCH Pharmacy and 40 vials at Harley Street Pharmacy) with the new expiry date: March 2010.**

Blinding of the study	DAMPA, the catabolic product of glucarpidase action on MTX, is known to cross-react with MTX in most commercial immunological MTX assays. Consequently MTX levels determined by commercial laboratories are unreliable following treatment with glucarpidase. In this study plasma MTX levels are measured by both immunoassay and high performance liquid chromatography (HPLC). Plasma DAMPA levels are measured by HPLC. Following each of the four MTX doses (on days 1 & 8 of cycles 1 and 2), blood samples are collected for MTX and DAMPA HPLC analysis at the following time points: Prior to starting MTX At the end of MTX infusion i.e. at 4 hours after starting MTX At 24 hours after starting MTX At 24.20 hours after starting MTX, i.e. 15 minutes after glucarpidase/placebo administration At 48 hours after starting MTX At 72 hours after starting MTX and then daily until MTX plasma levels <0.2 µmol/L. At 24 hours after the administration of MTX patients receive either glucarpidase or placebo. Glucarpidase rapidly converts MTX to DAMPA. Current literature suggests that 15 minutes after glucarpidase administration, plasma MTX concentrations decrease by >95%. When patients receive glucarpidase both MTX and DAMPA are identified and measured by HPLC. When patients receive placebo, only MTX is identified and measured. DAMPA is not present. Therefore it is obvious when patients receive glucarpidase and when they receive placebo. Therefore the study is not actually blind to the investigators. **We suggest that the design of the study is changed from "double-blind randomised" to "unblind randomised". In order to minimize any bias both "Day 15" assessments need to be performed by the on duty registrars and not the study chief or co-investigator.**

Blood samples for anti-glucarpidase antibody assessment and enzyme neutralisation assay	Blood samples for anti-glucarpidase antibody qualitative assessment and enzyme neutralisation assay are collected on days 0, 1, 8 and 15 of cycle 1 and days 1, 8, 15 and 30 of cycle 2. If anti-glucarpidase antibodies are present on day 30 of cycle 2, patients are followed up for a further blood test, at 3 and 6 months after starting cycle 2. The samples are analysed by Covance Laboratories Limited, Otley Road, Harrogate, North Yorkshire, HG3 1PY. There is a limited supply of one of the reagents used in the anti-glucarpidase antibody qualitative assay. In order to get the most use out of this reagent Covance can assay 30 samples per assay run. This means that sometimes it is not possible to have the results for the first time points (days 0, 1, 8 and 15 of cycle 1 and days 1, 8, 15 and 30 of cycle 2) and we collect the two further blood samples (at 3 and 6 months after starting cycle 2). Further to the above a recent email from Protherics states that a new method has been developed by BioAnaLab for the qualification (screening) assay, and they (Protherics) would eventually like to have all antibody samples screened by BioAnaLab. This will mean Covance will ship one aliquot of each antibody sample to BioAnaLab. Any samples identified as positive on the qualification by BioAnaLab will then be confirmed by a confirmatory assay. The percentage of samples confirmed as positive will be sent to Covance to undergo neutralization analysis. BioAnaLab would report to UCL/UCLH on the qualification and confirmation results. Covance would report to UCL/UCLH on the neutralization assay results. **We suggest that the protocol is amended so that blood samples for anti-glucarpidase antibody qualitative assessment and enzyme neutralisation assay are collected on days 0, 1, 8 and 15 of cycle 1 and days 1, 8, 15 and 30 of cycle 2 and at 3 and 6 months after starting cycle 2. The updated arrangements for anti-glucarpidase antibody qualitative analysis and enzyme neutralisation assay will also be included in the forthcoming protocol amendment.**

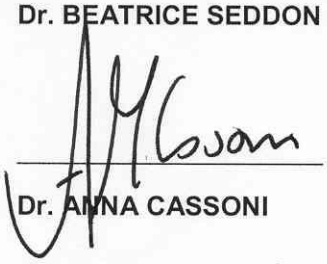
Forthcoming protocol amendment	The forthcoming protocol amendment will cover the following issues: Extension of IMP shelf life Amendment of the design of the study to unblind randomized Amendment of time points for collection of blood samples for anti-glucarpidase antibody assessment and enzyme neutralisation assay Administrative amendment: sponsor contact details for SAE submission Administrative amendment: new contact details for Protherics/BTG Administrative amendment: updated arrangements for anti-glucarpidase antibody qualitative assessment and enzyme neutralisation analysis



