STRATEGIES TO ENHANCE THE EFFICACY OF CHEMOTHERAPY

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By

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I confirm that the work presented in this thesis is my own.

Abstract

Chemotherapy, alongside radiotherapy and surgery remains the mainstay of treatment for many cancers. However as our understanding of the biology of malignancy improves targeted therapies are being developed, which need to be integrated with existing standard chemotherapeutics. This thesis discusses three studies that aim to address this challenge.

In the first part of this thesis, the activity of lomeguatrib, a novel agent that acts as a pseudosubstrate for the DNA-repair protein MGMT, was investigated in a range of solid tumours. Following a single administration, it was found to deplete MGMT in primary CNS, colorectal and prostate cancers. The lomeguatrib dose required to achieve this was independent of pre-treatment MGMT expression in these tumour types.

MGMT expression appears to correlate with sensitivity to irinotecan, a standard treatment for metastatic colorectal cancer. Combination treatment with lomeguatrib and irinotecan was investigated in patients with metastatic colorectal cancer. Treatment with this couplet was tolerable, no pharmacokinetic drug interactions were seen and complete MGMT depletion was observed. In this heavily pre-treated group of patients, there was no increase in efficacy with treatment with the couplet over irinotecan alone.

The latter part of the thesis describes an ongoing early phase trial investigating combination treatment with decitabine, a hypomethylating agent and standard ECF chemotherapy in patients with advanced oesophagogastric cancer. The maximum tolerated dose of decitabine in this combination was established, the main toxicity myelosuppression was manageable. Preliminary tumour DNA pyrosequencing results confirmed changes in methylation of the *MAGE1A* gene in tumour tissue associated

with immunohistochemical changes in protein product expression of a number of genes following decitabine treatment.

These studies have described early phase work for two very different targeted treatment approaches that may be used alongside standard chemotherapy. Clinical studies to explore both of these further are planned.

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Abbreviations

A	adenine
ADP	adenosine diphosphate
AE	adverse event
ALT	alanine transaminase
AML	acute myeloid leukaemia
APS	adenosine 5' phosphosulphate
AST	aspartate transaminase
ATP	adenosine triphosphate
ATP7A	adenosine triphosphatases 7A
ATP7B	adenosine triphosphatases 7B
AUC	area under plasma concentration-time curve
5-aza-dCMP	5-aza-deoxycytidine monophosphate;
5-aza-dCTP	5-aza-deoxycytidine triphosphate
BCNU	1, 3-bis- (2-chloroethyl)-1-nitrosurea
BCRP	breast cancer resistance protein
BER	base excision repair
BSA	body surface area
BSA	bovine serum albumin
BSC	best supportive care
C	cytosine
CBG	coomassie brilliant blue G250
CCNU	1-(2-chloroethyl)-3-cyclohexyl-1-nitrosurea
cdks	cyclin dependent kinases
cDNA	complementary DNA
CE	carboxyl esterase
CEA	carcinoembryonic antigen
CI	confidence interval
CIMP	CpG-island methylation phenotype
C_{max}	peak plasma concentration
CML	chronic myeloid leukaemia
cMOAT	cannalicular multispecific organic anion transporter/MRP2
CNS	central nervous system
CpG	cytosine-guanine repeats

cpm	counts per minute
CR	complete response
CsCl	caesium chloride
CT	computerised tomography
CTC	National Cancer Institute Common Toxicity Criteria
CV%	coefficient of variation
DAB	diaminobenzidine
DEPT	directed prodrug enzyme therapy
dl	decilitre
DLT	dose limiting toxicity
DNA	deoxyribonucleic acid
DNMT	DNA methyltransferase enzymes
DNMTIs	DNA methyltransferase enzyme inhibitors
DPD	dihydropyrimidine dehydrogenase
dTMP	deoxythymidine monophosphate
dTTP	deoxythymidine triphosphate
dUMP	deoxyuridine monophosphate
dUTP	deoxyuridine triphosphate
ECF	epirubicin, cisplatin and 5-FU
ECOG	Eastern Cooperative Oncology Group
ECX	epirubicin, cisplatin and capecitabine
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EORTC	European Organisation for Research and Treatment of Cancer
EOX	epirubicin, oxaliplatin and capecitabine
ER	oestrogen receptor
FdUMP	5-fluoro-2'-deoxyuridine monophosphate
FdUTP	5-fluoro-2'-deoxyuridine triphosphate
fmol	femtomoles
5-FU	5-fluorouracil
FUDP	fluorouridine diphosphate
FUMP	fluorouridine monophosphate
FUTP	fluorouridine triphosphate
g	gravitational force
g	grams

G	guanine
GICNO	Gruppo Italiano Cooperativo di Neuro-Oncologia
Gmean	geometric mean
GOJ	gastro-oesophageal junction
HATs	histone acetyltransferases
HCl	hydrochloric acid
HDAC	histone deacetylase
HDACi	histone deacetylase inhibitor
HDGC	hereditary diffuse gastric cancer syndrome
HIV	human immunodeficiency virus
HMTs	histone methyltransferases
HNPCC	hereditary non-polyposis colon cancer
HP-1	heterochromatin protein-1
HR	homologous repair / hazard ratio
HRP	horseradish peroxidase
IAP	inhibitor of apoptosis
IBSA	bovine serum albumin in buffer I
ICE assay	topoisomerase I cleavage complex formation assay
ICF	immunodeficiency centromeric instability and facial abnormalities syndrome
IL-2	interleukin-2
I.P.	intraperitoneal
IU	international units
i.v.	intravenous
KCl	potassium chloride
kg	kilogram
l	litre
LD	longest diameter
LV	leucovorin
MAGE1A	melanoma antigen family 1A
MBD	methyl-CpG-binding domain proteins
MDR	multidrug resistance phenotype
MDS	myelodysplastic syndrome
mg	milligram
MgCl	magnesium chloride

MGMT	O^6-methylguanine-DNA-methyltransferase
$MgSO_4$	magnesium sulphate
ml	millilitre
mM	millimolar
mm	millimetre
MMR	mismatch repair
mRNA	messenger ribonucleic acid
MRP	multidrug resistance phenotype–associated proteins
MSI	microsatellite instability
MS-PCR	methylation specific polymerase chain reaction
MT	metallothionein
MTD	maximum tolerated dose
NaOH	sodium hydroxide
NCIC	National Cancer Institute of Canada
NHEJ	non-homologous end joining
µg	micrograms
µm	micrometre
µmol	micromolar
µl	microlitre
NPC	7-ethyl-10-[4-(1-piperidino)-1-amino]carbonyloxycamptothecin
O^6-BG	O^6-benzylguanine
O^6-MG	O^6-methylguanine
PBMCs	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PBS-Tween	phosphate buffered saline and Tween-20
Pc	Polycombe
PCA	perchloric acid
PCR	polymerase chain reaction
PD	pharmacodynamic / progressive disease
PICC	percutaneous indwelling central catheter
PPE	palmar plantar erythema
PPi	pyrophosphate
PR	partial response
PS	performance status
pRB	retinoblastoma gene

RECIST	response evaluation criteria in solid tumours
RNA	ribonucleic acid
rpm	revolutions per minute
RR	response rate
SAHA	suberoylanilide hydroxamic acid
s.c.	subcutaneous
SD	stable disease / standard deviation
SET	Su(var)3-9, Enhancer-of-zeste, Trithorax domain
SI	small intestine
SN-38	7-ethyl-10-hydroxycamptothecin
T	thymidine
TE	trizma and 1mM EDTA
TE*	trizma and 10mM EDTA
t_{max}	time point at C_{max}
TMZ	temozolomide
TNE	tris base, EDTA and sodium chloride
TNF-α	tumour necrosis factor- α
TP	thymidine phosphorylase
TS	thymidylate synthase
TTP	time to progression
UK	United Kingdom
ULN	upper limit of normal
UV	ultra-violet
VHL	Von-Hippel Lindau gene
vs.	versus
w/v	weight per volume
WHO	World Health Organisation

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1. Introduction

1.1 How chemotherapy works

A myriad of chemotherapeutic agents are now available for the treatment of cancer including DNA damaging agents, hormones, targeted treatments and immunotherapy. Since the discovery and application of the first cytotoxic agent, nitrogen mustard, in the early 1940s, these drugs have been used to treat a wide variety of solid malignancies with varying levels of success. Classical cytotoxic agents act through damaging cellular DNA via a range of different mechanisms (see next section). The main classes of cytotoxic agents include: anthracyclines and other antitumor antibiotics, alkylating agents, antimetabolites, spindle poisons, platinums and topoisomerase inhibitors.

As our knowledge of the molecular basis of cancer has increased, cytotoxic agents have been combined with a number of other therapeutics to try to improve efficacy. Endocrine therapy is an important and effective modality in the treatment of a number of hormonally sensitive tumours including cancers of the breast [anti-oestrogens (e.g. tamoxifen), aromatase inhibitors (e.g. anastrazole)], endometrium [progestogens (e.g. megestrol acetate)] and prostate [antiandrogens (e.g. biclutamide), luteinizing hormone-releasing hormone analogues (e.g. goserelin) and oestrogens (e.g. diethylstilboestrol)]. Corticosteroids have an important role in the management of haematological malignancies e.g. lymphoid leukaemias and in supportative care as anti-emetics and appetite promoters.

Drug therapy may be targeted against cancer cells via a number of different mechanisms. Passive targeting based on, for example the rate of blood flow or vascularity of a tumour, occurs with all systemic therapy. Regional chemotherapy, such

as intrahepatic arterial chemotherapy, may allow the delivery of higher concentrations of chemotherapy locally than would be tolerated if given systemically. True targeting may be achieved with the use of monoclonal antibodies, for example bevacizumab and rituximab, or radiolabelled antibody therapy for example iodine-131-labelled tositumomab (Bexxar™). Directed produg enzyme therapy (DEPT) involves a 2-step approach for targeted therapy. In VDEPT (virus-directed enzyme prodrug therapy), a replication defective virus encoding an enzyme is administered directly into a tumour followed by a prodrug which is selectively converted to an active cytotoxic drug by the enzyme, for example the adenovirus encoding nitroreductase (CTL102) and the prodrug CB1954 (Palmer *et al.*, 2004). Several other prodrug/enzyme systems for cancer therapy may also be used including gene-directed enzyme prodrug therapy (GDEPT) and antibody-directed enzyme prodrug therapy (ADEPT). Targeted therapy may be pathway-defined, for example imatinib and tyrosine kinase dependent-pathways including bcr-abl in chronic myeloid leukaemia. Microenvironment defined therapies are under development: AQ4N, an aliphatic amine N-oxide is bioreduced selectively in hypoxic regions of solid tumours to generate a cytotoxic metabolite. Antisense oligonucleotides for example G3139 (Oblimersen™) against bcl-2 are another form of targeted therapy.

Immunotherapeutic agents produce anti-tumour effects by modifying the host response to tumour cells or by other biological effects on tumour viability or growth. Approaches used in biotherapy include: active non-specific immunotherapy using interferon and interleukins, adoptive immunotherapy (cellular therapy) with the delivery of antigen specific or non-specific lymphocytes and other cells such as dendritic cells to the cancer host and specific immunotherapy with cancer vaccines. There are a variety of vaccines

in clinical use including peptides, whole proteins, DNA vaccines, cell lysates and whole cell products.

1.2 DNA-damaging agents

Over 50 drugs are in routine clinical use today. Space does not permit a discussion of all their properties, and the following concentrates on the drugs used to treat upper gastrointestinal cancers and melanoma relevant to the research presented in this thesis.

1.2.1 Methylating and chlorethylating agents

Cytotoxic methylating agents, including temozolomide, streptozotocin, procarbazine and dacarbazine, are used in the treatment of melanoma, gliomas, carcinoid tumours and Hodgkin's lymphoma. They damage DNA by forming adducts with bases (Frei *et al.*, 1978), the most common of which occur at the N7 position of guanine and the N3 position of adenine, which are repaired by the base excision repair (BER) mechanism. However, the most important lesion formed, in terms of mutagenesis, carcinogenesis and cytotoxicity, is O^6-methylguanine (O^6-MG).

O^6-chloroethylguanine adducts are formed by 1, 3-bis- (2-chloroethyl)-1-nitrosurea (BCNU), 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosurea (CCNU) and related compounds (Gonzaga & Brent, 1989). BCNU is widely used to treat gliomas, lymphomas and for high dose therapy with autologous transplantation for lymphomas, CCNU is used in the treatment of brain tumours and Hodgkin's disease. Most O^6-chloroethylguanine lesions are converted to G-C inter-strand cross-links, blocking DNA replication. Both O^6-MG and O^6-chloroethylguanine adducts, the latter prior to cross-linking, may be repaired by O^6-methylguanine-DNA-methyltransferase (MGMT). If un-repaired, these adducts

trigger futile DNA mismatch repair, G2 arrest, and ultimately cell death by a combination of senescence and mitotic catastrophe.

1.2.2 Platinums

Cisplatin, one of the most widely used anticancer drugs, is routinely used to treat a wide range of malignancies, including upper gastrointestinal, ovarian, lung, head and neck and bladder cancer. Cisplatin forms strong covalent bonds by displacing nucleophilic atoms from DNA preferentially at the N7-postion of guanine and adenine and forming intra-strand cross-links the extent of which correlate with cytotoxicity (Eastman, 1986; Zwelling *et al.*, 1979). DNA platinum mono-functional adducts are formed initially, that may react further to form intra-strand cross-links between adjacent guanine DNA bases, inter-strand cross-links to complementary guanine DNA bases located on the opposite strand (rarer) or DNA-platinum-protein cross-links. Structural analysis of the cisplatin guanine-guanine intra-strand cross-link, the main cytotoxic lesion, has shown DNA distortion and unwinding. These intra-strand DNA adducts lead to replication arrest, activation of the mismatch repair (MMR) pathway, cell cycle checkpoint activation, sustained G2 arrest and cell death.

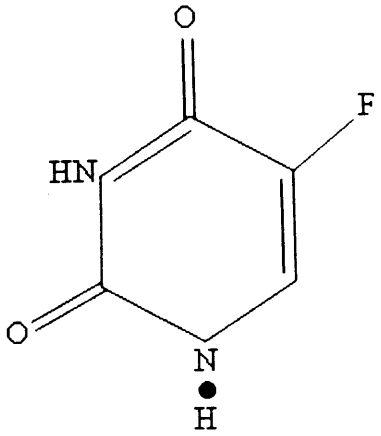
1.2.3 Fluoropyrimidines

Pyrimidine antagonists act as pseudo-nucleotides, interacting with thymidylate synthetase and folate cofactors, resulting in their incorporation into DNA and RNA. Cells exposed to pyrimidine antagonists demonstrate reduced DNA synthesis, reduced RNA processing and reduced protein synthesis, ultimately resulting in inhibition of cell growth. 5-fluorouracil (5-FU) has an important role in the treatment of gastrointestinal cancer and squamous cell carcinomas of the head and neck. Synergistic reactions have

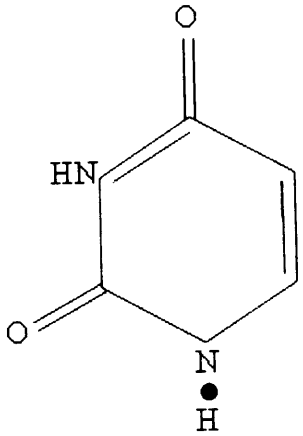
also been seen between 5-FU and other antitumor agents, irradiation and physiological nucleosides.

5-fluorouracil is based on the chemical structure of uracil (Figure 1) and acts as a false-pyrimidine base. It enters cells via a carrier-mediated process and subsequently undergoes phosphorylation by thymidine phosphorylase, followed by thymidine kinase resulting in the formation of the active metabolite 5-fluoro-2'-deoxyuridine monophosphate (FdUMP) (Figure 2). In the presence of the reduced folate cofactor 5, 10-methylenetetrahydrofolate, FdUMP forms a stable covalent complex with thymidylate synthase (TS). TS catalyzes the only intracellular *de novo* formation of deoxythymidine monophosphate (dTMP). Inhibition of TS leads to depletion of deoxythymidine triphosphate (dTTP), thereby interfering with DNA synthesis and repair.

Figure 1. 5-fluorouracil and uracil



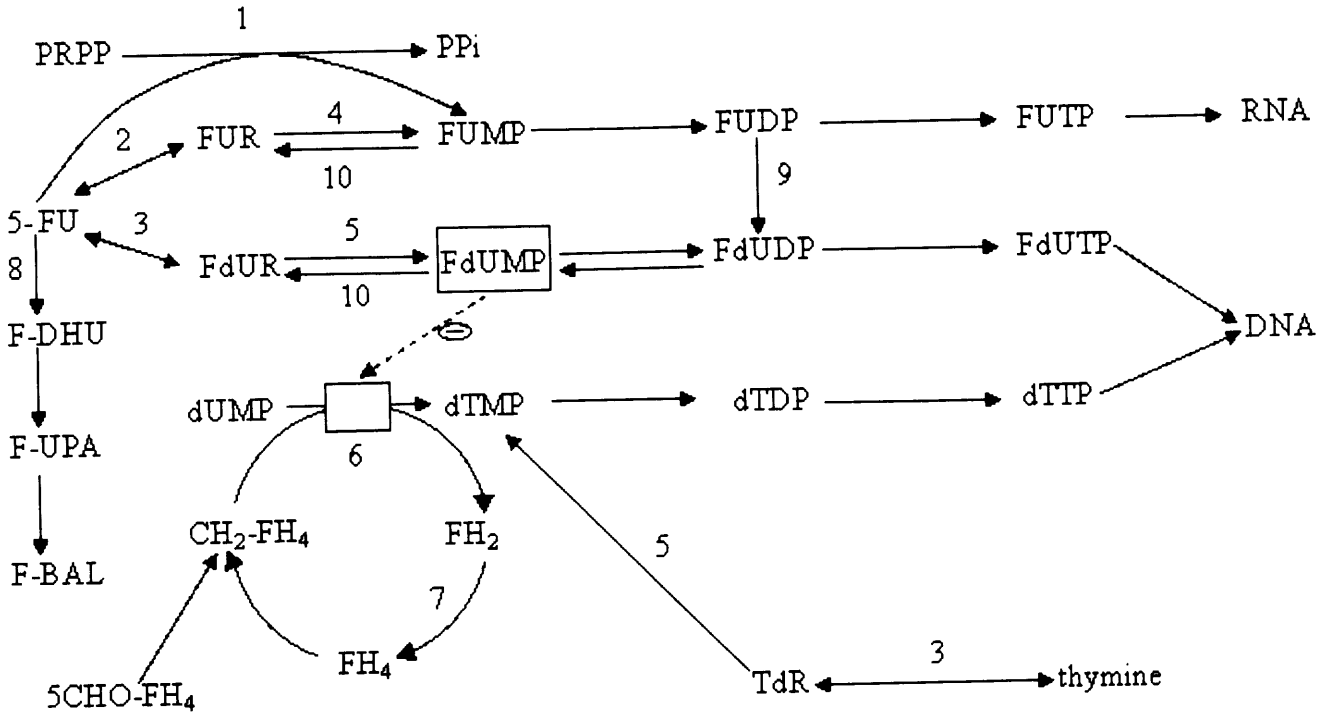
5- fluorouracil



Uracil

Figure 2. Schematic representation of 5-fluorouracil metabolism

Key: 5-FU=5-fluorouracil; FdUR= 5-fluoro-2`-deoxyuridine; FdUMP= 5-fluoro-2`-deoxyuridine monophosphate; dUMP= deoxyuridine-5`-monophosphate; dTMP= deoxythymidine monophosphate; dTDP= deoxythymidine diphosphate; dTTP= deoxythymidine triphosphate; FUMP= fluorouridine monophosphate; FUDP= fluorouridine diphosphate; FUTP= fluorouridine triphosphate; FUR= 5-fluorouridine; F-DHU= 5-fluorodihydrouracil; F-UPA= fluoroeidopropionate; F-BAL= α-fluoro-ß-alanine; PRPP= 5-phosphoribosyl-1-pyrophosphate; TdR=thymidine; Enzymes catalysing reactions: 1, orotic acid phosphoribosyltransferase; 2, uridine phosphorylase; 3, thymidine phosphorylase.; 4, uridine kinase; 5, thymidine kinase; 6, thymidylate synthase; 7, dihydrofolate reductase; 8, dihydropyrimidine dehydrogenase; 9, ribonucleotide reductase; 10, 5`-nucleotidases and phosphatases. Inhibition of thymidylate synthase by FdUMP is represented by ⊖.



5-FU is also metabolized to fluorouridine monophosphate (FUMP) through the sequential action of uridine phosphorylase and uridine kinase. In the presence of 5'-phosphoribosyl-1-pyrophosphate, orotic acid phosphoribosyltransferase directly converts 5-FU to FUMP. This is further metabolized to fluorouridine diphosphate (FUDP) and then to the triphosphate form (FUTP), which is subsequently incorporated into both nuclear and cytoplasmic RNA interfering with normal RNA processing and function (Longley *et al.*, 2003).

The cytotoxicity of 5-FU is due to the inhibition of TS by FdUMP. The TS-FdUMP-folate ternary complex is slowly dissociable, and the intracellular level of 5, 10-methylenetetrahydrofolate is critical for ternary complex formation as well as for maintaining enzyme inhibition. Leucovorin (5-formyltetrahydrofolate) enhances the cytotoxicity of 5-FU by expanding the intracellular pool of 5, 10-methylenetetrahydrofolate and thereby increasing the extent and duration of TS inhibition.

Inhibition of TS leads not only to depletion of dTTP, but also to the accumulation of deoxyuridine monophosphate (dUMP). Subsequently both dUMP and FdUMP may be metabolized to their respective triphosphate forms. Incorporation of deoxyuridine triphosphate (dUTP) and fluorodeoxyuridine triphosphate (FdUTP) into cellular DNA, with resultant inhibition of DNA synthesis and function, may represent another mechanism of cytotoxicity. Deoxyuridine triphosphate hydrolase degrades triphosphate nucleotides and limits the intracellular accumulation of (F)dUTP. The nucleotide excision repair enzyme uracil-DNA glycosylase may repair DNA that contains uracil and 5-FU; however, this will be unsuccessful if the intracellular nucleotide ratio favours (F)dUTP over dTTP.

The combined effects of dTTP depletion and (F)dUTP-DNA incorporation result in inhibition of nascent DNA chain elongation, altered DNA stability, production of DNA single-strand breaks, and interference with DNA repair. The genotoxic stress resulting from TS inhibition may also activate programmed cell death pathways in susceptible cells, which leads to induction of parental DNA fragmentation. Factors operating downstream from TS, e.g., Bcl-2 and p53, also influence the cellular responses to such genotoxic stress (Fisher *et al.*, 1993; Lowe *et al.*, 1993).

1.2.4 Anthracyclines

Anthracyclines are widely used anti-neoplastic agents, commonly used in the management of carcinomas of the upper gastrointestinal tract, breast, lung, ovary, sarcomas, childhood malignancies and haematological malignancies. Their precise mechanism of action however remains controversial because of their complex pharmacology. Early studies demonstrated that the bulk of the intracellular drug is nuclear and the flat, planar anthracycline molecule is intercalated into DNA thereby inhibiting DNA and RNA synthesis (Zunino *et al.*, 1975). Later studies suggested that the quinone structure of anthracyclines enhanced the catalysis of reduction-oxidation reactions, promoting the generation of oxygen free radicals that also contributed to cytotoxicity unrelated to strand cleavage (Muller *et al.*, 1997). This has been confirmed in the clinical setting, where oxidised DNA bases have been found in the lymphocytes of patients treated with epirubicin (Olinski *et al.*, 1997).