Dr. BEATRICE SEDDON

17th June 2009

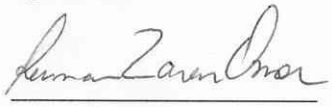
DATE



Dr. ANNA CASSONI

17/6/09

DATE



Dr. RUMANA OMAR

18/6/09

DATE

APPENDIX 16. ABSTRACTS – POSTERS

a. CTOS 2006 Oral Presentation Abstract

#611. INCIDENCE OF DELAYS IN CHEMOTHERAPY DUE TO METHOTREXATE TOXICITY IN TREATMENT OF OSTEOSARCOMA

M Perisoglou; * B Seddon; S Daniels; N Mayne; * J Whelan
University College Hospital, London, , United Kingdom

OBJECTIVES: To determine the incidence of delays in chemotherapy due to methotrexate (MTX) toxicity in patients with osteosarcoma, treated with doxorubicin, cisplatin and high-dose methotrexate (PAM).

METHODS: The medical records of 56 patients with osteosarcoma, treated with PAM in a single institution, were reviewed. Data were collected on chemotherapy dates, reasons of chemotherapy delays and folinic acid (FA) rescue regimens.

RESULTS: Fifty two percent of chemotherapy cycles (92/175) were delayed due to MTX toxicity (median 7 days, range 1-28). Causes of delay included mucositis in 51% of cycles, bone marrow suppression in 28%, infection (12%), nephrotoxicity (8%) and elevated liver enzymes (1%). Of 350 planned MTX courses, 5% (19/350) were omitted due to previous MTX toxicity. Treatment with MTX was discontinued in 10% (6/56) of patients. Two FA rescue regimens were used. In regimen A, FA dose was first adjusted according to 48 hour MTX levels. In regimen B, which replaced regimen A, FA was adjusted based on 24 hour MTX levels. Regimen A was used in 98 cycles of which 57% (56/98) were delayed due to MTX toxicity (median 7 days, range 1-28). Regimen B was used in 77 cycles of which 47% (36/77) were delayed (median 7 days, range 3-27).

CONCLUSIONS: The incidence of chemotherapy delays due to MTX toxicity in patients with osteosarcoma is high. However, early adjustment of folinic acid dose according to MTX levels at 24 hours appears to reduce the incidence of chemotherapy delay. Nevertheless, improving rescue from MTX toxicity is a worthwhile goal.

b. SIOP 2006 poster

INCIDENCE OF DELAYS IN CHEMOTHERAPY DUE TO METHOTREXATE TOXICITY IN TREATMENT OF OSTEOSARCOMA

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BACKROUND

High-dose methotrexate (MTX) at a dose of 12 g/m², in combination with vigorous hydration and urinary alkalinisation along with a pharmacokinetically guided folinic acid (FA) "rescue" schedule, is an essential component of osteosarcoma treatment.

It is given in combination with cisplatin and doxorubicin [PAM regimen: cisPlatin, doxorubicin (Adriamycin), Methotrexate].

Despite current supportive measures, MTX-induced toxicity (myelosuppression, mucositis, hepatic and renal toxicity) still occurs and results in increased morbidity, patient discomfort, increased costs and potentially reduced treatment efficacy, due to delays in chemotherapy.

Several studies[1-4] have indicated better outcome for these patients with less MTX toxicity. However, the true incidence of MTX toxicity which results in chemotherapy delays is unknown.

OBJECTIVES

To determine the incidence of delays in chemotherapy due to MTX toxicity in patients with osteosarcoma, treated with doxorubicin, cisplatin and high-dose methotrexate (PAM).

METHODS

The medical records of 56 patients with osteosarcoma, treated with PAM in the University College Hospital, London, UK, over the last 2 years, were reviewed. Data were collected on chemotherapy dates, reasons of chemotherapy delays and folinic acid (FA) rescue regimens.