An important advance in understanding anthracycline-DNA interactions came with the discovery that the anthracyclines inhibited topoisomerase II re-ligation by trapping DNA strand passage intermediates resulting in the accumulation of protein-linked

double- and single-strand DNA breaks (cleavable complexes), which ultimately resulted in cell death (Pommier, 1993). These seminal observations subsequently led to the general acceptance of this molecular mechanism as being most important for anthracycline-induced antitumor activity. However, the precise mechanism by which the anthracyclines stabilize DNA topoisomerase II cleavable complexes has not been fully defined and may be independent of DNA intercalation. Furthermore, the pattern of cleavable complex formation in DNA induced by specific anthracyclines can vary, suggesting that differences may exist between these drugs at the molecular level (Doroshow, 2006).

Anthracyclines such as daunorubicin and doxorubicin can also directly inhibit cellular helicases, enzymes that unwind DNA into single strands, thereby limiting replication (Bachur *et al.*, 1992) and they may also have direct inhibitory effects on topoisomerase II independent of cleavable complex stabilization acting in part as true topoisomerase enzyme inhibitors (Doroshow, 2006).

Anthracyclines are membrane-active compounds that produce a wide range of effects at the cell surface, which may be related to cytotoxicity and DNA damage as well as affecting signal transduction pathways. These pleiotropic effects have been linked to the induction of apoptosis through a number of mechanisms including activation of p53. As a consequence of their diverse molecular effects, the ultimate mechanism of cytotoxic action of the anthracyclines may involve multiple different pathways.

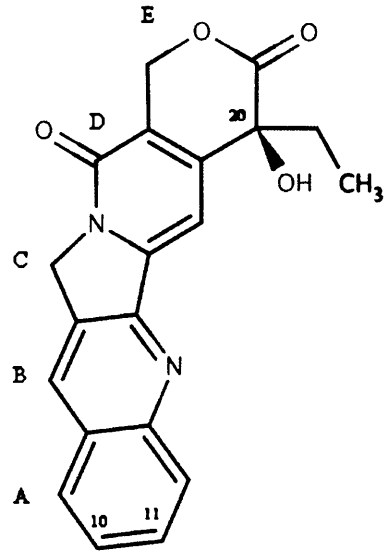
1.2.5 Topoisomerase-I inhibitors

Irinotecan, a camptothecin analogue, is a topoisomerase I-targeting agent, commonly used in the treatment of colorectal cancer. All camptothecin analogues have a basic 5-

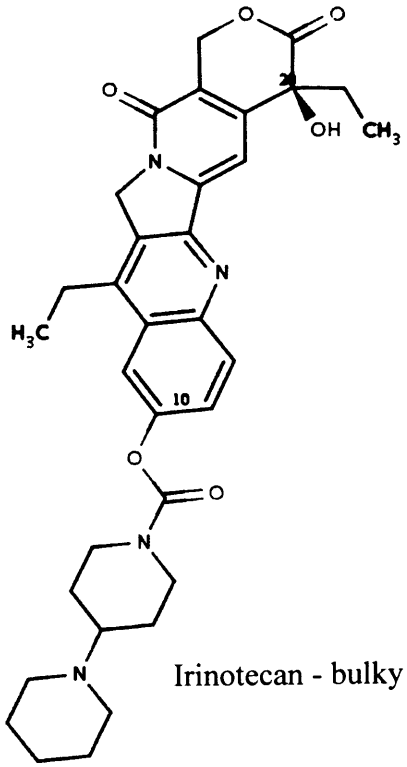
ring structure (A-E). The lactone form, with a closed E-ring, is the active form and replacement of the α-hydroxyl group at position 20 on the E-ring is essential for cytotoxicity (Figure 3). The pro-drug irinotecan is unique amongst camptothecin derivatives because of the bulky piperidine side chain at the 10 position on the A-ring, which is enzymatically cleaved by various types of carboxylesterases to generate an active water-insoluble metabolite, SN-38 (Arbuck & Takimoto, 1998; Slichenmyer *et al.*, 1993).

The principal target of camptothecin analogues is DNA topoisomerase I, a ubiquitous enzyme primarily localised in the nucleolus, that catalyses the relaxation of super-coiled DNA during replication, transcription and recombination (Arbuck & Takimoto, 1998; Slichenmyer et al., 1993). Camptothecin analogues induce DNA damage by interfering with the catalytic cycle of topoisomerase I; reversibly binding to the topoisomerase-I-DNA complex, stabilising the intermediate complex and in effect slowing religation. Stabilization of the cleavable complex in itself is not sufficient to induce cell death, but the interaction between the advancing replication fork with the drug-stabilized topoisomerase-I-DNA complex results in lethal irreversible arrest of DNA replication, a DNA double strand break at the fork and apoptosis. This would suggest that camptothecin analogues are selectively cytotoxic during the S-phase of the cell cycle, but S-phase independent cytotoxicity is also seen which may be due to the inhibition of transcription during G1/G2 phases (Bendixen *et al.*, 1990; Goldwasser *et al.*, 1996).

Figure 3. Chemical structures of Camptothecin analogues



Camptothecin - 5-ring structure (A-E)



Irinotecan - bulky piperidine side chain at position 10 of ring A

1.3 Resistance to treatment

As our understanding of the mechanisms of action of chemotherapeutic agents has increased it has become possible to identify intracellular processes that contribute to cellular resistance to cytotoxics. In simple terms, a drug must first get to the cell, be transported across the cell membrane, it may then require activation and finally bind with a target, which may require processing in order for the drug to have its lethal effect. Drug resistance may arise from modification of any or all of these steps.

1.3.1 Upstream resistance

1.3.1.1 Impaired transport to the cell

Impaired drug absorption may result in inadequate cellular concentrations of cytotoxics resulting in drug resistance. This is exemplified by etoposide which has a very variable pharmacokinetic profile when administered orally resulting in suboptimal dosing in a proportion of patients and drug resistance. Drug transport may also be impaired by poor vascular access. The microvasculature of tumours is not highly organised; some areas may have relatively poor blood supply and therefore the cellular concentrations of cytotoxics achieved in these areas may be low and result in drug resistance.

1.3.1.2 Impaired transport of drug across cell membrane

Intracellular access may occur via facilitated transport, whereby the drug binds to a protein receptor on the cell membrane, the protein-drug complex is then translocated and the drug is released into intracellular space. Alterations in the structures of protein receptors or reduction in number of receptors will therefore significantly impair active

transport of the drug. This will result in lower intracellular drug concentration, which may then not be sufficient to cause cytotoxicity.

Low intracellular drug concentrations are also seen with active efflux of drugs. Platinums undergo active efflux via the P-glycoprotein–associated multidrug resistance (MDR) phenotype–associated proteins (MRP) e.g. cMOAT (cannalicular multispecific organic anion transporter/MRP2) and copper-transporting P-type adenosine triphosphatases 7A and 7B (ATP7A, ATP7B). Independent over-expression of cMOAT, ATP7A and ATP7B has been shown in some cisplatin-resistant human cancer cell lines exhibiting decreased intracellular platinum accumulation and acquired cisplatin resistance. Transfection of an antisense cMOAT complementary DNA into HepG2 cells results in decreased cMOAT protein levels and a five-fold increase in cisplatin sensitivity (Koike *et al.*, 1997). Conversely, transfection of epidermoid carcinoma cells with ATP7B led to a nine-fold decrease in cisplatin sensitivity (Komatsu *et al.*, 2000).

Irinotecan and its metabolite SN-38 do not appear to be substrates for the MDR drug efflux and cross-resistance to irinotecan is not seen in MDR-over-expressing cell lines that are highly resistant to vincristine and doxorubicin (Tsuruo *et al.*, 1988). However, the MDR–associated protein-1 (MRP-1) has been associated with resistance to SN-38 and 9-aminocamptothecin (Rasheed & Rubin, 2003) . The breast cancer resistance protein (BCRP) has also been implicated in resistance to irinotecan *in vivo* (Candeil *et al.*, 2004; Doyle & Ross, 2003). All anthracyclines are substrates for the P-glycoprotein–mediated drug efflux pump, and MPR-1–associated pleiotropic drug resistance may be an important determinant of clinical drug sensitivity (Doroshow, 2006). Other drug efflux pumps such as BCRP can also reduce intracellular

anthracycline accumulation and may contribute to clinical drug resistance (Doyle & Ross, 2003).

1.3.1.3 Reduced intracellular activation

A number of anti-neoplastic agents, primarily purine or pyrimidine analogues, must be metabolised to function as cytotoxics. Resistance to these drugs commonly arises through the loss of enzymes responsible for drug conversion to the active form. Deletion or diminished activity of thymidine or uridine phosphorylase, thymidine or uridine kinase, and orotic acid phosphoribosyltransferase interferes with the metabolic activation of 5-FU. A relative deficiency of the reduced folate substrate 5, 10-methylenetetrahydrofolate may also compromise the cytotoxic action of FdUMP on TS. This may result from low extracellular levels of reduced folates, decreased membrane transport of reduced folates, or reduced activity of folylpolyglutamate synthase, which thereby prevents its polyglutamation (Grem, 2001). Camptothecin sensitivity may be affected by the decreased intra-tumoral production of SN-38 by the irinotecan carboxyl esterase-converting enzyme (Kanzawa *et al.*, 1990).

1.3.1.4 Altered or increased intracellular target

Many anti-neoplastic agents exert their cytocidal effects by binding to a normal cell enzyme and rendering it non-functional. Cellular resistance may develop through increased production of a particular enzyme or by the production of a variant that is no longer inhibited by the drug. With these changes a much higher intracellular concentration of the drug is required effectively to inhibit the entire enzyme and this may not be achievable.

Alterations in the target enzyme TS represent the most commonly described mechanism of resistance to 5-FU. This may occur through 3 distinct mechanisms. Firstly, point mutations in the protein-coding region of the TS gene A can give rise to a decrease in binding affinity of the 5-FU metabolite FdUMP to TS. Secondly, *in vitro*, *in vivo*, and clinical model systems have shown a strong correlation between higher levels of TS enzyme activity secondary to amplification of the TS gene, raised levels of TS protein and reduced sensitivity to 5-FU (Grem, 2001). Thirdly, in several *in vitro* and *in vivo* model systems, TS protein levels and therefore TS enzyme activity increase after exposure to 5-FU, other specific TS inhibitor compounds, or both. Increases in the expression of TS protein have been identified in the clinical setting in paired tumour tissue biopsy specimens obtained from patients before and during therapy with 5-FU. This induction of TS protein in response to drug exposure is mediated by two different mechanisms: enhanced stability of the protein via a post-translational event and a translational regulatory mechanism; TS protein, in its unbound or free state, is capable of specifically repressing the translation of its own mRNA. However, when TS protein is bound to either nucleotide or anti-folate inhibitors, or both, it is unable to repress TS mRNA translation, and the rate of TS protein synthesis increases.

Cellular resistance to epirubicin resulting from topoisomerase II point mutations or from down-regulation of this enzyme has been shown *in vitro* (Son *et al.*, 1998). However, the relevance of these mechanisms to clinical drug resistance is not clear, as in general, the absolute expression of topoisomerase II in tumours does not correlate well with drug efficacy (Kaufmann *et al.*, 1994).

In vitro studies have identified a number of different mechanisms of camptothecin resistance in cell lines. Point mutations of topoisomerase I have been characterized *in*

vitro that confer relative resistance to camptothecins by decreasing topoisomerase I catalytic activity or impairing binding of camptothecins to topoisomerase I. However, the identification of topoisomerase I mutations in clinical specimens has been exceedingly rare (Rasheed & Rubin, 2003). Camptothecin resistance can also result from down-regulation of topoisomerase I due to chromosomal deletions or hypermethylation of the topoisomerase I gene. However, the absolute level of topoisomerase I expression in tumour tissues has not been highly predictive of drug efficacy in experimental studies (Goldwasser *et al.*, 1995).

1.3.1.5 Increased intracellular drug detoxification

The glutathione S-transferase system contains isoenzymes that may conjugate glutathione reducing the cytotoxicity of a variety of chemotherapeutics. Glutathione-platinum complexes have been demonstrated in cultured cells, and glutathione has been shown to bind to platinum-DNA mono-adducts *in vitro*, preventing them from being converted to potentially cytotoxic cross-links (Mistry *et al.*, 1993). A number of studies have reported a strong association between platinum drug sensitivity and glutathione levels but disappointingly reduction of intracellular glutathione levels has resulted in, at best modest potentiation of cisplatin sensitivity (Smith & Brock, 1988).

Another non-protein thiol that may be involved in cisplatin resistance is cysteinylglycine, which is generated during glutathione catabolism by the enzyme, α-glutamyltransferase. The affinity of cysteinylglycine for cisplatin is significantly higher than that of glutathione, and transfection studies have demonstrated that over-expression of α-glutamyltransferase confers resistance to cisplatin (Daubeuf *et al.*, 2002). Inactivation of platinum drugs may also occur through binding to the metallothionein

(MT) proteins. In some cell lines, elevated MT levels have been shown to be associated with cisplatin resistance, whereas in others, they have not (Kojima *et al.*, 1994; Mistry *et al.*, 1991). In the clinical setting, a significant correlation between MT over-expression, tumour response and patient survival has been reported in urothelial transitional cell carcinoma patients (Siu *et al.*, 1998).

Irinotecan resistance may involve the innate or acquired capacity to detoxify SN-38 by glucuronidation to SN-38 glucuronide (Takahashi *et al.*, 1997). 5-FU resistance may arise from decreased accumulation of FUTP, FdUMP, and (F)dUTP resulting from increased activity of catabolic enzymes [acid and alkaline phosphatases, dUTP hydrolase, and dihydropyrimidine dehydrogenase (DPD)].

1.3.2 DNA damage processing

All cells exhibit a variety of repair mechanisms following cellular injury including damage by chemotherapeutics. The cytotoxic effects of a drug often represent a balance between cellular damage and repair. Impaired DNA damage recognition, repair mechanisms or tolerance of DNA damage may account for cellular resistance to certain drugs. The capacity to rapidly and efficiently repair DNA damage plays an important role in determining a tumour cell's sensitivity to a DNA- damaging drug.

1.3.2.1 Mismatch Repair (MMR)

The DNA mismatch repair system (MMR) recognises and repairs base mismatches after DNA replication, inhibits recombination between non-identical DNA-sequences and initiates both checkpoint and apoptotic responses following certain types of DNA damage. Defects in MMR are associated with an increased risk of cancer as cells

deficient in MMR phenotypically have a greatly elevated rate of spontaneous mutations. Inherited mutations in MMR genes cause hereditary non-polyposis colon cancer (HNPCC), whilst somatic mutations of MMR genes and epigenetic silencing of *MLH1* expression are observed in a significant proportion of sporadic cancers (Palombo *et al.*, 1995).

MSH2 alone, and in combination with MSH6, has been shown to bind to cisplatin-induced guanine-guanine intra-strand adducts with high efficiency (Duckett *et al.*, 1996; Mello *et al.*, 1996), which may trigger programmed cell death rendering cells with intact MMR more sensitive to cisplatin-induced DNA damage (Fink et al., 1996). Alternatively, cytotoxicity may occur through repeated rounds of DNA synthesis past the platinum-DNA lesions followed by recognition and subsequent removal of the newly synthesized strand by the MMR system. This futile cycling may generate DNA strand gaps and breaks that trigger programmed cell death (Karran & Bignami, 1994).

DNA damage tolerance due to loss of function of the DNA MMR system has been observed in cisplatin-resistant cells. Tumour cell lines selected *in vitro* for cisplatin resistance often have lost the expression of MLH1 or MSH2 (Strathdee *et al.*, 1999), becoming 2-4 fold more resistant to cisplatin (Brown *et al.*, 1997). Similarly, in the clinical situation, initially cisplatin or carboplatin chemosensitive tumours such as testicular or ovarian cancers, have acquired resistance to chemotherapy through the loss of MMR during treatment (Gifford *et al.*, 2004; Mayer *et al.*, 2002). The predominant mechanism for loss of MMR in acquired platinum resistance of ovarian tumours appears to be the loss of MLH1 expression due to promoter CpG island hypermethylation and epigenetic silencing (Gifford et al., 2004; Strathdee et al., 1999). Twenty five percent plasma samples in patients relapsing after platinum based treatment

of their ovarian cancer had acquired promoter methylation of *MLH1* compared to pre-chemotherapy plasma samples. Furthermore, *MLH1* methylation was significantly associated with increased microsatellite instability in plasma DNA at relapse (Gifford et al., 2004). MMR also contributes to the ability of tumour cells to withstand the cytotoxic effects of anthracyclines, as seen with the loss of *MLH1* (Nielsen *et al.*, 1996). Cytotoxicity of monofunctional alkylating agents requires intact MMR. Mammalian cells proficient in MMR are generally 100 times more sensitive to alkylating agents than their MMR-deficient counterparts (Karran, 2001). Resistance to O^6-alkylating agents, such as temozolomide and dacarbazine, is associated with loss of expression or function of MMR genes, particularly in the absence of MGMT (Branch *et al.*, 1993), which results in the accumulation of DNA damage but not cell death. The accumulation of O^6-methylguanine (O^6-MG) in MMR-deficient cells has been termed alkylation or methylation tolerance, rather than resistance (Karran, 2001).

1.3.2.2 Nucleotide excision repair (NER)

The repair of platinum-DNA adducts occurs predominantly by nucleotide excision repair (NER). Increased expression of proteins involved in platinum adduct recognition and incision, including ERCC1 and XPE, may enhance NER activity and cisplatin resistance (Chu & Chang, 1990; Ferry *et al.*, 2000). Evaluation of ERCC1 levels in tumour samples from patients with metastatic lung cancer by immunohistochemical analysis, have been shown to correlate inversely with response and survival. The first randomised prospective trial using ERCC1 mRNA levels to assign chemotherapy in patients with advanced non-small lung cancer has recently been completed. Patients in the control arm received cisplatin-based chemotherapy, in the study arm patients were genotypically assigned chemotherapy; those with high ERCC1 levels received a non-cisplatin-containing regimen, those with low ERCC1 expression received

cisplatin/docetaxel. The study found an objective response rate of 39.4 % in patients in the control arm vs. 50.7 % in patients genotypically assigned chemotherapy (p=0.02), suggesting that customising chemotherapy based on ERCC1 mRNA levels may improve response to treatment (Cobo *et al.*, 2007).

1.3.2.3 Base excision repair (BER)

This process is responsible for the repair of the N7-methylguanine and N3-methyladanine lesions formed by temozolomide and other methylating agents. The nuclear enzyme poly (ADP-ribose) polymerase-1 (PARP) has a key role in facilitating this process through the detection of single strand breaks and subsequent recruitment of the appropriate repair enzymes (de Murcia & Menissier de Murcia, 1994). PARP-1 inhibitors have been shown *in vitro* (Boulton *et al.*, 1995; Delaney *et al.*, 2000; Miknyoczki *et al.*, 2003) and *in vivo* (Calabrese *et al.*, 2004; Tentori *et al.*, 2002) to potentiate temozolomide cytotoxicity through the inhibition of BER and incomplete processing of N7-methylguanine and N3-methyladanine. A recently reported phase I study of a first-in-class PARP inhibitor AG014699 combined with temozolomide in patients with advanced malignancy demonstrated that combination therapy is well tolerated and pharmacodynamic assessments confirmed proof of principle of the mode of action of this new class of agents (Plummer *et al.*, 2008).

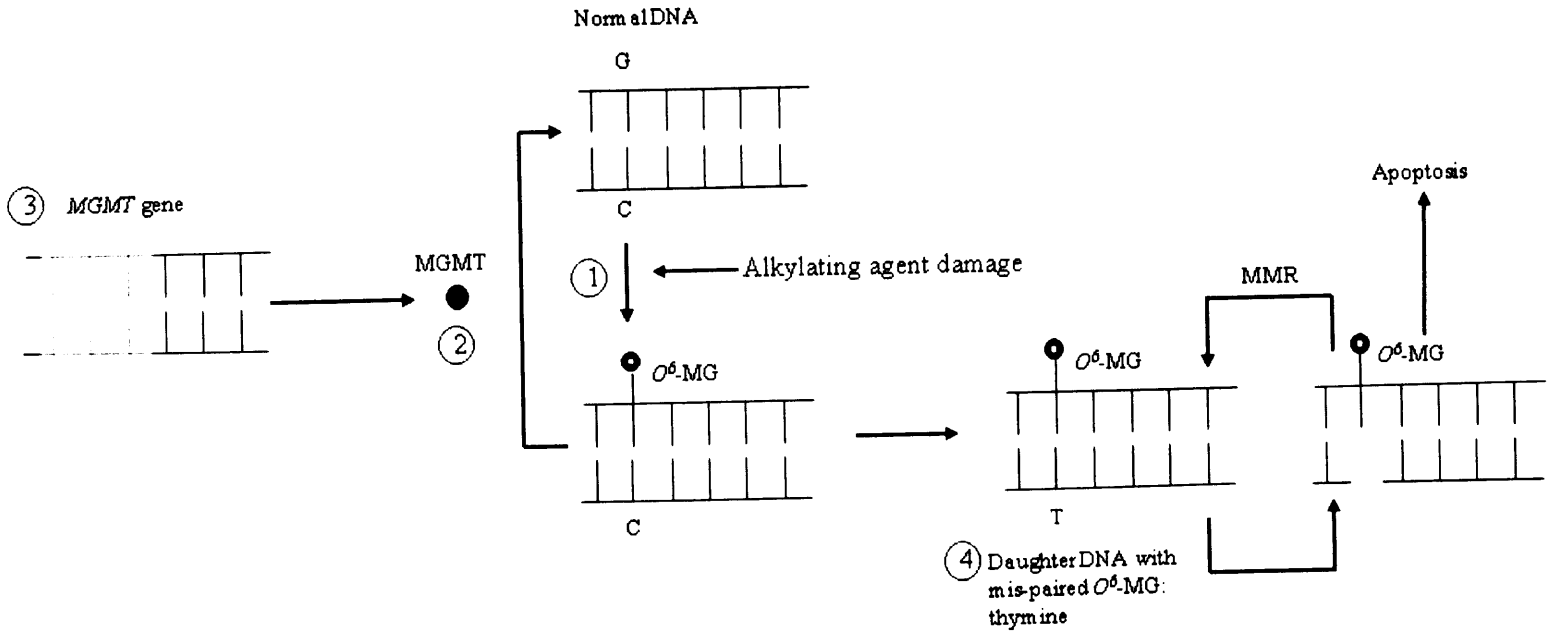
1.3.2.4 MGMT

O^6-methylguanine-DNA-methyltransferase (MGMT) repairs potentially mutagenic and cytotoxic alkylation damage, primarily by removing adducts from the O6 position on guanine but also at the O4 position on thymine. It does this through a stoichiometric auto-inactivating reaction, covalently transferring the alkyl group to its active site

cysteine before chloroethylguanine cross-links can form or O^6-methylguanine: thymine (O^6-MG: T) mispairing results from further rounds of replication. O^6-MG: T mispairing may be recognised by MMR, which incorrectly targets the newly synthesised strand containing thymine, leading to futile rounds of repair, persistent single-strand breaks and ultimately apoptosis (Figure 4). A clear relationship between failure of mismatch repair and resistance to alkylating agents has been demonstrated (Friedman *et al.*, 1998b; Kat *et al.*, 1993; Koi *et al.*, 1994).

Figure 4. Overview of DNA repair by MGMT

Alkylating agents cause DNA damage by forming O^6-methylguanine adducts (O^6-MG) (1). These may be repaired by O^6-methylguanine-DNA-methyltransferase (MGMT) (2), a DNA repair protein encoded by the MGMT gene (3). If this damage is not repaired before the next round of cellular replication, O^6-MG adduct may be incorrectly paired with thymine (T) rather than cytosine (C) (4). This results in several rounds of futile DNA replication as mismatch repair (MMR) incorrectly targets the newly synthesized thymine for repair resulting ultimately in apoptosis.



MGMT protects cells from exogenous carcinogens, and also cancer cells from chemotherapeutic agents. The protein is unique in its ability to remove DNA adducts independently rather than via multi-enzyme complexes, a feature common to all other DNA repair systems.