RESULTS

- Fifty two percent of chemotherapy cycles (92/175) were delayed due to MTX toxicity (median 7 days, range 1-28) (table 1).
- Causes of delay included mucositis in 51% of cycles, bone marrow suppression in 28%, infection (12%), nephrotoxicity (8%) and elevated liver enzymes (1%) (figure 1).
- Of 350 planned MTX courses, 5% (19/350) were omitted due to previous MTX toxicity. Treatment with MTX was discontinued in 10% (6/56) of patients.
- Two FA rescue regimens were used. In regimen A, FA dose was first adjusted according to 48 hour MTX levels. In regimen B, which replaced regimen A, FA was adjusted based on 24 hour MTX levels.
- Regimen A (late FA rescue) was used in 98 cycles of which 57% (56/98) were delayed due to MTX toxicity (median 7 days, range 1-28) (tables 1, 2a, 2b).
- Regimen B (early FA rescue) was used in 77 cycles of which 47% (36/77) were delayed due to MTX toxicity (median 7 days, range 3-27) (tables 1, 2a, 2b).

CONCLUSIONS

- The incidence of chemotherapy delays due to MTX toxicity in patients with osteosarcoma is high.
- However, adjustment of folinic acid dose according to MTX levels at 24 hours appears to reduce the incidence of chemotherapy delay by 20%, compared with adjustment at 48 hours (47% vs. 57%).
- Nevertheless, improving rescue from MTX toxicity is a worthwhile goal.

INCIDENCE OF DELAYED CHEMOTHERAPY CYCLES DUE TO METHOTREXATE TOXICITY (table 1)

	Total number of cycles	Delayed cycles	Incidence of delay	Median delay (range)
Regimen A (Late rescue)	98	56	57%	7 (1-28)
Regimen B (Early rescue)	77	36	47%	7 (3-27)
Both regimens	175	92	52%	7 (1-28)

INCIDENCE OF CHEMOTHERAPY DELAY PER CYCLE (table 2a)

BOTH REGIMENS

Cycles	Incidence of delay	Median delay (range)
Cycle 2	52% (29/56)	7 (3-28)
Cycle 3	64% (14/21)	7 (1-28)
Cycle 4	39.5% (15/38)	7 (3-17)
Cycle 5	57% (17/31)	8 (2-27)
Cycle 6	80.7% (17/28)	7 (3-16)

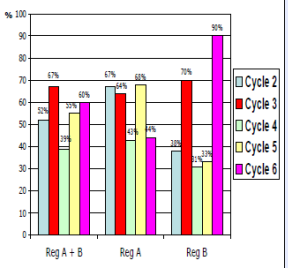
REGIMEN A (late adjustment)

Cycles	Incidence of delay	Median delay (range)
Cycle 2	66.6% (18/27)	7 (3-13)
Cycle 3	63.6% (7/11)	7 (3-10)
Cycle 4	43.5% (10/23)	7 (3-17)
Cycle 5	68.5% (13/19)	9 (4-27)
Cycle 6	44.5% (8/18)	7 (4-13)

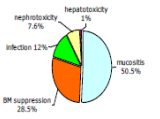
REGIMEN B (early adjustment)

Cycles	Incidence of delay	Median delay (range)
Cycle 2	38% (11/29)	7 (5-28)
Cycle 3	70% (7/10)	7 (1-28)
Cycle 4	31% (5/16)	9 (6-15)
Cycle 5	33% (4/12)	6.5 (2-17)
Cycle 6	90% (9/10)	7 (3-16)

INCIDENCE OF CHEMOTHERAPY DELAY PER CYCLE (table 2b)



CAUSES OF DELAY (figure 1)



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38th Congress of SIOP, 17-21 September 2006, Geneva, Switzerland

c. CTOS 2011 Oral Presentation Abstract

2011 Combined Meeting of the
CONNECTIVE TISSUE ONCOLOGY SOCIETY
and the MUSCULOSKELETAL TUMOR SOCIETY

Friday, October 28, 2011 5:40 PM MALIGNANT BONE TUMORS II

PAPER #82

A RANDOMISED, CROSS-OVER, PHASE II STUDY, TO INVESTIGATE THE EFFICACY AND SAFETY OF GLUCARPIDASE FOR ROUTINE USE AFTER HIGH DOSE METHOTREXATE IN PATIENTS WITH BONE SARCOMA: INTERIM ANALYSIS

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Objective: High dose methotrexate (HD-MTX) is a critical component of adjuvant chemotherapy for osteosarcoma but, despite adequate folinic acid rescue (FAR), toxicity is common and frequently causes delay in subsequent chemotherapy. Glucarpidase (GLU) rapidly cleaves MTX into inactive metabolites and has been used extensively in the emergency management of acute severe MTX toxicity. We are conducting a phase II study to investigate whether adding GLU to routine rescue after HD-MTX reduces delay to subsequent chemotherapy in patients with bone sarcoma.