MGMT is ubiquitously expressed in human tissue, but levels vary considerably between organs and between individuals (Belanich *et al.*, 1996; Gerson *et al.*, 1986; Povey *et al.*, 2000; Silber *et al.*, 1996). Mean MGMT activity is generally higher in malignant tissue compared to corresponding normal tissue. This has been observed in colon cancer, lung tumours and gliomas (Belanich et al., 1996; Citron *et al.*, 1991; Povey et al., 2000) . A direct relationship between MGMT activity and resistance to chlorethylating and methylating agents in tumour cells *in vitro* and in xenografts has been established, with depletion or knockdown of MGMT resulting in sensitisation of hitherto resistant cells (Gerson *et al.*, 1992; Gerson & Willson, 1995). Cell lines deficient in MGMT have developed alkylating agent resistance following transfection with a vector containing MGMT cDNA (Passagne *et al.*, 2006). The clinical data for MGMT is discussed in a later section.

1.3.2.5 Non-homologous end joining (NHEJ)

NHEJ is involved in a number of different processes including the repair of double-strand DNA breaks. It involves a series of proteins; the Ku heterodimer (Ku70 and Ku80) initiates NHEJ by binding to the broken DNA ends. It forms a complex with DNA -dependent protein kinase (DNA-PK) and recruits the DNA-PK catalytic subunit, which phosphorylates other nuclear proteins. Terminal processing of the DNA ends is

required before ligation can occur. The DNA polymerases Pol λ and Pol μ can synthesis new DNA to fill in gaps creating blunt ends, alternatively DNA can be trimmed off via nuclease activity which may involve the Artemis protein. Once blunt ends are in place, DNA Ligase IV and its XRCC4 are recruited to perform ligation. This is an inherently inaccurate process.

1.3.2.6 Homologous recombination (HR)

Homologous recombination (HR) is involved in a number of processes including the repair of double strand-DNA breaks by replacing the DNA regions flanking each double-strand break by a faithful copy from a sister chromatid or homologous chromosome. It requires resection of the 5'ends of the double-strand break, association of replication protein A complexes with the resulting single-stranded DNA, D-loop formation with strand invasion and branch migration as the complementary duplex DNA strand progressively pairs with the invading single-strand sequence, repair synthesis and resolution of the D-loop structure through resolution of Holliday junctions (DNA crossovers) or synthesis dependent strand annealing. As NHEJ and HR primarily repair double stranded-DNA breaks which are not caused by the chemotherapeutics relevant to this thesis, they will not be discussed in further detail.

1.3.3 Downstream resistance

1.3.3.1 Cell cycle control and Apoptosis

Apoptosis is activated through two major signalling pathways- intrinsic and extrinsic. The intrinsic pathway is triggered from within the cell following severe cell stress, such as DNA damage by chemotherapy. The extrinsic pathway is activated by pro-apoptotic ligands for example tumour necrosis factor-α (TNF-α) binding to proapoptotic cell

membrane receptors such as DR4. Both pathways converge via activation of the caspase cascade that ultimately triggers cell death. Caspase function is regulated by a number of proteins including the Inhibitors of apoptosis (IAPs) e.g. XIAP.

Chemotherapeutic agents commonly cause cell death by triggering apoptosis therefore defects in this process may also contribute to drug-resistance. DNA damage tolerance may be the result of decreased expression or inactivation of one or more components of the programmed cell death pathway. A number of pro-apoptotic and anti-apoptotic signalling pathways have been implicated in determining an individual's sensitivity to cytotoxic therapy.

The tumour suppressor gene *p53* has an important role in apoptosis and can stimulate the process in cells that have sustained damage to DNA or following activation of proapoptotic receptors. Inactivation of *p53,* a commonly observed genetic lesion seen in approximately half of human malignancies, may inhibit apoptosis. Inactivation occurs through a variety of mechanisms including mutation of the gene itself, the inappropriate expression of certain oncogenes or by the action of gene products produced by a variety of oncogenic viruses (Martin & Green, 1995). There is substantial evidence that the p53 protein may influence response to anticancer drugs, and several mechanisms of drug resistance are probably influenced by it. For example, induction of wild type *p53* in colon cancer cells with mutant endogenous p53 protein was found to increase sensitivity to 5-FU and camptothecin (Yang *et al.*, 1996).

Cell death may be influenced by expression of members of the BCL-2 gene family, key components of the intrinsic apoptotic pathway. These pro-apoptotic (BAX, BAK) and anti-apoptotic proteins (BCL-2, BCL-X_L, MCL-1) regulate mitochondrial function, cell

survival and cell death. On activation by BH3 domain containing proteins (BID, BAD, BIM), pro-apoptotic BAX/BAK may be activated or alternatively, BH3-members may be inactivated by anti-apoptotic BCL-2, BCL-X_L and MCL-1. If pro-apoptotic activation is in excess of anti-apoptotic production BAX/BAK, via other proteins including MOMP induces permeabilization of the mitochondrial membrane and the release of cytochrome c and SMAC/DIABLO. Cytochrome c prompts the assembly of the apoptosome complexes that catalyze activation of effector caspases and cell death. A small percentage of BAX/BAK acts on the endoplasmic reticulum modifying the amount of calcium ions released on a death stimulus.

Changes or mutations in any part of the complex apoptotic pathway may result in insensitivity/resistance to a cytotoxic agent. For example, over-expression of BCL-2 or BCL-X_L has been shown to prevent disruption of the mitochondrial transmembrane potential and to prolong cell survival in some cells after exposure to cisplatin and other anticancer drugs (Minn *et al.*, 1995; Miyashita & Reed, 1993). The activity of these proteins is negated however, in the presence of high levels of the pro-apoptotic protein BAX therefore, the relative intracellular levels of these proteins may also confer resistance to cytotoxic drugs.

1.4 Tumorigenesis and Epigenetics

Traditionally malignant change has been thought to arise from progressive genetic changes, including mutations in tumour-suppressor genes and oncogenes, and chromosomal abnormalities. As our understanding of the molecular basis of cancer has evolved, it has become apparent that malignant transformation and progression may be driven by patterns of altered gene expression that are mediated by mechanisms that do not affect the primary DNA sequence- so called epigenetic changes. Epigenetic

silencing is seen throughout the genome of cancer cells, affecting genes involved in regulating all cell functions. Aberrant hypermethylation of promoter DNA and transcriptional repression resulting in the loss of gene function has been shown to contribute to tumorigenesis (Herman & Baylin, 2003; Jones & Baylin, 2002; Jones & Laird, 1999). The apparently contrasting phenomenon of global hypomethylation is also seen in cancer cells, causing structural changes in chromosomes, genomic instability and increase in gene expression. Histone modification is another key event resulting in gene silencing in conjunction with CpG island (defined below) hypermethylation.

Epigenetic modifications of DNA and histones are stable and heritable or acquired but also reversible (Bird, 2002). This reversibility renders them attractive targets for therapeutic interventions. Pharmacological reversal of epigenetic modifications can directly change gene expression and therefore cellular characteristics, behaviour and potentially clinical outcomes.

1.4.1 DNA methylation

1.4.1.1 Mechanism of physiological DNA methylation

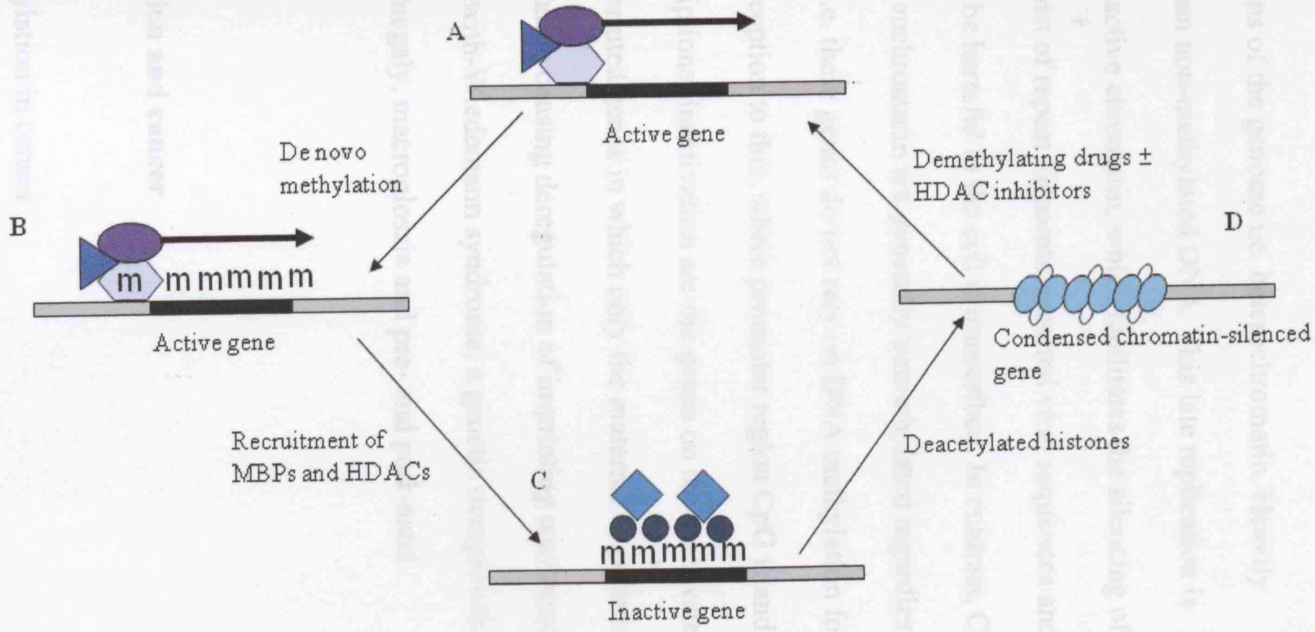
The majority of DNA methylation occurs at CpG islands, non-CpG sequences may also exhibit methylation but this generally occurs at a much lower frequency. CpG islands are GC (guanine cytosine) - and CpG (cytosine guanine)-rich areas of approximately 1 kb, usually found in the vicinity of genes and often found within the promoters of widely expressed genes. DNA methyltransferase enzymes (DNMTs) covalently add a methyl-group, using s-adenosylmethionine as the methyl group donor, to the 5-position of cytosine, resulting in 5-methylcytosine, altering the major groove of DNA. Proteins characterised by the methyl-CpG-binding domain (MBPs) are responsible for binding to

methylated CpG dinucleotides. MBPs mediate the silencing of gene expression by functioning as adaptors between methylated DNA and histone-modifying enzymes inducing the formation of chromatin structures that repress gene transcription (Figure 5).

De novo DNA methylation by DNMT3a and DNMT3b primarily results in cytosine methylation at previously unmethylated CpG sites. Global *de novo* methylation has been well documented during germ-cell development and early embryogenesis, but the mechanism by which DNMT enzymes are recruited to targeted DNA sequences has been difficult to establish (Klose & Bird, 2006). *De novo* methylation is a rare occurrence in normal somatic tissues (Jones & Laird, 1999). DNA methylation is maintained by the maintenance enzyme DNMT1, which copies pre-existing methylation patterns onto the new DNA strand during DNA replication.

Figure 5. Effects of methylation and histone deacetylation on gene expression and silencing

A Initially genes are unmethylated and the promoter region (hexagon) is occupied by transcription factors (ovals) that direct the production of mRNA (arrows). **B** De novo methylation, by itself, has a minimal effect on gene expression. **C** However, methylated DNA (m) attracts methyl-binding proteins (MBPs, circles), such as MeCp2. These in turn attract a protein complex that contains histone deacetylases (HDACs, squares). At this point, synthesis of mRNA is inhibited with the loss of protein production. **D** Through the action of MBPs and HDACs, the DNA structure changes to a compact 'condensed chromatin' configuration, which results in permanent inhibition of mRNA and protein production (silencing). Hypomethylating agents can reverse this silenced state and restore mRNA and protein expression (**A**). HDACis act synergistically with hypomethylating agents to restore gene expression. Adapted from Santini et al., (Santini *et al.*, 2001).



1.4.1.2 Physiological role of DNA methylation

DNA methylation is a powerful mechanism that maintains transcriptional silence in non-expressed or non-coding regions of the genome i.e. heterochromatin. Heavily methylated DNA replicates later than non-methylated DNA. This late replication is associated with the formation of inactive chromatin, which facilitates the silencing of large parts of the genome that consist of repeat elements, inserted viral sequences and transposable elements, which may be harmful to the cell if transcribed. In contrast, CpG island sites in promoter regions of euchromatin are generally unmethylated regardless of the transcription state of the gene i.e. these genes do not rely on DNA methylation for control of their expression. The exception to this, where promoter region CpG islands are methylated to maintain transcriptional inactivation are the genes on the inactivated X chromosome in females and imprinted genes in which only the maternal or paternal allele is expressed. Loss of methylation causing deregulation of imprinting may result in an inherited disorder such as Beckwith-Wiedemann syndrome, a genetic overgrowth syndrome associated with visceromegaly, macroglossia and pre- and post-natal overgrowth.

1.4.2 CpG island hypermethylation and cancer

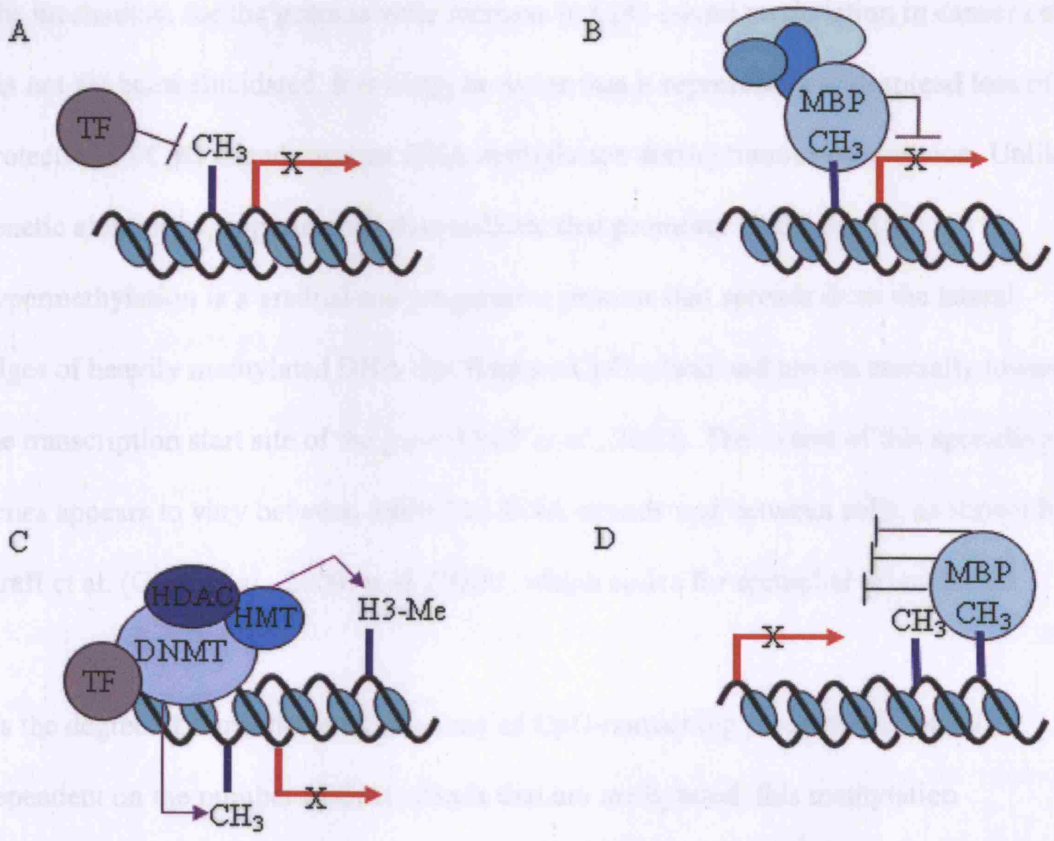
1.4.2.1 Mechanism of DNA methylation in cancer

The mechanism by which DNA methylation causes transcriptional silencing of associated genes in malignancy has been extensively studied and three models have emerged: first, DNA methylation may directly repress transcription by blocking transcriptional activators from binding to associated DNA sequences (Clark *et al.*, 1997; Watt & Molloy, 1988); second, methyl-CpG-binding proteins (MBPs) may recognise

methylated DNA and act as transcriptional repressors (Asiedu *et al.*, 1994; Nan *et al.*, 1997) (Figure 6).Thirdly, for some genes, repression mediated by DNA methylation may be dependent on chromatin modification (Kass *et al.*, 1997). This is supported by the observation that MBPs are associated with chromatin remodelling co-repressor complexes including histone deacetylases, leading to alteration of chromatin configuration and preventing binding of transcription factors (Yoon *et al.*, 2003). This latter mechanism is now recognised as being the most likely.

Figure 6. Mechanisms of DNA-methylation-mediated repression

A DNA methylation in the cognate DNA-binding sequences of some transcription factors (TF) can result in inhibition of DNA binding. By blocking activators from binding target sites, DNA methylation directly inhibits transcriptional activation. **B** Methyl-CpG-binding proteins (MBPs) directly recognise methylated DNA and recruit co-repressor molecules to silence transcription and to modify the surrounding chromatin. **C** In addition to their DNA methyltransferase activities, DNMT enzymes are also physically linked to histone deacetylase (HDACs) and histone methyltransferase (HMT) activities. In this case, the addition of methyl groups to DNA is coupled to transcriptional repression and chromatin modification. **D** DNA methylation within the body of genes can also have a dampening effect on transcriptional elongation. MBPs may be involved in inhibiting elongation, either directly or by their effects on the surrounding chromatin structure. Adapted from Klose et al., (Klose & Bird, 2006).



As well as methylation of DNA, following recruitment by transcriptional repressors during gene silencing, DNMTs, which are expressed at higher levels in tumours than in normal tissue (Esteller, 2005a), also have a non-enzymatic-role in transcriptional silencing. This mechanism appears to be chromatin- modification dependent as the DNMTs interact with histone methyltransferases and histone deacetylases (Geiman *et al.*, 2004). DNMTs therefore appear to achieve gene silencing through an indirect approach with transcriptional repression secondary to chromatin modification and directly via the methylation of cytosine.

The mechanism for the genome wide increase in CpG-island methylation in cancer cells has not yet been elucidated. It is likely however that it represents a widespread loss of protection of CpG islands against DNA methylation during tumour progression. Unlike genetic alterations, experimental data indicate that promoter CpG island hypermethylation is a gradual and progressive process that spreads from the lateral edges of heavily methylated DNA that flanks a CpG island and moves centrally towards the transcription start site of the gene (Graff *et al.*, 2000). The extent of this spreading in genes appears to vary between individual DNA strands and between cells, as shown by Graff et al. (Graff et al., 2000) with *CDH1*, which codes for epithelial (e)-cadherin.

As the degree of transcriptional silencing of CpG-containing promoters is usually dependent on the number of CpG islands that are methylated, this methylation heterogeneity from one DNA strand to another and from one cell to another, can lead to different populations of cells within a tumour, with respect to their gene expression levels. This may contribute to the heterogeneous phenotype for key neoplastic properties seen in tumour cells.

1.4.2.2 Knudson and epigenetic silencing

According to Knudson's two hit theory of carcinogenesis, loss of function of both alleles in a tumour suppressor gene is required for malignant transformation (Knudson, 2001). Promoter hypermethylation may constitute the initial hit in many somatic cancers, with subsequent mutations or deletions eliminating the second gene copy. Epigenetic changes have been reported to cause the second hit in familial cancers, the first occurring through a germ line mutation (Esteller *et al.*, 2001b). Moreover, hypermethylation of both alleles has been noted in non-familial tumours in the absence of mutations or other genetic mechanisms (Herman & Baylin, 2003). Promoter hypermethylation of the *retinoblastoma* gene (*pRB*) and the *von Hippel Lindau* (*VHL*) gene have been detected in familial cases of unilateral retinoblastoma and renal cancer, respectively where promoter hypermethylation is the primary inactivating event (Baylin & Herman, 2000; Jones & Laird, 1999).

1.4.2.3 Other mechanisms of tumorigenicity by cytosine methylation

5-methylcytosine is itself mutagenic, undergoing spontaneous hydrolytic deamination that can lead to cytosine (C) to thymidine (T) transitions. Up to 50 %, of the inactivating point mutations seen in the coding region of *p53* in somatic cells occur at methylated cytosines (Rideout *et al.*, 1990). The presence of a methyl group in the promoter region of this gene significantly increases the rate at which mutations are induced by ultraviolet (UV) light during the development of skin cancers (Pfeifer *et al.*, 2000), as methylated cytosine is affected by UV light at frequencies occurring in natural light, as opposed to cytosine. Methylated CpG dinucleotides are also the preferred targets of G to T transversion mutations, which are induced by tobacco carcinogens (Yoon *et al.*, 2001).

1.4.3 DNA hypomethylation in cancer

Across the genome, tumour cells are hypomethylated compared to wild-type cells. Loss of transcriptional repression in normally silent regions of the genome may be harmful, via expression of inserted viral genes, repeat elements or normally silenced genes such as imprinted genes and genes on the inactive X chromosome. Loss of imprinting in the case of the *IGF2* gene, which is normally expressed from the paternal copy, results in bi-allelic expression of the gene, which is associated with an increased incidence of colorectal cancer (Cui *et al.*, 2003). Hypomethyation of non-promoter regions of DNA and of structural elements such as centromeric DNA leads to genomic instability. This is seen in germ line mutations of DNMT3B, which leads to the Immunodeficiency Centromeric instability and Facial abnormalities (ICF) syndrome. Similarly, pericentric chromosomal instability is seen in Wilms tumours with loss of DNA methylation (Qu *et al.*, 1999).

1.4.4 Histone modification in malignancy

Histones have a key function in epigenetic modifications in cancer cells. They have a primary role in control of gene expression and chromatin structure, working in concert with DNA methylation as described above. Specific modifications have been shown to be markers of malignant transformation for example, the overall loss of monoacetylation of lysine 16 and trimethylation of lysine 20 in the tail of histone H4 (Fraga *et al.*, 2005). Histone post-translational modification may occur in a number of ways including acetylation and methylation, via histone acetyltransferases (HATs) and histone deacetylases (HDACs) and histone methyltransferases (HMTs) respectively. Most modifications localise to the amino- and carboxy-terminal histone tails, and a few localise to the histone globular domains.

1.4.4.1 Histone acetylation and methylation

Histone acetylation is required to maintain chromatin in an open, transcriptionally active state. This allows binding of transcription factors, histone acetylases and other regulatory co-activators that promote gene expression. Conversely HDACs deacetylate these residues, maintaining transcriptional silencing. Binding of HDACs to hypermethylated chromatin is directed by DNMTs and MBPs, which form a complex with other regulatory proteins to block access of transcriptional machinery to the promoter (Bird, 2002), demonstrating the intimate connection between methylation of DNA and modification of histones. In the collaboration between DNA methylation and histone deacetylation that silence gene expression, DNA methylation appears to be dominant. Drugs that inhibit histone deacetylases can increase the expression of genes without methylated promoters, but cannot induce the expression of hypermethylated genes in cancer cells. However, if demethylation occurs first, by using low doses of 5-azanucleosides, histone deacetylase inhibitors will synergistically increase re-expression of the silenced genes (Suzuki et al., 2002).

Methylation of histones occurs at lysine or arginine residues on their amino-terminal tail domains. It is crucially important for normal gene regulation as it may allow the perpetuation of epigenetic silencing through cell divisions (Rea *et al.*, 2000). Histone methylation is catalysed by histone methyltransferases, many of which contain a conserved SET (Su(var)3-9, Enhancer-of-zeste, Trithorax) domain (Schneider *et al.*, 2002). These 'methyl marks' are recognised by binding proteins, heterochromatin protein-1 (HP-1) and Polycombe (Pc), which perpetuate the cycle by recruiting further histone methyltransferases. Histone methylation may be a marker of active and inactive regions of chromatin depending on which amino-acid residues are involved. Methylation may occur several times (mono-, di- or trimethylation) on one lysine side

chain and each level of modification can have different biological outcomes. In the histones H3 and H4, arginine residues can also be mono-or di-methylated, but arginine methylation seems to be strictly activating to transcription.