Methods: Patients are randomised to receive two courses of HD-MTX with FAR (cycle M) followed by two courses of HD-MTX with GLU and FAR (cycle GluM), or to receive cycle GluM followed by cycle M. The primary endpoint is assessment of fitness to receive chemotherapy on day 15 of each cycle ('Day 15' criteria). An interim analysis was carried out after data for all courses of treatment for 50% of the planned number of patients were obtained.

Results: Prior to interim analysis sixteen patients [median age: 19 years (range: 13-47 years)] were enrolled and 27 treatment cycles administered. Among them, fifteen cycles (56%) were given with GLU and FAR and twelve cycles (44%) with FAR. 'Day 15' criteria were met in 8/15 (53%) of the cycles given with GLU and FAR, and in 3/12 (25%) cycles with FAR alone. Reasons for not meeting the 'day 15' criteria included impaired renal function, delayed methotrexate elimination and mucositis. No adverse events related to GLU occurred.

Conclusion: GLU in combination with FAR may have a favourable effect in reducing delays to further chemotherapy after HD-MTX. Accrual to the study continues and will identify the degree of benefit. Additional analyses of quality of life and economic savings are incorporated.

♦ Indicates those faculty presentations in which the FDA has not cleared the drug and/or medical device for the use described (i.e., the drug or medical device is being discussed for an "off label" use) and •FDA information not available at the time of printing. *For full information, refer to inside back cover.*

d. SIOP 2012 poster

1st Category: d Bone tumours

2nd Category: 2 Clinical Research

Title: EFFICACY AND SAFETY OF GLUCARPIDASE FOR ROUTINE USE AFTER HIGH DOSE METHOTREXATE IN PATIENTS WITH OSTEOSARCOMA

Author(s): **Martha Perisoglou**[1,2,3], Leonardo Trani[1], Sandra Strauss[1], John Hartley[3], Janet Hartley[3], Beatrice Seddon[1], Emma Thomas[4], Jeremy Whelan[1]

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Text: **Purpose:** High-dose methotrexate (HD-MTX) is an essential component of osteosarcoma treatment. Despite supportive measures MTX-related toxicity results in delays in subsequent chemotherapy administration and potentially reduced treatment efficacy. It is essential to explore alternative rescue regimens such as routine use of glucarpidase.
Method: GLU 1, a phase II randomised cross-over clinical trial, was set up to examine the efficacy and safety of routine use of glucarpidase after HD-MTX. Patients were randomised to receive two HD-MTX courses with FA rescue (cycle FA) followed by two HD-MTX courses with FA and glucarpidase (cycle glu/FA), or cycle glu/FA first followed by cycle FA. The primary objective of the trial was to examine whether glucarpidase rescue after HD-MTX reduces delay to subsequent cycles of chemotherapy due to MTX toxicity. The data of sixteen patients enrolled up to the interim analysis of the trial were analysed.
Result: MTX toxicity resulted in delays in 47% of glu/FA cycles and 75% of FA cycles. There was no difference in MTX peak plasma concentrations between the two rescue regimens. More importantly the use of glucarpidase was not associated with a statistically significant reduction of MTX AUC. Severe mucositis-clinical (CTCAE v3.0, grade ≥ 3) complicated 23% and 7% of FA and glu/FA cycles, respectively. Mucositis-functional (CTCAE v3.0, grade ≥ 3) complicated 8% FA treatment cycles and none of glu/FA cycles. The incidence and severity of MTX-related nephrotoxicity was similar in both rescue regimens. No glucarpidase toxicity was observed.
Conclusion: Glucarpidase offers a promising opportunity for rescue from MTX toxicity. The GLU 1 clinical trial is ongoing.

Author Keywords: methotrexate, glucarpidase, toxicity, osteosarcoma, rescue, folinic acid