With so many possible combinations of modifications that can occur on a variety of sites on histones, it has been proposed that different combinations of histone modifications may result in distinct outcomes in terms of chromatin regulated functions. This has been called the Histone Code Hypothesis (Jenuwein & Allis, 2001).

1.4.4.2 Other histone modifications

Sumoylation and ubiquitiylation of the lysine residues of histones involves the addition of large moieties, resulting in profound changes in chromatin structure. Sumoylation of lysine appears to be repressive to transcription; by contrast ubiquitiylation has variable effects depending on the precise histone residue and context affected. For example, the two ubiquitiylation sites in the C termini of the histone subunits H2B and H2A correlate with active and repressed transcription, respectively. Serine/threonine phosphorylation is also involved in transcription. Histone H3 phosphorylation is well characterised and correlates with both activated transcription and mitotic chromosomal condensation.

Phosphorylation and ADP-ribosylation of histones play a part in tumorigenesis. Aurora kinase B has been shown to phosphorylate serines in histone H3 and act as an oncogene in human cancers (Balmain *et al.*, 2003). Increased ADP-ribosylation of histones with increase in malignancy has been seen in human oral tumours (Das, 1993).

1.4.5 Effects of epimutations in cancer

Epigenetic gene inactivation is thought to be as common, if not more common, than genomic mutational events in the development of cancer although in contrast to gene mutations, epigenetic changes are not random (Jones & Baylin, 2002). A large number of genes involved in regulating cell growth, differentiation, signal transduction, DNA repair, tumour metastases and angiogenesis, are found to undergo hypermethylation in a variety of malignancies (Table 1). The profile of promoter hypermethylation for genes varies between cancer types, providing a tumour-type and gene-specific profile. Gastrointestinal tumours e.g. colon and gastric cancers share a set of hypermethylated genes characterised by $p16^{INK4a}$, $p14^{ARF}$, *MGMT* and *hMLH1*. Some genes such as the cell cycle inhibitor $p16^{INK4a}$ are hypermethylated across many tumour types (An *et al.*, 2005; Esteller, 2005b). It would appear that unlike genetic mutations in a single tumour which are usually unique in a given cell pathway, multiple epigenetic events may effect a single cell pathway resulting in a complex, integrated network of disrupted pathways fostering tumorigenesis (Jones & Baylin, 2007).

Table 1. Examples of Pathways and Genes Hypermethylated in Cancer

Pathway	Gene	Function	Tumors affected (Examples)
Cell cycle	pRB	Inhibitor of cyclin-dependent kinase	Retinoblastoma
	$p16^{INK4a}$	Inhibitor of cyclin-dependent kinase	Stomach, oesophagus, colon, lung, breast
	$p15^{INK4b}$	Inhibitor of cyclin-dependent kinase	Leukaemia, lymphoma
	p73	Cell cycle checkpoints	Leukaemia, lymphoma, neuroblastoma
	$p14^{ARF}$	Inhibitor of cyclin-dependent kinase	Stomach, colon, breast, lung
Apoptosis	DAPK	Pro-apoptotic serine/threonine kinase	Stomach, lymphoma, lung, colon
	TMS1	Pro-apoptotic CARD domain family	Breast, ovary, neuroblastoma
DNA repair/DNA damage response	hMLH1	Mismatch repair	Stomach, colon, endometrium
	MGMT	DNA alkylation repair	Stomach, oesophagus, colon, lung
	BRCA1	DNA damage response	Breast
Angiogenesis/hypoxia	THBS1	Angiogenesis inhibitor	Colon, neuroblastoma
	VHL	Ubiquitin ligase	Kidney
	BNIP3	Hypoxia-mediated apoptosis	Stomach, pancreas, colon
Chromatin regulation and transcription	HLTF	Helicase-like transcription factor	Colon
	RIZ1	Histone methyltransferase	Stomach, breast, colon
Signal transduction	RASSF1	RAS effector	Lung, colon, breast, ovary
	SOCS3	Inhibitor of JAK/STAT pathway	Lung
Hormone receptor	ER	Estrogen receptor	Colon, breast
	PGR	Progesterone receptor	Breast
Cell attachment and invasion	CDH1	Cell adhesion	Stomach, oesophagus, breast, lung

Adapted from Toyota, M. et al., (Toyota & Issa, 2005).

Concordant methylation of multiple genes is termed CpG-island methylation phenotype (CIMP) and is seen in certain cancers such as those of the colon, which have substantially more frequent CpG island methylation than others. This phenotype provides strong evidence that a specific defect leads to aberrant methylation in certain cancers and provides evidence that hypermethylation is selected for and therefore contributes to neoplasia. Tumours affected by this phenotype have distinct clinical and molecular features. For example, in colon cancer, CIMP+ tumours tend to be proximal and occur more frequently in women and older people (Toyota et al., 1999; Whitehall *et al.*, 2002). They have a distinct pathology (Whitehall et al., 2002), a distinct molecular genetic profile with frequent *KRAS* and *BRAF* mutations and infrequent *p53* mutations (Kambara *et al.*, 2004; Toyota *et al.*, 2000). They have a specific clinical course with a good prognosis if *hMLH1* is affected and a poor prognosis otherwise.

The presence of epigenetic lesions is often an early event in the natural history of cancer. Promoter hypermethylation affecting $p16^{INK4a}$, $p14^{ARF}$ and *MGMT* occurs in colorectal adenomas (Esteller *et al.*, 2000b; Esteller *et al.*, 2000c; Esteller *et al.*, 2000d) and *hMLH1* promoter hypermethylation can be detected in ulcerative colitis and may predispose to colorectal tumours (Fleisher *et al.*, 2000). An increase in the number of methylated genes has been seen in the transition from non-neoplastic gastric mucosa to intestinal metaplasia, as well as from pre-malignant gastric lesions to frank carcinomas (Kang *et al.*, 2001). This suggests that CpG island hypermethylation can occur early in multi-step gastric carcinogenesis and tends to accumulate.

It would appear that epigenetic abnormalities may even play a significant role in cancer initiation. Abnormal gene imprinting and/or silencing may allow early aberrant clonal

cell expansion, which is at risk of subsequent genetic and epigenetic alterations fostering tumour progression (Baylin & Ohm, 2006).

Hypermethylation based silencing has allowed the detection of hundreds of previously unknown possible tumour suppressor genes via DNA-based or RNA reactivation- based approaches. The functional importance of these loci is likely to be variable. Some oncogenes such as *TERT* are hypermethylated in cancer and other genes that are hypermethylated are not expressed in normal tissue, making the matter of silencing irrelevant (Jones *et al.*, 1990). Methylation, even though detectable by sensitive techniques such as methylation specific polymerase chain reaction (MS-PCR), may be relatively sparse and not lead to substantial decreases in gene expression (Santini *et al.*, 2001).

1.4.5.1 Effects of epigenetics on the cell cycle

Cell cycle progression is determined by cell cycle checkpoints, which ensure the integrity of the genome is maintained, by activating cellular repair or death pathways on detecting DNA damage. Deregulation of this process is a characteristic feature of tumour cells and mutations of the genes involved in controlling the cell cycle are very common in human cancer. Several studies have demonstrated epimutations in cell cycle genes such as $p16^{INK4a}$, part of the *pRb*/$p16^{INK4a}$/cyclin D1 pathway, and $p14^{ARF}$, part of the $p14^{ARF}$/mdm2/*p53* pathway (Esteller, 2003a; Jones & Baylin, 2002). $p14^{ARF}$ and $p16^{INK4a}$ are encoded for by the *CDKN2A/INK4A* locus (9p21). This locus is unusual as it encodes for two proteins, $p14^{ARF}$ and $p16^{INK4a}$ which activate two different tumour suppressors, *p53* and *pRb* respectively. The locus is often silenced or mutated in tumours and both the *p53* and *pRb* pathways are found to be directly or indirectly compromised in a large number of tumours.

1.4.5.1.1 p16 ^{INK4a}

Key checkpoints exist at the G1/S and G2/M transitions of the cell cycle that are monitored by cyclins and cyclin dependent kinases (cdks). Cyclin D1/cdk 4/6 regulates mid to late G1 by phosphorylating *pRb*, which allows E2F to activate genes for progression to S-phase. p16 ^{INK4a} inhibits the association of cdk 4/6 and cyclin D1, by binding to cdk 4/6 causing it to adopt an inactive conformation. This prevents the cyclin D1/cdk 4 phosphorylation of *pRb* and instead results in the formation of a *pRb*/E2F-recessive complex inhibiting cells from entering the S-phase. Dysregulation of the cell cycle at the G1-S transition point is a common event in tumorigenesis, such that most cancers have alterations in at least one of the main regulators of this transition, including p16^{INK4a}.

Alterations to *p16 ^{INK4a}* may be inherited, as in familial melanoma (Hussussian *et al.*, 1994; Kamb *et al.*, 1994), occur somatically in sporadic tumours (Caldas *et al.*, 1994) or as a result of inactivation through gene promoter hypermethylation.

Emphasising its role in tumorigenesis, deletion of *p16 ^{INK4a}* in mice results in the development of spontaneous tumours at a young age, and a high sensitivity to carcinogenic treatments (Serrano *et al.*, 1996). Allelic loss of *p16 ^{INK4a}* may be one of the earliest events in the development of gastro-oesophageal cancer and is more prevalent than the loss of *p53* in Barrett's metaplasia and dysplasia (Galipeau *et al.*, 1999). Promoter hypermethylation of *p16 ^{INK4a}* is seen with increasing frequency in Barrett's metaplasia (3 %), to low-grade dysplasia (56 %) to high-grade dysplasia (75 %), whilst point mutations and homozygous deletions occur only in a minority of cases (Lin & Beerm, 2004). Similarly, increasing levels of *p16 ^{INK4a}* gene methylation are seen in gastric dysplasia (4 %), adenomas un-associated with carcinoma (18 %), gastric

adenomas/dysplasia associated with adenocarcinoma (29 %), and adenocarcinoma (44 %) (Lee *et al.*, 2004). Therefore the methylation of $p16^{INK4a}$ may play a role in the malignant transformation of oesophago-gastric precursor lesions.

Aberrant methylation of $p16^{INK4a}$ has also been shown in a wide variety of other tumours such as lung, breast, bladder, head and neck, and colon (An *et al.*, 2005; Esteller, 2005b). Inactivation of the $p16^{INK4a}$ gene in non-small cell lung cancer is a predictor of clinical outcome and is significantly related to a poor prognosis (Wang *et al.*, 2004). Similarly, hypermethylation of the $p16^{INK4a}$ promoter and down-regulated tumour expression of $p16^{INK4a}$ has been associated with poor patient survival in sporadic colorectal cancer (Cui *et al.*, 2004). Hypermethylation inactivates $p16^{INK4a}$ in approximately 40 % of oesophageal cancers (Xing *et al.*, 1999) and correlates with cyclin D1 protein over-expression and a poor prognosis. Restoration of $p16^{INK4a}$ expression in oesophageal cell lines, via $p16^{INK4a}$ gene transfer in cells with $p16^{INK4a}$ depletion due to promoter hypermethylation, has been shown to inhibit proliferation (Schrump *et al.*, 1996).

A close link has been established between CpG island methylation of the $p16^{INK4a}$ promoter and loss of $p16^{INK4a}$ mRNA/protein, as well as gene reactivation following treatment with demethylating agents (Fang *et al.*, 2005a; Fang *et al.*, 2003; Shim *et al.*, 2000). Treatment with 5-aza-2'deoxycytidine and trichostatin A, a histone deacetylase inhibitor (HDACi) has a synergistic effect in inducing $p16^{INK4a}$ expression in cancer cells (Cameron *et al.*, 1999; Magdinier & Wolffe, 2001).

1.4.5.1.2 p14 ARF

The *p14 ARF*/mdm2/*p53* pathway appears to play a major role in mediating oncogenic-induced apoptosis. The suppression of apoptosis by inactivation of this pathway has an important role in tumour development. *p53*, a key regulator of cell cycle checkpoints, is an inducer of cell cycle arrest and apoptosis. Its turnover is regulated by ubiquitination through mdm2 binding, leading to proteosomal degradation and limiting p53 accumulation. *p53* also activates mdm2 transcription, ensuring negative feedback regulation. The tumour suppressor p14 ARF arrests the cell cycle in G1 and G2 phases in a *p53*-dependent manner. p14 ARF binds to mdm2, which is associated with mdm2 modification promoting its rapid degradation, and concurrent p53 stabilisation and accumulation.

Progressively increasing levels of p14 ARF methylation have been detected in oesophageal hyperplasia, dysplasia and carcinoma and once hypermethylated in an earlier stage lesion, p14 ARF hypermethylation was always present in later stage lesion from the same individual (Nie *et al.*, 2002). Similarly, methylation frequency of p14ARF has been shown to increase with some fluctuation in chronic gastritis (29.7 %), intestinal metaplasia (31.6 %), gastric adenomas (75.9 %) and gastric carcinomas (62.5 %) (Kang *et al.*, 2003a). Analysis of primary colorectal adenomas and carcinomas has shown p14 ARF methylation in 28 % and 32 % respectively. This was independent of p16 INK4a promoter methylation in 52 % of colorectal carcinomas (Esteller et al., 2000c). p14 ARF methylation was absent in a report of 50 Barrett's oesophagus associated adenocarcinomas (Sarbia *et al.*, 2004).

Hypermethylation of *p14 ARF* has been detected in primary breast, bladder, lung, ovarian and brain tumours (Dominguez *et al.*, 2003; Yang *et al.*, 2006b; Yoshino *et al.*, 2007;

Zheng *et al.*, 2000), but is more prevalent in gastrointestinal tumours (Esteller et al., 2000b; Esteller et al., 2000c). Hypermethylation of *p14 ARF* has been shown to inversely correlate with p14 ARF expression in cell lines. Retrospective analysis has demonstrated *p14 ARF* hypermethylation is associated with a worse prognosis in colon, bladder and breast cancers (Dominguez et al., 2003).

Adenovirus- mediated transfer of *p14 ARF* in pancreatic cells deficient in *p14 ARF* has shown significant inhibition of cell growth compared with control vector *in vivo* and *in vitro*. These effects are partly dependent on the status of *p53* gene within these cells (Chen *et al.*, 2005). Demethylation and restoration of p14 ARF mRNA expression has been observed *in vitro* in human colon cancer cell lines using low dose decitabine (Zheng et al., 2000). Combination therapy with decitabine and irinotecan produced marked suppression in tumour growth compared with either agent alone, both *in vivo* and *in vitro* (Ishiguro *et al.*, 2007).

1.4.5.2 Effects of epigenetics on metastasis/ invasion

Cell adhesion molecules have an essential role in tissue formation and maintenance during embryological development, and in the maintenance of normal architecture and function in normal tissues. Loss of this activity can promote tumour invasiveness and metastatic potential. The cadherins including epithelial (e)-cadherin, are a family of integral membrane glycoproteins that are the prime mediators of calcium dependent cell-cell adhesion in normal cells. The cytoplasmic domain of e-cadherin is closely related to β-catenin, which tethers it to the actin cytoskeleton.

Reduced e-cadherin expression, by cell transfection with specific anti-sense RNA, has been shown to promote epithelial invasiveness, dedifferentiation and metastasis in

various human carcinomas, supporting a role for this protein as an invasion suppressor molecule (Vleminckx *et al.*, 1991). *In vitro*, loss of expression of the e-cadherin gene (*CDH1*) located on chromosome 16q22.1 has been shown to be associated with hypermethylation of its promoter region (Graff et al., 2000) Methylation-associated silencing of e-cadherin has been observed in cancers of the liver, prostate, breast, oral cavity and stomach (Graff *et al.*, 1995; Kanai *et al.*, 1997; Saito *et al.*, 1998; Tamura *et al.*, 2000). Conversely, transfection of malignant epithelial tumour cells with wild-type *CDH1* may restore normal phenotype (Vleminckx et al., 1991).

Expression of e-cadherin is reduced in 60-80 % of oesophageal cancers (Bongiorno *et al.*, 1995). Reduced expression has been shown to correlate with worse clinicopathological features in oesophageal squamous cell cancer including tumour grade, size, clinical staging, lymph node metastases, venous invasion and prognosis (Zhao *et al.*, 2003).

Mutations of *CDH1* are rare in adenocarcinoma of the oesophagus but aberrant methylation of the 5' CpG island of *CDH1* occurs in Barrett's oesophagus and the majority of oesophageal adenocarcinomas, leading to reduced e-cadherin expression (Corn *et al.*, 2001). Reduced expression in oesophageal adenocarcinoma is associated with a greater frequency of lymph node metastasis and shorter patient survival (Bailey *et al.*, 1998; Krishnadath *et al.*, 1997). Promoter hypermethylation of many genes including *CDH1*, *MGMT* and $p16^{INK4a}$ in oesophageal adenocarcinoma is a predictor of poor prognosis (Brock *et al.*, 2003).

The 2 histological variants of gastric adenocarcinomas are intestinal and diffuse-type. Intestinal-type lesions appear to have an environmental aetiology, predominate in

geographic regions with a high incidence of gastric carcinoma and have a falling incidence. Diffuse type lesions are associated with a worse prognosis than the intestinal type and have a genetic basis. *CDH1* mutations are considered to be the commonest somatic mutation in diffuse gastric cancer and are detectable in about 50 % of cases. *CDH1* germ-line mutations accompanied by loss of the wild-type allele have only been found in the hereditary diffuse gastric cancer (HDGC) syndrome (Berx *et al.*, 1998). In contrast, hypermethylation of the *CDH1* promoter has been found in 40-80 % of sporadic diffuse gastric cancers and identified as the cause of inactivation of the remaining wild-type allele in 50 % of HDGC cases. The incidence of *CDH1* promoter hypermethylation is significantly less in gastric carcinomas of the intestinal subtype (34 %) (Tamura et al., 2000).

1.4.6 DNA repair gene expression, methylation and malignancy

1.4.6.1 *MGMT*

The *MGMT* promoter region contains several transcription-factor-recognition sequences, including Sp1, glucocorticoid receptor binding elements, and activator protein-1 and 2 (AP-1, AP-2) binding sites (Figure 7). Activation of these sites causes modest *MGMT* induction in rodents, which may be achieved with glucocorticoids, cyclic AMP and protein kinase C activators (Biswas *et al.*, 1999; Grombacher *et al.*, 1996; Pegg, 2000) as well as DNA damage.

MGMT expression may be attenuated by methylation of its CpG-rich promoter region. Hypermethylation of the *MGMT* promoter results in a global alteration in heterochromatin and a 'closed' nucleosome structure, limiting transcription-factor

binding (Patel *et al.*, 1997; Watts *et al.*, 1997) (Figure 8). This has been observed *in vitro* in human tumours lacking MGMT activity e.g. gliomas, lymphoma, breast and prostate tumours and retinoblastomas (Esteller *et al.*, 2000a; Kang *et al.*, 2002; Watts et al., 1997). *MGMT*-silenced cells lack detectable levels of MGMT mRNA and protein. No gross genetic abnormalities of the gene have been reported, suggesting the absence of mRNA is due to regulation at the transcriptional level. A number of reports have shown that *MGMT* suppression is strongly linked to CpG methylation in the 5' region of the gene (Esteller *et al.*, 1999b). Primary malignant tumours with silenced *MGMT* are hypersensitive to cancer therapeutic and carcinogenic alkylating agents *in vitro*.

Figure 7. *O^6-methylguanine-DNA-methyltransferase* (*MGMT*) gene and its promoter region

The *MGMT* gene is located on chromosome 10q26 and is approximately 300 kilobases (kB) long, including 5 exons. *MGMT* gene expression is under the control of its promoter region. This contains several transcription factor binding sites including glucocorticoid receptor binding elements (GC), activator protein-1 (AP1), activator protein-2 (AP2), and Sp-1. Activation of these sites causes *MGMT* induction.

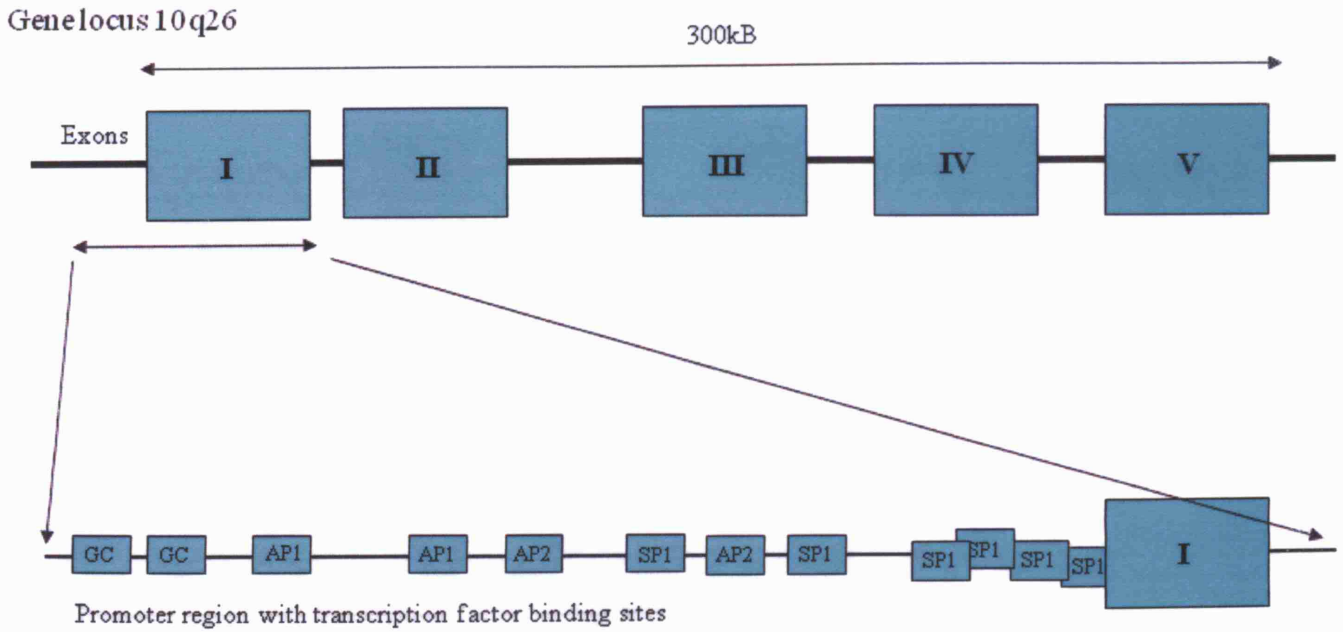
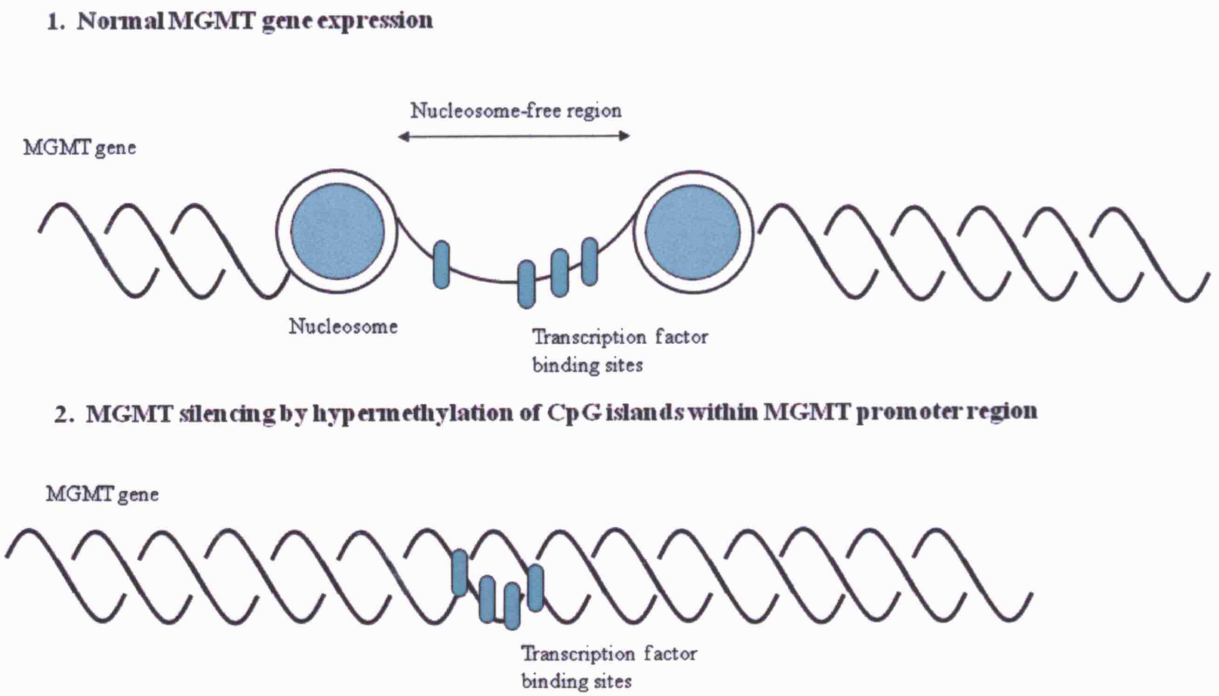


Figure 8. Schematic model for *MGMT* silencing following methylation of its promoter region

1. The *MGMT* gene contains a nucleosome-free area, with transcription factor binding sites available in the promoter region to allow gene transcription.
2. Following hypermethylation of CpG islands within the *MGMT* promoter region, the gene adopts a 'closed' nucleosome structure. The nucleosome positioning is altered and transcription factor binding sites are no longer available for binding inhibiting *MGMT* expression.



As expected. reduced *MGMT* expression and consequently loss of mutagenesis policing is associated with an increase in point mutations in key oncogenes and tumour suppressor genes. For example, *KRAS* in colon cancer, non-small cell lung cancer and gastric cancer (Esteller et al., 1999b; Esteller et al., 2000d; Park *et al.*, 2001) and *p53* in astrocytomas, colon cancer and non-small cell lung cancer (Esteller *et al.*, 2001c; Nakamura *et al.*, 2001; Wolf *et al.*, 2001). It may also be associated with a poorer prognosis as seen in lung cancer, hepatobiliary, gastric and breast tumours (Hayashi *et al.*, 2002; Koga *et al.*, 2005; Kohya *et al.*, 2002; Matsukura *et al.*, 2001).

Epigenetic inactivation of *MGMT* may be involved in premalignant change associated with colorectal cancer as it has been shown to create a field defect from which sporadic colorectal cancers may arise (Shen *et al.*, 2005). An increasing prevalence of DNA hypermethylation of the *MGMT* promoter region has been shown in one series from normal oesophageal epithelium (29 %), to hyperplasic epithelium (50 %), dysplastic epithelium (67 %) and finally carcinoma (72 %) (Fang *et al.*, 2005b). In this study, *MGMT* promoter hypermethylation highly correlated with the frequency of loss of MGMT mRNA and protein expression. MGMT levels are also reduced in gastric cancers with promoter hypermethylation of the *MGMT* gene (Esteller et al., 1999b). Aberrant methylation of *MGMT* has been detected in almost two-thirds of oesophageal adenocarcinomas and gastric carcinomas (Carvalho *et al.*, 2003; Eads *et al.*, 2001).

Transcriptional silencing of *MGMT* appears to be dependent on histone acetylation as well as DNA methylation (Danam *et al.*, 2005). *MGMT*-silenced cell lines strongly up-regulated MGMT expression following treatment with 5-aza-2'deoxycytidine, whereas Trichostatin A, an inhibitor of histone deacetylase, only weakly induced MGMT. A synergistic reaction, with significantly increased expression of MGMT, was seen when

combination treatment was given with 5-aza-2'deoxycytidine followed by Trichostatin A, indicating that histone deacetylation plays a role in *MGMT* silencing but that CpG methylation has the dominant effect. Methyl-CpG binding domain containing proteins (MBPs) were also found bound to the *MGMT* promoter in *MGMT*-silenced cells indicating they play a role in methylation-mediated transcriptional silencing (Danam et al., 2005).

Phase III studies in malignant gliomas have shown that *MGMT* promoter methylation is associated with improved disease response to alkylating agent chemotherapy and improved survival independent of treatment. Glioma patients with *MGMT* promoter methylation, as measured by methylation specific polymerase chain reaction, were shown retrospectively to have a better response and survival with BCNU, CCNU and temozolomide chemotherapy compared to those who did not (Esteller et al., 2000a; Hegi *et al.*, 2005; Paz *et al.*, 2004).

In the latest of these studies, conducted by the European Organisation for Research and Treatment of Cancer (EORTC) and the National Cancer Institute of Canada (NCIC), glioblastoma patients were randomised to radiotherapy alone or radiotherapy with concurrent and adjuvant temozolomide. Irrespective of treatment, *MGMT* promoter methylation was an independent favourable prognostic factor ($p<0.001$ by log-rank test; hazard ratio (HR), 0.45; 95 % confidence interval (CI), 0.32-0.61). For those with *MGMT* promoter methylation, a survival benefit was seen in those receiving combination treatment with radiotherapy and temozolomide: their median survival was 21.7 months (95 % CI, 17.4-30.4) as compared with 15.3 months (95 % CI, 13.0-20.9) in those receiving radiotherapy alone ($p=0.007$ by log-rank test). Conversely, in those patients who did not have methylation of the *MGMT* promoter there was a smaller,

statistically insignificant, difference in survival between the treatment groups (Hegi et al., 2005).

1.4.6.2 *hMLH1*

Although a number of genes are involved in the MMR pathway, the work in this thesis focuses on *hMLH1*. DNA methylation, as opposed to gene mutation, is an important mechanism in the transcriptional silencing of the MMR gene *hMLH1* on chromosome 3p21 (Kane *et al.*, 1997). Methylation of *hMLH1* is seen in sporadic tumours with microsatellite instability (MSI) including colon, endometrial, pancreatic, gastric and multiple primary tumours (Horii *et al.*, 1994).

Epigenetic changes can predispose to mutational events during tumour progression. Changes in the methylation of the 5' region of *hMLH1* have been seen in the apparently normal colonic epithelium of patients that have colorectal cancer with MSI (Nakagawa *et al.*, 2001), in hyperplastic regions preceding the development of endometrial cancers (Esteller *et al.*, 1999a) and in intestinal metaplasia and adenomas (Kang *et al.*, 2003b; Kang et al., 2001) that may precede the development of gastric cancer.

Treating colorectal cancer cell lines containing hypermethylated *hMLH1* alleles with the DNA methyltransferase inhibitor, 5-aza-2'deoxycytidine has resulted in re-expression of *hMLH1* and partial restoration of mismatch repair ability (Herman *et al.*, 1998). In MMR-deficient, drug-resistant ovarian and colon tumor xenografts that do not express hMLH1 because of gene promoter hypermethylation treatment with 5-aza-2 deoxycytidine induces *hMLH1* expression, with a decrease in *hMLH1* gene promoter methylation and sensitizes the xenografts to cisplatin, carboplatin, temozolomide, and

epirubicin. Sensitization is comparable with that obtained by reintroduction of the *hMLH1* gene by chromosome 3 transfer (Plumb *et al.*, 2000).

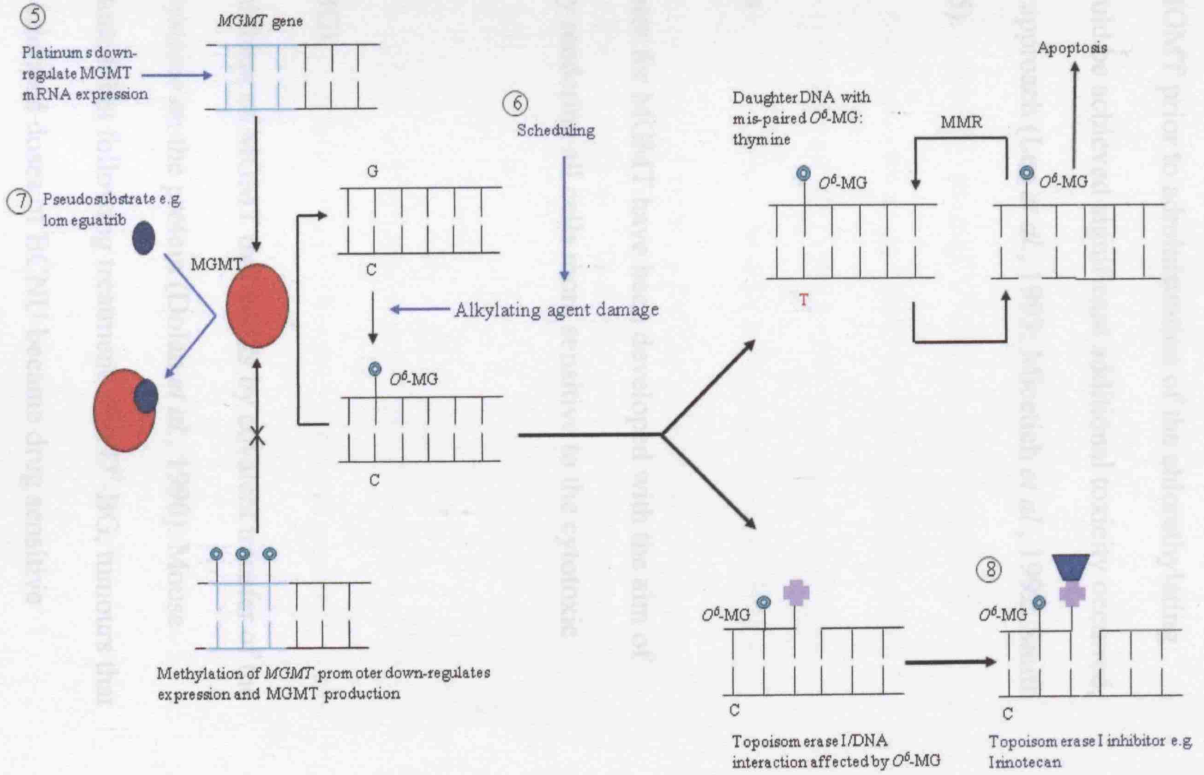
hMLH1 hypermethylation in gastric cancer has been found to be more prevalent in lower pathological tumour and nodal categories (Pinto *et al.*, 2000). This is reflected in the better survival of these patients and has lead to the concept of a distinct oncogenic pathway in MSI tumours (Yamamoto *et al.*, 1999). In most studies the frequency of high MSI oesophageal squamous cell carcinoma tumours is <10 % and the MSI pathway considered to be unlikely to be involved in their carcinogenesis (Kubo *et al.*, 2005). *hMLH1* hypermethylation can be seen with increasing frequency in normal oesophageal mucosa, Barrett's oesophagus and oesophageal adenocarcinomas indicating *hMLH1* hypermethylation may play an early part in the stepwise progression to the development of adenocarcinoma (Eads et al., 2001).

1.5 Manipulation of MGMT in Cancer Therapy

A number of different approaches may be taken to target the MGMT DNA damage pathway (Figure 9).

Figure 9. Schematic overview of DNA repair by MGMT, highlighting mechanisms for therapeutic intervention to improve alkylating agent efficacy.

This DNA repair process may be used to predict response to therapy, or targeted in several ways to increase chemotherapeutic efficacy. MGMT mRNA expression is down- regulated by platinums thereby reducing MGMT expression (5). The schedule of administration of alkylating agents may be modified to cause maximal DNA damage (6). Pseudosubstrates e.g. lomeguatrib can bind to MGMT inhibiting its repair function (7). Finally, O^6-MG adducts affect the kinetics of topoisomerase I cleavage complex formation increasing the cytotoxicity of subsequently administered topoisomerase I inhibitors (8).



1.5.1 Combination therapy with Chlorethylating and methylating agents

Several studies have sought to deplete MGMT by giving a methylating agent before the administration of a chlorethylating agent. The initial DNA methylation by the methylating agent inactivates MGMT prior to administration of the chlorethylating agent. Although inactivation could be achieved significant additional toxicity occurred, preventing development of this approach (Lee *et al.*, 1993; Micetich *et al.*, 1992; Smith *et al.*, 1996; Willson *et al.*, 1995).

1.5.2 Direct Protein Inhibition

A group of novel pseudosubstrates for MGMT have been developed with the aim of inactivating MGMT and thereby rendering all cells more sensitive to the cytotoxic effects of O^6-alkylating agents.

1.5.2.1 O^6-benzylguanine (O^6-BG)

O^6-BG is a potent, non-toxic, inhibitor of MGMT that works by covalent transfer of the benzyl group to the active site cysteine on the protein (Dolan *et al.*, 1990). Mouse xenograft models have demonstrated that following treatment with O^6-BG, tumours that had been resistant to maximum tolerated doses of BCNU became drug sensitive (Gerson *et al.*, 1993; Kreklau *et al.*, 1999).

In clinical trials human tumour MGMT activity can be completely suppressed by O^6-BG in a number of different tumour types (Dolan *et al.*, 2002; Friedman *et al.*, 1998a; Schilsky *et al.*, 2000; Schold *et al.*, 2004; Spiro *et al.*, 1999). However the dose of O^6-

BG required to inactivate MGMT and the duration of this inactivation has varied both between and within tumour types. Friedman et al found a dose of 100 mg/m^2 O^6-BG reliably depleted MGMT activity to undetectable levels in gliomas 18 hours after administration (Friedman *et al.*, 1998a). At 6 hours Schold et al found that 120 mg/m^2 O^6-BG was needed (Schold et al., 2004).

Phase I studies have established the maximum tolerated dose (MTD) of BCNU to be 40 mg/m^2 when administered with an inhibitory dose of O^6-BG, i.e. 20 % of the MTD for BCNU alone, (Friedman *et al.*, 2000; Schilsky et al., 2000) because of dose-limiting myelosuppression. Studies have combined O^6-BG with BCNU in myeloma patients (Bahlis *et al.*, 2003) and in cutaneous T-cell lymphoma (Apisarnthanarax *et al.*, 2003). In both of these studies O^6-BG inactivated tumour MGMT as determined on repeat biopsy and biochemical assay. As with the phase I trials, the main toxicity seen was myelosuppression.

However, the use of O^6-BG (100 mg/m^2) with reduced dose of BCNU (40 mg/m^2) was not effective against BCNU-resistant glioblastoma multiforme (Quinn *et al.*, 2002) or in melanoma (Gajewski *et al.*, 2005) in phase II studies. Although in the latter paper, MGMT activity was not measured within tumour but in peripheral blood mononuclear cells, which have previously been shown to correlate poorly with tumour MGMT levels (Spiro et al., 1999). The profound reduction in BCNU dose required may also be a factor in the failure of this combination in these studies.

A phase I trial of temozolomide plus O^6-BG in recurrent or progressive glioma was conducted in 2 stages by Quinn et al (Quinn *et al.*, 2005). The first stage was to determine the dose of O^6-BG effective at producing complete depletion of tumour

MGMT at 48 hours. The second stage was designed to define the MTD of a single dose of temozolomide when combined with O^6-BG. An intravenous dose of 120 mg/m^2 O^6-BG given over 1 hour followed by a continuous infusion of 30 mg/m^2/day for 48 hours was found to be effective in depleting MGMT levels at 48 hours. The MTD of temozolomide with this regimen was 472 mg/m^2, with dose limiting toxicity being myelosuppression. A single dose of temozolomide was used in this study, but the ideal temozolomide administration schedule with O^6-BG is still to be determined.

1.5.2.2 O^6- (4-bromothenyl) guanine (lomeguatrib)

Lomeguatrib is a more potent pseudosubstrate of MGMT than O^6-BG and is orally bioavaliable. Covalent transfer of the bromothenyl group to the active site cysteine residue on the protein results in inactivation of MGMT. This then undergoes ubiquitin dependent proteolysis.

Xenograft studies with melanoma, prostate and breast cancers have shown that a daily dose of lomeguatrib (20 mg/kg) administered with temozolomide over 5 days delays tumour growth significantly longer than can be achieved by treatment with temozolomide alone (Middleton *et al.*, 2000b; Middleton *et al.*, 2002).

A phase I clinical trial of lomeguatrib co-administered with temozolomide has established that 10 mg/m^2 intravenous lomeguatrib depletes MGMT in tumour (Ranson *et al.*, 2006b). The MTD of temozolomide in combination with this dose of lomeguatrib was 150 mg/m^2/day orally using the standard 5-day schedule of administration. The side effects of the combination with temozolomide were no different from those observed for temozolomide alone. These results are encouraging as at tumour depleting doses of lomeguatrib, more than 60 % of the standard single agent dose of temozolomide could

still safely be administered. The oral lomeguatrib MGMT depleting dose schedule was 40 mg daily for 5 days. To permit ease of administration this dose schedule in combination with temozolomide has been suggested for further evaluation.

A phase II trial of combination treatment of oral lomeguatrib with temozolomide in over 100 patients with metastatic melanoma has also recently been completed. Patients were given 40 to 80 mg lomeguatrib with 125 mg/m^2 temozolomide or 200 mg/m^2 temozolomide alone orally on days 1 through 5 of up to six 28-day treatment cycles. Patients on temozolomide alone were offered combination treatment at progression, if fit enough, to assess the propensity for lomeguatrib to reverse resistance to the methylating agent.

The efficacy of lomeguatrib and temozolomide with this dosing schedule was found to be similar to that of temozolomide alone (response rates 13.5 % vs. 17.3 % respectively; median time to disease progression was 65.5 vs. 68 days respectively). No patient responded to combination treatment having progressed through temozolomide alone. Rapid recovery of MGMT was seen in tumour biopsies after combination treatment indicating dosing with lomeguatrib would need to be continued beyond that of temozolomide to maintain MGMT depletion (Ranson *et al.*, 2007).

A phase II trial to examine extended dosing with lomeguatrib in combination with temozolomide in metastatic colorectal cancer has recently been reported (Khan *et al.*, 2008). Patients received lomeguatrib (40 mg) and temozolomide (50-200 mg/m^2) orally for 5 days every 4 weeks. Despite consistent depletion of MGMT in peripheral blood mononuclear cells (PBMCs), no clinical responses to treatment were seen. This may have been due to a number of reasons: the wrong doses of temozolomide and lomeguatrib may have been given, colorectal cancer may be inherently insensitive to temozolomide, and deficient mismatch repair (MMR) may be present allowing

tolerance of methylation damage. Ranson et al reported tumour biopsies from patients treated with the same regimen with melanoma showed early recovery of MGMT activity (within 24 hours) even when 60 and 80 mg of lomeguatrib were given daily (Ranson *et al.*, 2006a).

1.5.3 Topoisomerase I inhibitors and MGMT

Irinotecan (CPT-11) is a camptothecin derivative that produces anti-tumour activity by inhibiting topoisomerase I. Preclinical data indicates that MGMT has a role in resistance to irinotecan and its active metabolite SN-38 (Okamoto *et al.*, 2002). MGMT expression was found closely to correlate with sensitivity to the irinotecan derivative and inhibition of MGMT by O^6-BG augmented drug activity in a number of cell lines. Transfection studies showed that increasing MGMT expression decreased the sensitivity to irinotecan and SN-38, whereas repression of MGMT expression sensitised cells to the drug (Okamoto et al., 2002). There is also pre-clinical evidence that temozolomide can enhance the activity of irinotecan in a schedule-dependent manner. This occurs through a change in the kinetics of the formation of topoisomerase I-DNA covalent complexes induced by O^6-MG adducts formed by the temozolomide when it is administered prior to irinotecan. In the presence of nearby O^6-MG topoisomerase I is 'trapped' on DNA (Friedman et al., 2000; Patel et al., 1997; Pourquier *et al.*, 2001).

A number of studies have been conducted to try to demonstrate the enhanced activity of irinotecan with prior alkylating agent administration. A phase I trial of irinotecan (Day 1, 8, 15 and 22) and BCNU (100 mg/m^2 once every 6 weeks) in patients with recurrent glioma established the MTD of irinotecan for patients not receiving anticonvulsants as 125 mg/m^2 and for those who were receiving anticonvulsants as 225 mg/m^2.

Myelosuppression was the main toxicity. The subsequent phase II study conducted by the same group found a response rate of 14 % (95 % CI, 5-29 %) in newly diagnosed patients and 13 % (95 % CI, 4-27 %) in those with recurrent disease, with a 40 % stable disease rate for all patients. Median time to progression was 11.4 weeks (95 % CI, 6.0-14.4 weeks) (Quinn *et al.*, 2004; Reardon *et al.*, 2004). A second phase II study by the Gruppo Italiano Cooperativo di Neuro-Oncologia (GICNO) using the same combination and schedule of administration in glioblastoma multiforme patients receiving second-line chemotherapy, reported 21.4 % partial response rate (95 % CI, 9-34 %), 50 % stable disease rate (95 % CI, 35-65 %) and median time to progression of 17 weeks (95 % CI, 11.9-23.9) (Brandes *et al.*, 2004b).

In a phase I trial of irinotecan (Day 1, 8, 22 and 29) and temozolomide (200 mg/m^2 days 1-5) once every 6 weeks in patients with recurrent malignant glioma, patients were stratified based on concurrent administration of enzyme-inducing anti-epileptic drugs (Reardon *et al.*, 2005). MTD of irinotecan for patients concurrently receiving and not receiving anti-epileptics was 325 mg/m^2 and 125 mg/m^2, respectively and 14 % (15/107) of patients achieved a complete or partial radiological response. Dose limiting toxicities were primarily haematological and gastrointestinal.

In all four of these studies, the overall results have been very similar to those achieved with irinotecan alone in progressive or recurrent malignant glioma. In a phase II trial of irinotecan (Day 1, 8, 15 and 22) in these patients 15 % (95 % CI, 6-24 %) achieved a partial response whereas 55 % achieved stable disease for >12 weeks (Friedman *et al.*, 1999). Median time to tumour progression was 12 weeks (range 6-68 weeks). Toxicity was limited and concentrations of irinotecan and its active metabolites were lower in patients with concurrent steroid and anti-convulsant use. The disappointing results for

combination therapy with the absence of synergism may be due to ineffective scheduling of the combination of drugs. In all of these studies, the alkylating agent was not administered prior to irinotecan despite the preclinical findings. More encouraging results have been seen when drug scheduling is optimised in accordance with preclinical data and the alkylating agent is administered prior to irinotecan. In a phase I study in recurrent malignant glioma in which patients were given temozolomide (days 1-5) and irinotecan (day 6), 31 % (10/32) patients achieved a complete or partial response (Gruber & Buster, 2004).

The role that MGMT plays in modulating the effect of this synergistic interaction has not yet been elucidated. However cell line work in MGMT-expressing glioma cells adding O^6-BG to the combination of temozolomide and irinotecan showed a dramatic increase in growth delay compared with either temozolomide or irinotecan alone or together (Friedman *et al.*, 2002). A phase I trial of this combination is currently ongoing in temozolomide resistant malignant glioma.

1.5.4 Other mechanisms for modulating MGMT in cancer therapy

1.5.4.1 Temozolomide scheduling

Temozolomide scheduling has been manipulated to maximise MGMT depletion and hence cytotoxicity. The standard 5-day dosing regimen of temozolomide (150-200 mg/m^2/day) has been shown deplete PBMC MGMT in a cumulative and progressive manner (Lee *et al.*, 1994), but partial MGMT recovery is seen 24 hours after dosing, i.e. when the next temozolomide dose is due.

Compressed temozolomide scheduling has been associated with enhanced MGMT depletion compared with standard once daily administration but this is at the expense of significantly increased myelotoxicity, limiting wider clinical application (Danson *et al.*, 2003; Middleton *et al.*, 2000a; Spiro *et al.*, 2001).

Extended administration aims to take advantage of the continued fall in MGMT seen with each successive day of temozolomide dosing. Two phase I trials using different temozolomide administration schedules have been shown to deplete MGMT levels *in vivo* and deliver twice the dose intensity of the conventional schedule (Brock *et al.*, 1998; de Bono *et al.*, 2001). Whether this translates into meaningful clinical benefit is yet to be established although this question has been evaluated by the recently completed EORTC 18032 study in stage IV melanoma that randomised patients to oral temozolomide for 7 days alternate weeks over 6 weeks or dacarbazine once every 3 weeks. The results of this study are awaited.

1.5.4.2 Platinums and MGMT

Platinums may reduce MGMT expression by down-regulating transcription of the *MGMT* gene (D'Atri *et al.*, 2000) thereby potentially enhancing sensitivity to alkylating agents. This has been shown in the preclinical setting with low dose cisplatin and nimustine hydrochloride (ACNU) in a gallbladder cancer cell line (Sato *et al.*, 2005) and with both cisplatin and carboplatin in combination with ACNU in gliomas (Tanaka *et al.*, 2005). However combination treatment in phase II/III clinical trials have shown limited benefit over single alkylating agent treatment despite appropriate drug scheduling (Brandes *et al.*, 2004a; Fletcher *et al.*, 1993; Bafaloukos *et al.*, 2005).

1.6 Modulation of epigenetic parameters

The concept that epigenetic abnormalities could be as important as genetic ones in determining the course of tumour development is an important consideration in developing new therapies. Whereas genetic changes have been reversed in the experimental setting, this has been difficult to accomplish *in vivo*. However, the chromatin changes caused by epigenetic modulation have the potential for drug targeting, as the silenced genes may be induced to re-express (Egger *et al.*, 2004; Issa *et al.*, 2005). Three groups of epigenetic drugs have been found to have impact to date: DNA-methyltransferase inhibitors (DNMTIs), histone deacetylase inhibitors (HDACis) and histone methylation inhibitors.

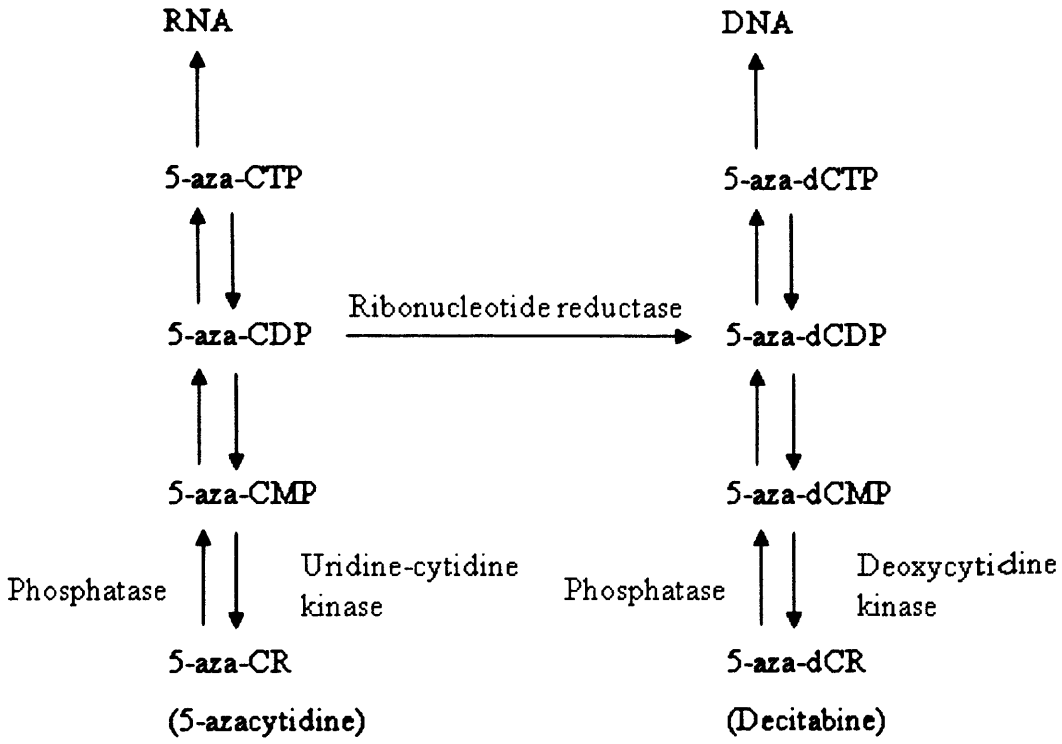
1.6.1 Nucleoside DNMTIs

These analogues of cytosine are phosphorylated and incorporated instead of cytosine into replicating DNA during S-phase. Once incorporated, they bind non-specific DNMTs inhibiting the methylation of DNA. Covalent arrest of DNMTs results in their cellular depletion and demethylation of genomic DNA occurs through continued DNA replication with re-expression of growth suppressor genes that ultimately may result in cell death. The covalent adducts formed between DNMTIs and DNMTs can lead to direct anti-tumour activity through the steric inhibition of DNA replication, transcription and repair (Ferguson *et al.*, 1997). Finally global inhibition of DNMTs resulting in DNA hypomethylation may induce cell-cycle arrest and apoptosis through activation of tumour suppressor genes such as *p53,* in a process independent of the methylation status of its promoter (Karpf *et al.*, 2001). DNMTIs are at a clinically advanced stage of development having been tested in a number of phase I-III trials.

1.6.1.1 5-Azacytidine (Vidaza)

5-azacytidine was first used clinically in the 1960s. It is a ribose nucleoside that must be converted chemically to a deoxyribonucleotide triphosphate to be incorporated into DNA. However, before this can occur, a proportion of the drug is incorporated into RNA, which affects a variety of RNA functions and therefore has cellular consequences independent of demethylation (Figure 10). Historically it was evaluated as an anti-leukemic drug before its demethylating activity was discovered. More recently, this drug has shown significant success in the treatment of myelodysplastic syndrome in phase II and III clinical trials resulting in its licensing for this condition in the USA (Silverman *et al.*, 2002).

Figure 10. Metabolism of 5-azacytidine & decitabine



Key:

(d)CR, (deoxy)cytosine ribose; (d)CMP, (deoxy)cytidine monophosphate; (d)CDP, (deoxy)cytidine diphosphate; (d)CTP, (deoxy)cytidine triphosphate. Adapted from Jones *et al.*, (Jones, 2004).

1.6.1.2 5-aza-2'-deoxycytidine (Decitabine)

Decitabine, first synthesised in 1964, was found to be cytotoxic at high doses, arresting DNA synthesis. In the early 1980s, however, decitabine was also shown to decrease DNMT activity in erythroleukaemic cells with resulting hypomethylation of the newly synthesised DNA and the induction of cellular differentiation (Creusot *et al.*, 1982). In the late 1990s, a clinical study of decitabine in patients with sickle cell anaemia confirmed the hypomethylating efficacy of low dose decitabine in the absence of cytotoxicity (Koshy *et al.*, 2000) resulting in a new role for the drug in the treatment of malignancy through epigenetic modification.

1.6.1.2.1 Metabolism and molecular mechanism of action

Decitabine is a deoxycytidine analogue in which the carbon at the 5 position of pyrimidine ring is replaced by nitrogen. For activation of decitabine to occur, it has to be phosphorylated by the enzyme deoxycytidine kinase to 5-aza-dCMP. This is rapidly converted by kinases to 5-aza-dCTP. 5-aza-dCTP is a good substrate for DNA polymerases allowing incorporation of the nucleotide analogue into DNA (Figure 10). Decitabine can undergo degradation and therefore inactivation by deamination by cytidine deaminase. It exerts a cytotoxic action leading to cell killing at high doses, but can also cause cell death via demethylation at lower doses.

Other mechanisms are also involved in the anti-tumour effects of low-dose decitabine. In addition to gene re-expression that occurs as a consequence of reversing promoter hypermethylation, indirect gene expression changes may also occur. Decitabine treatment has been shown to alter gene expression in human bladder cancer cells and normal human fibroblasts, but in this study, 60 % of the genes induced by decitabine did not contain CpG islands, implying that decitabine treatment regulates the expression

of many genes via other mechanisms (Liang *et al.*, 2002). These may include demethylation of the promoter sites of upstream genes resulting in their re-expression, which in turn, affects the regulation of the genes studied. In acute myeloid leukaemia cell lines treatment with decitabine increases the expression of the pro-apoptotic protein p21, arresting the cell lines in G1 of the cell cycle. Expression of *p21* was induced irrespective of the methylation status of its promoter, mediated instead via re-expression of the tumor suppressor *p73*, an upstream regulator of p21, which was found to have hypermethylation of its promoter region (Tamm *et al.*, 2005).

Global inhibition of DNMTs by decitabine results in genomic hypomethylation, which may be perceived as DNA damage and result in the activation of tumour suppressor genes as *p53*. Research using colon cancer cell lines has shown that cells expressing wild-type *p53* are more sensitive to decitabine mediated growth arrest and cytotoxicity; the response to decitabine treatment includes the induction and activation of wild-type but not mutant p53 protein; and the induction of the downstream *p53* target gene *p21* is partially *p53*-dependent. The induction of p53 protein after decitabine treatment did not correlate with an increase in p53 transcripts, indicating that hypomethylation at the *p53* promoter did not account for the *p53* response. (Karpf *et al.*, 2001)

Decitabine has also been shown to induce the expression of tumour-associated antigens at the cell surface. Induction of the cancer antigens MAGE-1 and NY-ESO-1 following treatment with decitabine has been demonstrated in renal cell carcinoma and melanoma (Coral et al., 2002; Coral et al., 1999). Decitabine therapy may therefore have a therapeutic role prior to active and/or passive specific immunotherapy in the treatment of these malignancies.

1.6.1.2.2 Preclinical studies

Decitabine appears to have better anti-tumour activity, with greater inhibition of DNA methylation and increased gene expression, than 5-azacytidine in experimental models (Carr *et al.*, 1988; Momparler et al., 1984). A number of studies have been performed assessing the role of decitabine in sensitising cancer cells to cytotoxic treatment by altering expression of DNA repair proteins, mediators of apoptosis and enzymes involved in cytotoxic drug metabolism via demethylation of their gene promoters.

Decitabine has been shown at non-toxic doses to decrease *hMLH1* gene promoter methylation, inducing re-expression of *hMLH1* in drug-resistant colon and ovarian cancer xenograft models. Decitabine treatment also sensitised the xenografts to chemotherapeutic agents dependent on MMR for cytotoxicity e.g. cisplatin and temozolomide. Sensitization was comparable with that obtained by the reintroduction of the *hMLH1* gene by chromosome 3 transfer (Plumb *et al.*, 2004; Plumb *et al.*, 2000; Strathdee et al., 1999). *In vitro* treatment with decitabine has been shown to down-regulate P-glycoprotein expression in multi-drug resistant (MDR) myeloid leukaemia cell lines as a result of demethylation at the repressor-binding site of the *MDR-1* gene and enhance sensitivity to daunorubicin (Ando *et al.*, 2000).

Melanoma cells with inactivated apoptosis mediator *apaf-1* through hypermethylation are resistant to chemotherapy. *In vitro* exposure of the resistant tumour cells to decitabine restored the apoptotic defects associated with loss of *apaf-1* and sensitivity to chemotherapy (Soengas et al., 2001). Demethylation by decitabine can also restore caspase-8 expression and enhance sensitivity of neuroblastoma and primitive neuroectodermal brain tumour cells to apoptotic agents (Grotzer *et al.*, 2000).

Abnormal methylation can also influence the efficacy of enzymes essential for the metabolism of chemotherapeutic drugs. The anti-folate pemetrexed has activity in mesothelioma, yet another anti-folate capecitabine has significantly less efficacy. The enzyme thymidine phosphorylase (TP) is crucial to the efficacy of anti-folates such as capecitabine. In a mesothelioma cell line, the gene coding for thymidine phosphorylase, *ECRF-1*, was found to be methylated thereby down-regulating TP and reducing its cytotoxic effect. Following therapy with decitabine, TP expression was increased 6-fold in the mesothelioma cells and increased cytotoxicity was seen (Kosuri *et al.*, 2006).

Aberrant methylation also affects other factors that influence the growth and sensitivity of tumour cells. The expression of hormone receptors in a variety of tumour types is suppressed by DNA methylation. Treatment with decitabine has been shown to restore oestrogen receptor expression in breast cancer cell lines lacking ER expression due to methylation (Ferguson *et al.*, 1995). Similarly, treatment with a DNMTI has restored expression of progesterone receptor-beta in endometrial cancer cells and androgen receptor in prostate cancer cells (Nakayama *et al.*, 2000).

1.6.1.2.3 Decitabine scheduling

It has become apparent that demethylation of tumour suppressor genes may occur at drug-concentrations significantly lower than that required for cytotoxic effect (Bender *et al.*, 1998). In addition, it has been shown by measuring gene products, that genes demethylated by DNMTIs may be re-methylated to pre-treatment levels after 13 days in dividing cells (Velicescu *et al.*, 2002). Additional limitations of single-agent aza-nucleoside therapies include low drug stability, poor bioavailability and rapid elimination.

In view of these limitations and in order to maximise the biological effects and minimise toxicity of decitabine, an alternative approach has evolved, using a period of demethylation, before re-methylation can occur, as a window of epigenetic sensitisation for combination therapy. Plumb *et al.* demonstrated that in xenograft models with hypermethylation of *hMLH1*, resistance to chemotherapy dependent on MMR reduced after adding non-toxic doses of decitabine. The therapeutic response was dependent on the timing of decitabine, which had to be given 6-12 days before the cytotoxic drug (Plumb et al., 2000).

1.6.1.2.4 Clinical studies

Single agent decitabine has shown activity in haematological malignancies including myelodysplastic syndrome (MDS), acute myelogenous leukaemia (AML) and chronic myelogenous leukaemia (CML) (Issa *et al.*, 2004; Kantarjian *et al.*, 2006; Kantarjian *et al.*, 2003; Wijermans *et al.*, 2000), but limited activity in solid malignancies (Aparicio & Weber, 2002; Momparler *et al.*, 1997; Schwartsmann *et al.*, 2000).

The optimal dose and schedule for administering decitabine to inhibit methylation has not been established. Several studies have shown that decitabine can inhibit DNA methylation *in vivo*. In a phase I study of varied low-dose, prolonged exposure schedules to decitabine in relapsed/refractory leukaemias (predominantly CML), patient cohorts received decitabine at 5, 10, 15, or 20 mg/m^2 intravenously (i.v.) over 1 hour daily, 5 days a week for 2 consecutive weeks. There were 2 groups that also received 15 mg/m^2 daily for 15 or 20 days. The dose of 15 mg/m^2 for 10 days appeared to induce the most responses (65 %), with fewer responses seen when the dose was escalated or prolonged (11 %) (Issa et al., 2004). In the subsequent phase II study, decitabine was administered at 15 mg/m^2 intravenously over 1 hour daily, 5 days a week for 2 weeks,

in patients with imatinib resistant-CML. This study showed that decitabine induced global hypomethylation associated with a clinical response (Issa et al., 2005).

Kantarjian et al. treated 130 patients with CML at different phases with decitabine 50, 75 or 100 mg/m^2 administered over 6 hours every 12 hours for 5 days. Decitabine appeared to have significant clinical activity in all three phases of disease (blastic, accelerated and chronic), with some complete responses. No dose response was seen but there was delayed, prolonged, dose dependent severe myelosuppression (Kantarjian et al., 2003). Yang et al. demonstrated DNA methylation changes in patients with myeloid leukaemias after decitabine treatment. Patients received either decitabine 50-90 mg/m^2 twice a day for 5 days or 5-20 mg/m^2 once a day for 10 days. Dose-dependent hypomethylation was only demonstrated after low dose decitabine with a plateau of response above 20 mg/m^2 (Yang *et al.*, 2006a).

Global DNA methylation and *p21* DNA methylation were shown to decrease in serial bone marrow samples from patients with MDS treated with decitabine 45 mg/m^2 a day for 3 days every 6 weeks (Mund *et al.*, 2005). This group of patients had an overall response rate of 49 % with decitabine treatment, with a 64 % response rate in the patients with high-risk MDS (Wijermans et al., 2000). In the following phase III study 170 patients with MDS were randomized to receive either decitabine at a dose of 15 mg/m^2 i.v. over 3 hours every 8 hours for 3 days (at a dose of 135 mg/m^2 per course) and repeated every 6 weeks, or best supportive care. The treatment group achieved a significantly higher overall response rate (17 %), including 9 % complete responses, compared with supportive care (0 %) ($p < 0.001$). This group also had a trend toward a longer median time to AML, progression or death compared with patients who received

supportive care alone (Kantarjian et al., 2006). On the basis of these results decitabine has been approved for the treatment of MDS in the USA.

Global DNA methylation and methylation of the *MAGE1* gene promoter, routinely methylated in peripheral blood cells (PBCs) in adults, decreased in the PBMCs of patients with refractory solid tumors treated with decitabine 2 mg/m^2 daily via continuous infusion for 7 days (Samlowski *et al.*, 2005). In another phase I trial in patients with advanced solid tumors, decitabine was given at escalating doses of 45, 67, 90 and 120 mg/m^2, as a 2 hour i.v. infusion with cisplatin at a fixed dose of 33 mg/ m^2 i.v. immediately after the end of decitabine infusion. Both agents were given on days 1-3 every 21 days. The maximum tolerated dose (MTD) of decitabine was 120 mg/ m^2, with dose-limiting toxicities including neutropenia, thrombocytopenia and mucositis. In the subsequent phase II trial in patients with inoperable non-small cell lung cancer (NSCLC), decitabine 67 mg/m^2 was administered with cisplatin as in the previous study. As in the phase I study, significant (Common Toxicity Criteria (CTC) Grade 3-4) neutropenia and thrombocytopenia were observed. No significant anti-tumor activity was seen however this could be due to scheduling of the drugs; cisplatin was administered immediately after decitabine not allowing time for demethylation to occur (Schwartsmann et al., 2000).

In another phase I study, 33 patients were treated with escalating doses of decitabine 45, 90 and 135 mg/m^2 i.v. over 6 hours on day 1 followed by carboplatin AUC 5 or 6 on day 8 of a 28 day cycle. Decitabine 90 mg/m^2/carboplatin AUC 6 was the recommended dose for future studies. The main toxicities seen were haematological. Decitabine induced dose-dependent, reversible demethylation in PBCs, maximally at day 10. Decitabine 90 mg/m^2 also induced demethylation of the *MAGE1A* gene in PBCs, buccal

cells and tumor biopsies as well as elevation of HbF expression. At the recommended dose, in this heavily treated patient group, 1 patient had a partial response and 1 patient had stable disease. An additional 2 patients had stable disease having received decitabine 45 mg/m^2/carboplatin AUC 6 (Appleton *et al.*, 2007). This schedule of a 6 hour decitabine infusion on day 1 appeared to produce a similar reduction in methylation levels in PBMCs as seen with a 7 day continuous low dose regimen and a 1 hour infusion daily over 10 days (Issa et al., 2004; Issa et al., 2005; Samlowski et al., 2005).

A phase I trial using sequential low dose decitabine 0.1 to 0.25 mg/kg daily subcutaneously (s.c.) 5 days a week for 2 weeks with high dose interleukin-2 (IL-2) 600,000 IU/kg i.v. every 8 hours for 14 doses) in patients with melanoma and renal cell carcinoma, found that decitabine decreased global DNA methylation with gene re-expression in a non-dose responsive manner (Gollob *et al.*, 2006).

1.6.1.3 Other DNMTIs

Zebularine, a cytosine analogue is chemically stable, of low toxicity and can be taken orally. It is unique as it is a potent inhibitor of DNMT and also inhibits cytidine deaminase as well as having its own cytotoxic properties. However, higher concentrations of zebularine are needed to achieve similar levels of demethylation in cells in comparison to decitabine (Yoo *et al.*, 2004). Zebularine has been used to treat human bladder carcinoma cells containing transcriptionally silent hypermethylated *p16^{INK4A}* gene promoter, successfully inducing p16 ^{INK4A} expression (Cheng *et al.*, 2003). It has been used in combination with decitabine to reduce the rapid inactivation of decitabine by cytidine deaminase, enhancing its activity in leukemic cell lines and xenografts (Lemaire *et al.*, 2005). It has yet to be evaluated in clinical trials.

S110, a second generation demethylating agent has recently been developed by incorporating decitabine into a guanine dinucleotide thereby improving stability by reducing deamination by cytidine deaminase. In colon cancer cell lines, S110 has shown equivalent activity to decitabine in inducing re-expression of a number of genes commonly methylated in malignancy including $p16^{INK4}$ and *hMLH1*. S110 demonstrated methylation inhibition by causing DNMT1 degradation and showed a longer half life than decitabine *in vivo* in rats. It has shown anti-tumour activity in a cisplatin-resistant ovarian xenografts model, restoring sensitivity to cisplatin (Clifford *et al.*, 2008). Phase I trials are just beginning with this agent.

1.6.2 Non-nucleoside DNMTI

This group of compounds directly block DNMT activity and therefore do not have the inherent myelotoxicity caused by covalent trapping of the enzymes. Examples include, (-)-epigallocatechin-3-gallate, the main polyphenol compound in green tea, RG108, a small molecule inhibitor of human DNMTs (Brueckner *et al.*, 2005), 4-aminbenzoic acid derivatives e.g. procainamide and procaine (Lin *et al.*, 2001; Villar-Garea *et al.*, 2003), psammaplins (Pina *et al.*, 2003) and oligonucleotides e.g. hairpin loops and specific anti-sense oligonucleotides (Flynn *et al.*, 2003; Robert *et al.*, 2003). However most of these are still in the very early stages of development and undergoing preclinical testing. Preliminary data suggests that this group of compounds are not as effective as decitabine in inhibiting DNA methylation and are associated with less gene reactivation (Chuang *et al.*, 2005).

1.6.3 HDACis

These have a wide range of activity against all HDACs leading to the accumulation of acetylated histone proteins in tumour cells (Marks *et al.*, 2000) and the induction of differentiation, growth arrest and/or apoptosis (Marks *et al.*, 2003). One important property of this group of drugs is their ability to induce G1 cell cycle arrest via induction of *p21* in tumours that have defective *p53* function. Also, HDACis are able to induce G2 checkpoints and apoptosis both via the death receptor and mitochondria-mediated apoptotic pathways. The specificities of the HDACis to the four classes of different HDACs vary e.g. depsipeptide inhibits HDAC1 and HDAC2 whereas the hydroxamic derivative PXD 101 inhibits all class I, IIa, IIb and IV HDACs (Table 2).

A number of clinical trials using these compounds alone or in combination with other anticancer agents are currently ongoing. Suberoylanilide hydroxamic acid (SAHA) has now been approved for the treatment of T-cell cutaneous lymphoma in the United States.

Table 2. HDACs and their Inhibitors

Class (localisation)	HDAC	Inhibitors			
		Specific		General	
I (nucleus)	HDAC1	Depsipeptide, SK-7041, SK-7068	MS-275	Sodium valproate, phenylbutyrate	Trichostatin A, suberoylanilide hydroxamic acid (SAHA), PXD101, LAQ-824, LBH-589, Trapoxin
	HDAC2				
	HDAC3				
	HDAC8	SB-379872-A			
IIa (nucleus&cytoplasm)	HDAC4				
	HDAC5				
	HDAC7				
	HDAC9				
IIb (mainly cytoplasm)	HDAC6				
	HDAC10	Tubacin			
IV (nucleus)	HDAC11				
III (nucleus)	SIRT 1-7	Nicotinamide, splitomicin			

(adapted from Grønbæk et al. (Gronbaek *et al.*, 2007))

1.6.3.1 Combination treatment with HDACis and DNMTIs

In vitro studies have shown HDACI/II inhibitors used as single agents do not turn on genes with promoter hypermethylation, which require combination treatment with a demethylating agent for their activation (Egger et al., 2004). Combination treatment with HDACis and DNMTIs, as compared with DNMTIs alone results in an additive or synergistic re-expression of transcriptionally silenced genes (Suzuki et al., 2002). A clinical trial in patients with advanced leukaemia showed combination therapy with decitabine and valproic acid resulted in transient DNA hypomethylation and global histone H3 and H4 acetylation, with associated *p15* reactivation. This combination of epigenetic therapy also appeared to be safe and clinically active (Garcia-Manero *et al.*, 2006). HDACis have also been investigated *in vitro* and *in vivo* in combination with a number of other anticancer agents with promising results, such as conventional chemotherapy e.g. doxorubicin (Sanchez-Gonzalez *et al.*, 2006) and radiotherapy (Sonnemann *et al.*, 2006).

1.7 Aims of the work in this thesis

The aim of the work in this thesis is to assess drugs that modulate DNA damage processing so as to improve the outcome of conventional cytotoxic therapy. The first part of this thesis focuses on DNA repair and its modulation, primarily looking at the development of lomeguatrib in the clinical setting. Phase II data has identified that established that a combination of oral lomeguatrib and temozolomide is tolerable and produces satisfactory MGMT depletion in melanoma and colorectal cancer (Khan et al., 2008; Ranson et al., 2007). In this thesis the ability of lomeguatrib to produce absolute MGMT depletion in solid tumours (primary breast, prostate, central nervous system and primary and secondary colorectal) will be investigated using a bioassay and

ELISA. These tumour types have been selected following promising pre-clinical data demonstrating lomeguatrib can enhance temozolomide activity in animal xenograft models (Clemons *et al.*, 2005; Middleton et al., 2000b; Middleton et al., 2002). The safety profile of lomeguatrib will also be evaluated.

In the following chapter the role of lomeguatrib in combination with irinotecan in locally advanced or metastatic colorectal cancer will be presented. Although significant advances have been made in the treatment of stage IV colorectal cancer over the last 5-years, prognosis remains poor with 5-year survival of < 5 %. Alkylating agents appear to have some activity in colorectal cancer and as discussed, there is a strong association between MGMT expression and resistance to alkylating agents, which may be attenuated by lomeguatrib. Preclinical evidence has also shown that MGMT may have a role to play in cellular resistance to irinotecan. Given this data and the early evidence of synergism for combination treatment with temozolomide and irinotecan, an assessment of the use of lomeguatrib with temozolomide and irinotecan should be undertaken. As an initial step towards this, lomeguatrib has been combined with irinotecan in stage IV colorectal cancer to assess the safety, toxicity and efficacy of the combination, which will be discussed. Pharmacokinetic and pharmacodynamic parameters will be presented.

The identification of epigenetic changes in malignancy has revealed a new approach for the pharmacological treatment of cancer. Epigenetic silencing due to gene promoter hypermethylation causes transcriptional inactivation of key genes important in all aspects of tumour biology. Increased understanding of the molecular mechanisms behind gene promoter hypermethylation and the discovery/development of agents that may reverse/antagonise this process has significantly advanced over the last decade. The DNMTIs are perhaps the most well developed agents in this group. The role of low

dose decitabine in the treatment of haematological malignancies has been studied quite extensively in the clinical setting, but little work has been done in solid tumours.

The second part of this thesis aims to explore the combination of standard chemotherapy for locally advanced or metastatic gastro-oesophageal cancer using epirubicin, cisplatin and infusional 5-flurouracil (ECF) chemotherapy and escalating doses of low dose decitabine. ECF chemotherapy is the standard chemotherapy for locally advanced or metastatic gastric and oesophageal cancers and has a response rate of 40-50 % in clinical trials (Ross *et al.*, 2002; Webb *et al.*, 1997). Despite this however, gastric and oesophageal cancers have a relatively poor prognosis with 5-year survival of only 8-15 % (http://info.cancerresearchuk.org/cancerstats/types/stomach/oesophagus/survival/).
In gastro-oesophageal cancer, several genes are inactivated by gene promoter hypermethylation including genes regulating the cell cycle and apoptosis ($p14^{ARF}$ and $p16^{INK4}$) as well as genes reducing tissue invasiveness and metastatic potential (*CDH1*) (Table 1, page 58). Reversal of hypermethylation by low dose decitabine may allow reactivation of cell cycle checkpoints and tumour suppressor genes (*p53* and *pRb*) inducing cellular apoptosis and re-expression of invasion suppressor molecules (e-cadherin). In addition, DNA repair genes e.g. *hMLH1* and *MGMT* silenced by hypermethylation may undergo transcription following low dose decitabine circumventing cancer cell drug resistance allowing greater ECF chemotherapy activity. The safety, toxicity and efficacy of low dose decitabine and ECF will be reported. The subsequent chapter will discuss the ability of decitabine to cause DNA demethylation and re-expression of candidate genes silenced by hypermethylation.

2. Methods

2.1. Patients

2.1.1 Selection and ethical considerations

Most patients described in this thesis were treated at the Churchill Hospital. In the case of the trial described in chapter 3, additional patients were also treated at the John Radcliffe Hospital and Radcliffe Infirmary, Oxford and Freeman Hospital, Newcastle upon Tyne; in chapter 4, additional patients were treated at the Addenbrookes Hospital, Cambridge. The clinical trials described in chapters 3, 5 and 6 were reviewed and approved by the Oxfordshire Research Ethics Committee; the clinical trial described in chapter 4 was reviewed by the Hammersmith, Queen Charlotte's and Chelsea Hospitals Research Ethics Committee. All studies were also approved by the Radcliffe Hospitals NHS Trust Research and Development department. The research nature of the studies was emphasised to patients before they gave their written informed consent to participation in the trials. Patient selection was according to the inclusion and exclusion criteria specific to each study and is described in the relevant chapters. All the studies were conducted according to the principles of the International Conference on the Harmonisation of Good Clinical Practice guidelines and the Declaration of Helsinki.

2.1.2 Clinical evaluation of response and toxicity

Response evaluation was undertaken according to the Response Evaluation Criteria in Solid Tumours [RECIST;(Therasse *et al.*, 2000)] where appropriate. Briefly, at baseline tumour lesions were categorised as measurable [lesions that can be accurately measured in at least one dimension (longest diameter (LD) recorded) as ≥ 20 mm with conventional techniques or as ≥ 10 mm with spiral CT] or non-measurable [all other

lesions including small lesions (LD <10 mm with spiral CT scan) and all other truly non-measurable lesions]. A complete response (CR) was defined as the resolution of all target lesions confirmed by two separate assessments at least four weeks apart. A partial response (PR) was recorded where there was at least a 30 % decrease in the sum of the LDs of the target lesions taking as reference the baseline sum LDs confirmed by two separate assessments at least four weeks apart. Progressive disease (PD) was defined as at least a 20 % increase in the sum of the LDs of the target lesions taking as reference the smallest sum of LDs recorded since the treatment started or the appearance of one or more new lesions. Stable disease was recorded where there was neither sufficient shrinkage to qualify for PR nor sufficient increase to qualify for PD taking as reference the smallest sum of LDs since the treatment started. Toxicity was evaluated and graded according to the National Cancer Institute Common Toxicity Criteria version 2.0 (CTC v.2).

2.2 Drugs and their administration

2.2.1 Lomeguatrib

Lomeguatrib was provided by KuDOS Pharmaceuticals Ltd, Cambridge, UK. It was administered orally, as 10 mg capsules, as a single daily dose irrespective of body surface area. Patients were requested to fast for two hours before and after taking lomeguatrib capsules and advised to swallow the capsules whole so not to interfere with the enteric coating.

2.2.2 Irinotecan

Irinotecan was purchased from Rhone-Poulenc Rorer, Vitry-sur-Seine, France. A fixed dose of irinotecan was administered between 250-350 mg/m^2 based on the patient's

body surface area. Forty and 100 mg vials were used to prepare the dose and diluted in 500 ml 5 % dextrose for intravenous administration over 90 minutes on the morning of day 4 of treatment. The drug was protected from exposure to ultraviolet light during infusion by covering the infusion set with a black plastic sheet.

2.2.3 Epirubicin, cisplatin and 5-fluorouracil

Clinical supplies of epirubicin were obtained from Pfizer Ltd., Kent, England. Epirubicin was administered at a dose of 50 mg/m^2 as an intravenous bolus, based on the patient's body surface area using 50 and 200 mg vials.

Cisplatin administration was preceded by pre-hydration with 1 l 0.9 % normal saline containing 20 mmol potassium chloride (KCl) and 8 mmol magnesium sulphate ($MgSO_4$) over 4 hours. Cisplatin produced by Teva Sicor Pharmaceuticals Ltd., Irvine, CA, was administered at a dose of 60 mg/m^2 according to body surface area, using 50 and 100 mg vials, diluted in 500 ml 0.9 % normal saline over 4 hours.
This was followed by post-hydration with 2 l 0.9 % normal saline, each litre containing 20 mmol KCl and 8 mmol $MgSO_4$, given consecutively over 4 hours each.

5-fluorouracil was produced by Baxter Healthcare Ltd, Thetford, England. The drug was administered at a dose of 200 mg/m^2/day according to body surface area using 5000 mg vials, in 252 ml 0.9 % normal saline as a continuous infusion for 7 days via a central venous catheter.

2.2.4 Decitabine

Clinical supplies of decitabine were supplied by SuperGen, Inc., Dublin, CA and MGI Pharma, Bloomington, MA, USA. Decitabine was administered at the protocol specified dose according to body surface area. The decitabine infusion was prepared just prior to administration to minimise drug decomposition, as its concentration in reconstituted solution falls by 10 % after storage for 24 hours at 4 °C. The total dose was divided in two, 50 mg vials were reconstituted with sterile water, and then diluted into each of two cold (2–8 °C) 500 ml 0.9 % normal saline bags. Each decitabine infusion bag ran over 3 hours with the second infusion bag stored at 2-8 °C until it was administered.

2.3 Peripheral blood and tissue samples

2.3.1 Sample collection

Blood samples from patients were drawn by vacutainer or, when repeated sampling was required, via a plastic intravenous cannula. All tissue samples were obtained under sedation or using local anaesthetic as described in individual chapters. Biopsy material was collected in single containers and with the exception of samples for immunohistochemical analysis; all were immediately frozen on dry ice before storage at -70 °C for further analysis. Samples collected for immunohistochemistry were placed in universal containers containing 20 mls formalin (formal saline 10 %, formaldehyde 3.8-4 % w/v) for fixing and storage.

2.3.2 Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood using density centrifugation (Boyum, 1968) in chapters 3 and 4 although the exact

methodology differed between studies. The techniques used have been described in detail in individual chapters.

2.4 MGMT assay

MGMT analysis was performed by Dr Geoff Margison and his team at the Patterson Institute for Cancer Research, University of Manchester.

2.4.1 Tissue extract preparation for MGMT analysis

The minced tissue (100-300 mg) or cell pellet were transferred to a 1.5 ml Eppendorf tube in ice and 1 ml of cold Buffer I (50 mM Tris-HCl, 1 mM EDTA, 3 mM dithiothreitol, pH 8.3) containing 5 μg/ml leupeptin were added. Samples were disrupted by sonication for 10 seconds at 216 μm (peak to peak amplitude) and returned to ice. Repeat sonication was used where necessary. Phenylmethylsulphonyl fluoride (50 mM in 100 % ethanol; Sigma, UK) was added to the sample immediately following sonication to inhibit proteases, so that the final concentration was 0.5 mM (i.e. 1/100 of volume). The sonicates were centrifuged at 13-17,000 rpm for 10 minutes at 4 °C to remove cell debris. The supernatants were transferred to clean Eppendorfs, on ice, for assays of MGMT activity, and of DNA and protein content.

2.4.2 Determination of MGMT activity in tissue extracts

The method involves measuring [^{3}H]methyl group transfer to MGMT protein from [^{3}H]methylated DNA substrate under protein limiting conditions (Watson & Margison, 2000). The DNA substrate was diluted to 200 μl in Buffer I. At room temperature, 100 μl of substrate was dispensed into scintillation mini-vials and varying amounts of

extract (up to 200 µl; prepared as above) were added. A final total volume of 300 µl was made up with 1 mg/ml bovine serum albumin (BSA) in buffer I (IBSA). The mini-vials were incubated at 37 °C until the reaction was complete (30-60 minutes). After incubation, 100 µl 10 mg/ml BSA with 100 µl 4 M perchloric acid (PCA) and 2 ml 1 M PCA were added and the mixture was heated at 75 °C for 50 minutes to ensure complete hydrolysis of the DNA substrate. Protein was recovered by centrifugation at 2800 rpm for 10 minutes at room temperature. The resulting pellet was re-suspended in 4 ml 1 M PCA and centrifuged at 2800 rpm for a further 10 minutes at room temperature. The protein pellet was dispersed in 300 µl 10 mM sodium hydroxide (NaOH), followed by 3 ml Ecoscint scintillation fluid (Mensura Tech). Samples were counted by averaging two 5 minute counts each on a Rackbeta (LKB) scintillation counter. Counts per minute (cpm) were plotted against volume of extract used and specific activity calculated from a minimum of three points on the linear part of the curve. Activity was expressed as fmoles [^{3}H] methyl transferred to protein per mg protein or µg DNA in the extract.

2.4.3 Protein concentration measurement

Estimates of the protein concentration in extracts was according to the colour change of Coomassie brilliant blue G250 (CBG) (Bradford, 1976). Bio-rad protein reagent (BioRad Laboratories) was diluted to 1 in 5 in distilled water, left at 4 °C overnight then filtered through 3 mm Whatman number 1 filter paper. A standard curve for optical density at 595 nm of protein concentrations in the range 0.1-1.0 mg/ml was constructed using BSA standards. Forty microlitres of extract, appropriately diluted in buffer I, was added to 2 ml Bio-rad and mixed and the absorbance at 595 nm determined. This was performed in duplicate for all samples. The concentration of protein was calculated by

extrapolation from the standard curve. Latterly, the volumes of extract and Bio-rad used were scaled down to permit these measurements to be determined using a plate reader.

2.4.4 DNA concentration measurement

Fresh fluorescent dye solution was prepared using Hoechst 33258 (bisbenzamide; Sigma, UK) 1 mg/ml diluted 1 in 100 in double distilled water and added to 1 ml 10 x TNE Buffer (100 mM Tris base, 10 mM EDTA, 2 mM NaCl, pH 7.4) and 9 ml double distilled water. Fluorescence was measured in a TKO 100 mini fluorometer, which was calibrated using a calf thymus DNA standard with each use. Two microlitres of extract was added to 2 ml of dye solution and mixed well before introduction to the sample chamber. Measurements were taken in duplicate for each sample, with the machine reading giving an estimate of DNA concentration in μg/ml.

2.4.5 ELISA

Total, i.e. active and inactivated, MGMT protein was determined in cleared tumour sonicates by ELISA. Briefly, 96-well plates were coated with mouse anti MGMT monoclonal antibody (ABCAM, Cambridge, UK) overnight at 4 °C. After washing with phosphate buffered saline (PBS), blocking buffer (10 % horse serum, 0.1 % Tween 20 in PBS) was added to each well and incubated for 2 hours at room temperature. Blocking buffer was removed and the following 1 hour room temperature incubations performed in sequence with washing between additions: tumour sonicate (Watson & Margison, 2000), rabbit anti MGMT polyclonal antibody (Lee *et al.*, 1996), goat anti rabbit HRP antibody (Ely, UK). Finally, plates were washed with PBS, Western Lightning reagent (Perkin Elmer, Beaconsfield, UK) added and luminescence detected

using a TECAN GENios plate-reader. MGMT values were extrapolated from a purified human MGMT standard curve using Magellan v.3 software.

2.5 Statistical Analysis

Statistical analysis was performed using Microsoft Excel (Microsoft Office 2007) and Analyse-it v2.11 (Leeds, UK). Continuous data was represented throughout either as a mean with standard deviation (SD) or as median with a full range. The geometric mean and coefficient of variation was calculated, where indicated, for the pharmacokinetic data. In addition, percentages were presented where relevant. Comparative testing of categorical data was analysed using Fisher's exact test; $p \leq 0.05$ was considered statistically significant.

3. A pharmacodynamic study of tumour O^6-methylguanine-DNA methyltransferase (MGMT) depletion by oral lomeguatrib in a variety of cancer types.

3.1 Introduction

The role of lomeguatrib, a pseudosubstrate for MGMT, in overcoming MGMT-mediated resistance to chlorethylnitrosureas and related methylating agents has already been discussed in detail. Lomeguatrib has shown promising activity in sensitising a variety of human tumour xenografts to the growth inhibitory effects of O^6-alkylating agents, including temozolomide, at the expense of only limited additional toxicity (Middleton et al., 2000b; Middleton et al., 2002). Following a Phase I study of combination treatment with lomeguatrib and temozolomide (Ranson et al., 2006b), a phase II study using this combination in patients with metastatic melanoma has now been reported (Ranson *et al.*, 2007). Patients were treated with 40 to 80 mg lomeguatrib with 125 mg/m^2 temozolomide or 200 mg/m^2 temozolomide alone orally on days 1 to 5 every 28 days for up to 6 cycles. The lomeguatrib dose was selected based upon tumour depletion at 4 hours in 6 melanoma patients included in the phase I trial. The efficacy of combination treatment with lomeguatrib and temozolomide was found to be similar to that of temozolomide alone in terms of response rates and median time to disease progression (13.5 % vs. 17.3 % and 65.5 vs. 68 days respectively). This may have been due to the scheduling of lomeguatrib, which permitted rapid recovery of tumour MGMT. Data is required on the effects of lomeguatrib in other tumour types to determine the doses needed for use in tumour specific combination studies.

This study focused on four tumour types, primary breast, prostate and central nervous system (CNS) cancers and primary or secondary colorectal cancers. These were selected on the basis of work done on human xenograft models, which had shown that lomeguatrib enhances the anti-tumour effects of temozolomide in these tumour types (Middleton, 2000) (Table 3) and the readily detectable levels of MGMT in human tumour samples (Table 4) (Chen *et al.*, 1992).

The primary aim of this study was to determine the dose of oral lomeguatrib required to render MGMT undetectable 12 hours after dosing in primary breast, prostate and CNS tumours and primary or secondary colorectal tumours as measured by biochemical assay. Undetectable MGMT levels were only accepted as such below 0.3 fmol/μg DNA or below 25 fmol/mg protein in tumour and peripheral blood mononuclear cells (PBMCs). We also sought to assess total MGMT expression (active and inactive) in tumours and to evaluate the safety of lomeguatrib as a single agent.

Table 3. Effect of temozolomide alone and temozolomide-lomeguatrib combination treatment on tumour doubling time in human xenograft models[Δ]

Cell line	MGMT expression (fmol/mg protein)	IP TMZ dose (mg/kg) [1]	IP Lomeguatrib dose (mg/kg) [2]	TMZ alone - Tumour quintupling time (days)	TMZ and lomeguatrib - Tumour quintupling time (days) *
MCF-7 (breast)	1300	100	20	20.4	42.2
DU-145 (prostate)	700	100	20	25.4	42.4
U87MG (glioma)	5	100	20	10.4	13.0

Key: IP- intraperitoneal; TMZ- temozolomide; [1] administered daily for 5 days; [2] lomeguatrib administered prior to temozolomide daily for 5 days; *statistically significant in all 3 cases; [Δ] human tumour cell lines inoculated within nude (ALPK) mice

Table 4. MGMT expression in tumour tissue

Tumour tissue	**MGMT level (fmol/mg protein) [Mean ±SD]**
Breast	1071 ± 374
Stomach	515 ± 107
Lung (small cell)	509 ± 251
Lung (non-small cell)	461 ± 227
Renal cell	329 ± 246
Oesophagus	273 ± 376
Brain	244 ± 175
Colon	242 ± 308
Melanoma	201 ± 161

3.2 Patients and Methods

3.2.1 Eligibility

Patients due to undergo elective surgery for removal of a primary breast, prostate or CNS tumours or primary or secondary colorectal cancer were identified at the Churchill and John Radcliffe Hospitals and the Radcliffe Infirmary and Freeman Hospital. To be eligible for the study, patients aged 18 or over with elective surgery for tumour removal scheduled within 14 days, required histological or cytological confirmation of cancer for breast and colorectal patients, in the absence of histological or cytological confirmation of cancer for CNS and prostate patients, radiological confirmation or a PSA diagnosis ($\geq$ 20 μg/l) were necessary for CNS and prostate patients respectively.

All patients enrolled in the study gave written informed consent. The study was conducted under the auspices of Cancer Research UK in accordance with the principles of the International Conference on Harmonisation of Good Clinical Practice guidelines and the Declaration of Helsinki. The trial was approved by the Cancer Research UK's Central Institutional Review Board, Oxfordshire Research Ethics Committee and the Oxford Research and Development Department.

3.2.2 Treatment and dose escalation

Lomeguatrib (KuDOS Pharma Ltd, Cambridge, UK) was administered orally once approximately 12 hours prior to tumour resection. Patients were requested to fast for two hours before and after taking the lomeguatrib capsules and advised to swallow the capsules whole so as not to interfere with the enteric coating. Five dose levels of lomeguatrib were studied in each tumour type: 20, 40, 80, 120 and 160 mg. These were

selected to include the dose range found from phase I work to completely deplete MGMT in a variety of solid tumours, i.e. 40 to 80 mg/day (Ranson et al., 2006a). Dose levels above and below this range were included to take into account the different levels of MGMT expression in the tumours studied. Central nervous system tumours express relatively little MGMT, colorectal and prostate cancers intermediate levels and breast cancers high levels, such that different doses of lomeguatrib might be required to cause complete MGMT depletion (Table 3 and Table 4). The dose range also reflected the observation from studies with O^6-benzylguanine that approximately three times the dose of inactivator is required to deplete MGMT in tumour as in peripheral blood mononuclear cells (PBMCs) (Spiro *et al.*, 1999).

Initially, patients were enrolled in cohorts of 3 to allow an assessment of the safety of each lomeguatrib dose. If any of the 3 patients had detectable tumour MGMT escalation continued. In the event of all 3 patients exhibiting 100 % depletion, a further 3 patients were to be recruited at the same dose level to confirm the observation. Dose escalation was to cease in the event of an adverse event (AE) greater than the National Cancer Institute Common Toxicity Criteria version 2.0 (CTC v.2) grade 1 that was probably, possibly, or almost certainly related to lomeguatrib (except for grade 2 nausea and vomiting). As safety data emerged from other studies dose escalation was permitted immediately any patient showed active residual tumour MGMT. Due to poor recruitment, the breast cancer arm of the study was discontinued early.

3.2.3 Toxicity and response evaluation

Pre-treatment evaluations including a complete medical history (especially any gastrointestinal disease that might impact on lomeguatrib absorption) and physical

examination including performance status and vital signs (blood pressure and pulse) were performed within the 2 weeks prior to the anticipated date of surgery. The results of pre-operative baseline blood tests performed within the same time interval (including haematology and biochemistry [renal and hepatic function, glucose, uric acid and bone profile]) were recorded from patient notes. Patients were assessed for safety and toxicity whilst on study; from the time of informed consent until end of treatment evaluation on day 28 following lomeguatrib administration and toxicities were graded according to the CTC v.2. Patients were formally assessed preoperatively (as outlined above), on the day after surgery and 28 days after dosing. On the latter 2 occasions a physical examination (including vital signs), full blood count and biochemistry profile were performed and adverse events and concurrent medication recorded. If any adverse events attributable to lomeguatrib occurred, the patient was followed-up monthly until resolution of this event. The safety profile at each dose level was reviewed before the next group of patients was recruited. In the event that surgery was delayed such that the tumour could not be removed within 6-18 hours of the lomeguatrib dose, that patient was replaced.

3.2.4 Pharmacodynamics

At the time of surgery, approximately 12 hours after lomeguatrib dosing, a representative sample from the primary/secondary tumours was collected and immediately frozen on dry ice prior to storage at -70 °C. Ten millilitres of blood were also collected in a universal tube containing 100 µl 0.5 M EDTA for PBMC isolation. Blood samples were layered onto 10 ml of Ficoll-Paque PLUS (Pharmacia Biotech Ltd) and centrifuged at 1800 rpm for 20 minutes at 4 °C. The PBMC layer at the interface between the Ficoll and plasma layers was collected, phosphate buffered saline added to a total volume of 20 ml and the mixture centrifuged for 10 minutes at 1400 rpm at 4 ° C.

The remaining cell pellet was snap frozen in dry ice and subsequently stored at -70 °C. Analysis of MGMT expression was performed by Dr Geoff Margison and his team at the Patterson Institute for Cancer Research as outlined in Chapter 2.

The efficacy of lomeguatrib was assessed by measuring active and total MGMT protein in each tumour type to assess the percentage MGMT inactivation. The MGMT activity assay was biochemical, measuring the ability of DNA substrate to transfer radio-labelled methyl groups to MGMT protein within the sample under standard conditions. Total MGMT protein (active and inactive) was also determined by a validated ELISA method to confirm protein expression in the tumour prior to lomeguatrib administration and to allow calculation of the percentage MGMT inactivation. MGMT activity was also measured in post treatment PBMC samples as a confirmation of lomeguatrib ingestion (Chapter 2).

3.3 Results

3.3.1 Patients and dose escalation

A total of 40 patients were enrolled into the study of which 37 were treated with lomeguatrib. Two patients registered failed to fulfil eligibility criteria, including a patient with colorectal cancer and liver metastases who had their hepatectomy cancelled due to the diagnosis of new lung metastases; A third patient failed to receive the study drug due to an omission by nursing staff.

All 37 patients treated with lomeguatrib were evaluable for toxicity but only 32 patients were evaluable for tumour MGMT i.e. 5 patients had tumour samples taken that were inadequate for analysis or tumours that did not express detectable MGMT prior to

treatment. Of the 37 patients treated with lomeguatrib, 9 entered the study at the 20 mg dose level of lomeguatrib, 4 at the 40 mg dose level, 10 at the 80 mg dose level, 12 at the 120 mg dose level and 2 at the 160 mg dose level (Table 5).

A waiver was granted for one patient to enter the study despite a persistent side effect (hearing loss) from previous chemotherapy, and for one patient who received the study drug twice as their surgery was postponed after dosing for the first time.

3.3.2 MGMT depletion

All patients treated with lomeguatrib exhibited total or near total MGMT depletion from PBMC MGMT levels assessment. Lomeguatrib depleted MGMT in all four tumour types (Table 5 and Table 6). Of the 10 CNS tumour samples received from patients treated with lomeguatrib, 9 were assessable for MGMT depletion. One sample was from a patient diagnosed post-operatively with a primary lymphoma and therefore ineligible for the study. In the 9 assessable CNS patient biopsies, the mean total MGMT was 89.8 fm/mg protein (SD 44.5). One hundred percent MGMT depletion was observed in the 2 tumour samples from patients treated with 160 mg of lomeguatrib.

Tumour samples were obtained from the primary tumour or metastatic lesions of 16 patients with colorectal cancer (Table 5 and Table 6). From these, 14 were assessable for MGMT depletion. The remaining 2 tumour samples were inadequate for analysis or did not express MGMT. Mean total MGMT in the assessable samples was 243.9 fm/mg protein (SD 180.8). Total MGMT depletion was reached in the biopsies of the 6 patients treated with 120 mg of lomeguatrib.

Table 5. Summary of lomeguatrib treatment and PD results for each tumour site

Tumour type	Dose level	Lomeguatrib Dose (mg)	No. of patients assigned dose level	No. of patients treated with lomeguatrib	No. of tumour samples suitable for PD analysis	% Mean MGMT depletion
CNS	1	20	3	3	3	43.0
	2	40	1	1	1	46.0
	3	80	2	2	1[1]	57.0
	4	120	2	2	2	73.4
	5	160	2	2	2	100
Colorectal	1	20	3	3	3	81.8
	2	40	2	2	2	60.8
	3	80	4	4	3[2]	97.7
	4	120	7	7	6[2]	100
Prostate	1	20	1	1	1	93.6
	2	40	1	1	1	100
	3	80	4	4	3[2]	98.4
	4	120	3	3	3	99.8
Breast	1	20	2	2	1[2]	100

[1] One patient ineligible as diagnosed with lymphoma post-operatively
[2] Inadequate tumour sample(s) for analysis or tumour negative for MGMT expression

Table 6. Tumour MGMT depletion data

Tumour type/Patient number	Dose (mg)	Mean active MGMT (fm/µg total DNA)	Mean active MGMT (fm/mg total protein)	Total MGMT (fm/mg total protein)	Percentage MGMT inactivation
N01	20	5.6	33	102	70
N02	20	13	91	123	25
N03	20	0.8	25	38	34
N04	40	17.4	93.3	165	46
N06	80	4.6	56	128	57
N07	120	2.5	31	98	61.9
N08	120	0.7	9	67	84.9
N09	160	ND	ND	40	100
N10	160	ND	ND	48	100
C01	20	3.7	110	475	76.5
C02	20	0.8	16.5	85	80
C03	20	1.2	60	547	89
C04	40	11.8	325	410	21.5
C05	40	ND	ND	55	100
C06	80	ND	ND	74	100
C07	80	ND	ND	47	100
C08	80	ND	ND	ND	Inadequate sample
C09	80	0.9	27	382	93
C10	120	ND	ND	ND	Inadequate sample
C11	120	ND	ND	251	100
C12	120	ND	ND	112	100
C13	120	ND	ND	362	100
C15	120	ND	ND	96.3	100
C16	120	0.43	ND	99.7	100
C17	120	ND	ND	419	100
P01	20	6	24	376	93.6
P02	40	ND	ND	653	100
P03	80	ND	ND	112	100
P04	80	ND	ND	ND	Inadequate sample
P06	80	ND	ND	366	100
P07	80	24.2	52.5	1064	95.1
P09	120	ND	ND	47	100
P10	120	4.1	8	1136	99.3
P11	120	ND	ND	678	100
B01	20	ND	ND	126	100

Key: Nxx- Primary CNS tumour, Cxx- primary or secondary colorectal cancer, Pxx- Primary prostate cancer, Bxx- Primary breast cancer; ND, not detected

The samples taken from the 9 patients with prostate cancers were variable in size and quality (Table 5 and Table 6). Eight samples were assessable from these for MGMT depletion. The remaining sample was inadequate for analysis. The mean total MGMT was 554.0 fm/mg protein (SD 404.2). Absolute depletion of MGMT was seen in 2 out of 3 patients treated with 80 and 120 mg of lomeguatrib in which MGMT was assessable. Only 1 tumour sample was received from the 2 patients with breast cancer treated with lomeguatrib. Complete MGMT depletion was seen in this tumour sample following treatment of the patient with 20 mg lomeguatrib.

3.3.3 Toxicity

Lomeguatrib was very well tolerated and only a few grade 1 adverse events were recorded following treatment that may have related to the drug. The adverse events did not appear to be dose related and all resolved spontaneously. They included: an abnormal sensation in the abdomen and raised liver enzymes (raised transaminases, gamma glutyltransferase and alkaline phosphatase) between 1 and 4 weeks after administration of the study drug.

A number of adverse events were recorded that were felt to be secondary to the surgical procedures the patients had undergone and not due to lomeguatrib administration. Of these 5 episodes were serious, by virtue of the requirement for or prolongation of hospitalisation of the patient. One patient experienced pain and blurred vision in their right eye (both grade 2), thought to be secondary to their right temporal craniotomy; grade 3 hepatic dysfunction (raised alanine transaminase) was seen in 2 patients following hepatic resections, one patient developed a grade 3 chest infection and grade 4 pulmonary embolus and one patient had a grade 3 ileus; These were thought to be

post-operative complications associated with the surgical procedures the patients had undergone and not related to lomeguatrib.

3.4 Discussion

The aim of this trial was to determine the dose of lomeguatrib that depleted tumour MGMT in patients with one of four cancers to inform phase II trial design.

The use of a pharmacodynamic end-point to determine the dose of lomeguatrib for further evaluation was based on the premise that MGMT depletion in the clinical setting was likely to be achievable at doses well below the maximum tolerated dose of lomeguatrib itself. The range of lomeguatrib dosing was selected based upon the findings of a phase I trial where 40 to 80 mg of lomeguatrib given orally produced absolute MGMT depletion in both tumour and PBMCs at 4 hours post dosing (Ranson et al., 2006a), and by the constraints inherent in the 10 mg capsule size available to us.

Both PBMC and tumour samples were used to determine the extent of MGMT depletion following lomeguatrib administration. Both samples were taken approximately 12 hours after lomeguatrib administration as previous data has shown it takes up to 6 hours to achieve maximal MGMT depletion with low dose oral lomeguatrib (20 mg) (Ranson et al., 2006a).

Substantial variation in MGMT protein levels were seen both between and within tumour types as previously reported (Table 4) (Chen et al., 1992). The mean total MGMT levels in the 3 main tumour types assessed (primary brain: 89.9 fm/mg protein [SD 44.5]), colorectal: 243.9 fm/mg protein [SD 180.8] and prostate cancer: 554.0 fm/mg protein [SD 404.2]) and the increase in total MGMT expression from primary

CNS tumours to colorectal cancer and prostate cancers were in keeping with previous results from xenograft models and tumour tissue from patients (Table 3 and Table 4).

Absolute MGMT depletion was seen in primary CNS tumour biopsies after treatment with lomeguatrib 160 mg and in colorectal and prostate cancer after lomeguatrib 40 mg. However consistent MGMT depletion was only seen in colorectal cancer with lomeguatrib 120 mg. The specified end-point of the study was not reached in the other 2 tumour types as the study closed prior to completion. This was due to slow recruitment and expiry of the lomeguatrib stock for the study.

This is the first study to report active and inactive MGMT in tumour biopsies. Of particular interest, prostate cancer appears either to be easier to deplete of MGMT than primary CNS or colorectal cancers or a greater proportion of MGMT within prostate cancers is inactive.

Oral lomeguatrib was well tolerated at the dose levels studied in keeping with previous findings (Khan et al., 2008; Ranson et al., 2007; Ranson et al., 2006a). It is likely, however, to have no single agent anti-tumour activity and will be used in combination with cytotoxic agents. To this end the results seen in primary brain tumours, the mainstay of treatment of which is temozolomide, are of particular interest. Studies have been conducted with lomeguatrib and temozolomide in a variety of solid tumours in the phase I setting. The main toxicity seen with combination treatment was haematological, which required the standard single agent dose of temozolomide to be reduced by ~40 %. A regimen of oral lomeguatrib 40 mg/day with temozolomide 125 mg/m^2 days 1 to 5 was suggested for further evaluation (Ranson et al., 2006a). The dose of temozolomide that could safely be administered with the comparatively high levels of lomeguatrib

(160 mg) required to achieve complete MGMT depletion in primary brain tumours seen in this study will need to be established. Ranson et al established that MGMT recovers rapidly after treatment with lomeguatrib and temozolomide in patients with metastatic melanoma (Ranson et al., 2007). However it may be that high doses of lomeguatrib (120-160 mg) are required to deplete tumour MGMT but lower doses are required to maintain depletion.

In summary, total MGMT depletion can be achieved in primary CNS, colorectal and prostate cancers with a single administration of lomeguatrib. The doses required to achieve this does not correlate with mean pre-treatment MGMT expression in the 3 tumour types studied. Lomeguatrib is well tolerated over the required dose range and doses of 120 – 160 mg/day will be administered in future studies.

4. Clinical efficacy, safety, toxicity and clinical pharmacology of combination treatment with lomeguatrib and irinotecan in metastatic colorectal cancer.

4.1 Introduction

Approximately one third of patients with colon or rectal cancer will present with metastatic disease, which cannot be cured by surgery, except for a small subset with liver-isolated disease. For all other patients with metastatic disease, treatment is palliative and likely to involve systemic chemotherapy, possibly with additional local measures such as surgery or radiotherapy.

Irinotecan, a camptothecin derivative, produces anti-tumour activity by inhibiting topoisomerase I and has been shown to have activity in colorectal cancer, both in the metastatic and adjuvant settings. Treatment with irinotecan has been shown, in two phase III trials in 5-fluorouracil (5-FU)/leucovorin (LV)-refractory patients with metastatic colorectal cancer, to provide a survival advantage compared to treatment with infusional 5-FU/LV or best supportive care (BSC). One year survival rates were 36.2 % with irinotecan and BSC vs. 13.8 % with BSC alone (p=0.0001); and 45 % with irinotecan vs. 32 % with 5-FU/LV (p=0.035) (Cunningham *et al.*, 1998; Rougier & Lepere, 2005; Rougier *et al.*, 1998) . The efficacy of single agent irinotecan has also been evaluated in the Intergroup study N9841, which compared second-line 5-FU/LV/oxaliplatin (FOLFOX4) and irinotecan alone in patients that had failed 5-FU-based therapy, subsequently allowing crossover to the other arm at progression. Initial data suggested patients receiving single agent irinotecan after failing FOLFOX4 had a

response rate (RR) of 4 %; median time to progression (TTP) from crossover was 2.7 months and median survival was 8.7 months (Rowland *et al.*, 2005). Subsequently, three pivotal phase III trials demonstrated a survival benefit for combined irinotecan and 5-FU/LV (bolus and infusional regimens) compared to 5-FU/LV alone as first line treatment for metastatic colorectal cancer (Douillard *et al.*, 2000; Kohne *et al.*, 2005; Saltz *et al.*, 2000).

Irinotecan is thought to exert its anti-tumour activity *in vivo* after enzymatic cleavage by a carboxyl esterase (CE) that generates the active metabolite SN-38 (7-ethyl-10-hydroxycamptothecin), which is at least 100-fold more cytotoxic than irinotecan (Kawato *et al.*, 1991). Irinotecan may also undergo cytochrome P-450 3A4 and 3A5-mediated oxidation of its bipiperidine side chain (Haaz *et al.*, 1998; Lokiec *et al.*, 1996). One of the main metabolites resulting from this, 7-ethyl-10-[4-(1-piperidino)-1-amino] carbonyloxycamptothecin (NPC) can be converted into SN-38 by liver CEs and may contribute to the overall production of the pharmacologically active species (Dodds *et al.*, 1998).

The efficacy of irinotecan in patients with metastatic colorectal cancer may be improved by targeting specific mechanisms of cellular resistance. Pre-clinical data appears to indicate that MGMT, by virtue of its effect on irinotecan and its active metabolite SN-38, may be one such target. On studying 17 cell lines including 4 colorectal cancer lines, MGMT expression was found to closely correlate with sensitivity to irinotecan and SN-38 and inhibition of MGMT by O^6-BG augmented drug activity. Transfection studies showed that increasing *MGMT* expression decreased the sensitivity to irinotecan and SN-38, whereas repression of *MGMT* expression sensitised cells to the drug (Okamoto *et al.*, 2002).

Lomeguatrib, an orally bioavaliable inactivator of MGMT has undergone early phase clinical trials. When co-administered with therapeutic doses of temozolomide, lomeguatrib has been shown to deplete tumour MGMT without significantly increasing treatment-associated side effects. Unfortunately significant clinical responses in patients with metastatic melanoma have not been seen with combination treatment compared with temozolomide alone (Ranson et al., 2007; Ranson et al., 2006b). No studies have previously been conducted with lomeguatrib in combination with irinotecan.

The primary aim of this study was to elucidate the maximum tolerated dose (MTD) of lomeguatrib in combination with irinotecan in patients with metastatic colorectal cancer. Secondary objectives included demonstrating whether prior administration of lomeguatrib improved the efficacy of irinotecan through its inactivation of MGMT and assessing the safety, toxicity and clinical pharmacology of combination treatment.

4.2 Patients and methods

4.2.1 Inclusion and exclusion criteria

Individuals over the age of 18 with Stage 4 histologically confirmed colorectal cancer, deemed to have the potential for clinical benefit from treatment and with a life expectancy of at least 12 weeks were eligible for the study. ECOG performance status of 2 or better, adequate hepatic (AST or ALT levels $\leq$ 2.5 x upper normal limit (ULN) and total bilirubin levels $\leq$ 1.5 x ULN), renal (serum creatinine $\leq$ 1.5 x ULN) and bone marrow function (absolute neutrophil count $\geq$ 1,500 /mm^3 and platelet count of $\geq$ 100,000 /mm^3) were required.

Patients were excluded if they had recent major thoracic or abdominal surgery, chemotherapeutic or investigational agents, or radiotherapy in the preceding 4 weeks; had mitomycin C or nitrosureas within previous 6 weeks; had an active infection or significant non-malignant intercurrent illness; had prior pelvic radiotherapy; with unresolved bowel obstruction, uncontrolled diarrhoea or known gastrointestinal fistulae; were pregnant or lactating; with known central nervous metastases; with history of seizures; were serologically positive for Hepatitis B, Hepatitis C or HIV; were unable to swallow orally administered medication; or were receiving concurrent antacid medication. A negative serum pregnancy test was required in women with child-bearing potential and all patients were required to use medically approved contraceptive precautions during the study and for 4 weeks afterwards.

All patients enrolled in the study gave informed consent. The study was approved by the Hammersmith, Queen Charlotte's and Chelsea Hospitals Research Ethics Committee and the Oxford Radcliffe Hospitals NHS Trust Research and Development Department. It was conducted in accordance with the Principles of the International Conference on Harmonisation of Good Clinical Practice guidelines and the Declaration of Helsinki.

4.2.2 Treatment

Lomeguatrib was supplied by KuDOS Pharmaceuticals Ltd, Cambridge, UK. It was administered at a starting dose in the first cohort of 10 mg/day on days 1 to 5 orally in the evening, with the patient fasting for 2 hours before and after taking the capsule. During the fasting period, patients were allowed to drink clear liquids only.

The fixed dose of irinotecan (Rhone-Poulenc Rorer, Vitry-sur-Seine, France) was 350 mg/m^2 (Cohort 1), 250 mg/m^2 (Cohorts 2-5) or 300 mg/m^2 (Cohort 6), based on body

surface area (BSA) as calculated from actual body weight prior to each cycle. It was administered by intravenous infusion over 90 minutes on the morning of day 4 (12 hours after day 3 administration of lomeguatrib), with cycles repeated every 21 days for a total of 6 cycles. As per standard protocol, atropine sulphate 0.25 mg was administered subcutaneously just prior to commencing the irinotecan infusion to counteract cholinergic symptoms which may be experienced with irinotecan. Systemic anti-emetics were administered prior to and for a few days after irinotecan administration and all patients were prescribed loperamide to take if they developed diarrhoea. Prophylactic ciprofloxacin was also prescribed in the event of diarrhoea associated with possible neutropenia.

A minimum of 3 patients were treated at each dose level and any patient withdrawn before day 21 was replaced. The first patient at each dose level was observed for at least 1 week following irinotecan administration i.e. until at least day 11 before enrolment of subsequent patients at that dose level. All 3 patients at a dose level were required to complete one 21-day treatment cycle before a decision to dose escalate lomeguatrib or irinotecan could be made. If 1 of 3 patients at a dose level developed a dose-limiting toxicity (DLT), up to 3 additional patients were treated at that dose level. If 1 of the 3 additional patients developed a DLT, dose escalation ceased and a total of 6 patients were treated at the preceding dose level. This lower dose was defined as the MTD unless $\geq$2 of 6 patients developed a DLT.

4.2.4 Dose escalations

In the initial study design a fixed dose of 350 mg/m^2 irinotecan was to be administered with an escalating dose of lomeguatrib. However on the basis of results obtained during

the course of the study it became apparent that this dose of irinotecan in combination with low dose lomeguatrib (10 mg daily for 5 days) was not tolerated. A protocol amendment was obtained and the irinotecan dose was reduced initially to 250 mg/m^2 (cohorts 2-5) and subsequently 300 mg/m^2 (cohort 6). The dose of lomeguatrib administered was escalated between patient cohorts from the starting dose of 10 mg/day. The dose of lomeguatrib was escalated by 100 % in successive patient cohorts to a maximum of 80 mg to define the MTD and DLT in combination with irinotecan.

4.2.5 Toxicity and response evaluation

Pre-treatment evaluations included a complete medical history and physical examination, full blood count, biochemical profile including carcinogenic embryonic antigen (CEA), urinalysis and electrocardiogram. Baseline CT scans to evaluate tumour sites were performed. Safety and toxicity were evaluated at least weekly during the study period and toxicities graded according to the National Cancer Institute Common Toxicity Criteria version 2 (CTC v.2).

A DLT was defined as any of the following study drug related events experienced during cycle 1: grade 4 haematological toxicity lasting >5 days; grade 3 or 4 febrile neutropenia; grade 3 or 4 non-haematological toxicity including diarrhoea, nausea or vomiting despite adequate treatment. The MTD was defined as the dose level of lomeguatrib below that at which $\geq$ 30 % of the population experienced DLT due to the drug combination. Tumour response was assessed using RECIST prior to commencing treatment, after 2 complete cycles, and at the end of 6 cycles of treatment. Patients were followed up for 30 days after the last dose of study drug.

4.2.6 Treatment delay, dose modification, and treatment withdrawal

A treatment delay of up to 2 weeks was allowed for the resolution of drug-related toxicity. Dose modification of irinotecan by 1 or 2 levels was permitted in this event or in the event of a DLT in the preceding cycle. Patients could be withdrawn from the study for progressive disease, unacceptable toxicity, serious violation of the study protocol, or withdrawal of consent.

4.2.7 Pharmacodynamics

Optional tumour core biopsies were obtained on day 1 pre-treatment with lomeguatrib and post-treatment with lomeguatrib on day 6. Tumour biopsies were taken under local anaesthetic, using up to 20 ml 2 % lidocaine (Hameln Pharmaceuticals). Samples were placed in 10 ml universal containers and immediately frozen on dry ice before storage at -70 °C before determination of MGMT activity (see Chapter 2).

Blood samples for peripheral blood mononuclear cells (PBMCs) isolation and MGMT assessment and Topoisomerase I cleavage complex formation (ICE assay) were obtained prior to treatment on day 1 of cycle 1, prior to irinotecan dosing on day 4 and at the end of treatment delivery on day 6. Eight millilitres of blood were taken in each of 2 tubes containing 320 μmol EDTA for PBMC MGMT activity analysis. Four millilitres of blood was collected and immediately added to 0.2 ml 20 % sarkosyl to rapidly denature the cells and trap topoisomerase I/DNA complexes and frozen at -20 °C for ICE assay.

4.2.7.1 PBMC isolation

PBMCs were isolated from whole blood using density centrifugation. Eight millilitres of cold phosphate buffered saline (PBS) was mixed with the blood collected and half of the well-mixed diluted blood carefully layered on top of 9 ml of Ficoll-Paque PLUS (Pharmacia Biotech Ltd) in 2 universal containers using a standard Pasteur pipette. These were both centrifuged at 400 *g* for 40 minutes at 20 °C. The PBMC layer, at the interface between the Ficoll and plasma was aspirated using a fine Pasteur pipette to a fresh 25 ml universal container. Twenty five millilitres of ice cold PBS was added to the transferred lymphocyte universal and this was centrifuged at100 *g* for 10 minutes at 4 °C. The supernatant was removed and the remaining pellet was re-suspended in 1 ml ice cold PBS in a 2 ml Eppendorf tube. This was then centrifuged at 100 *g* for a further 5 minutes at 4 °C. The supernatant was removed and the Eppendorf was snap frozen on dry ice and stored at -80 °C.

4.2.7.2 ICE assay

This assay to measure topoisomerase I/DNA complex levels was performed by Dr David Anderson, Department of Clinical Pharmacology, Oxford, according to the methodology as described by Subramanian *et al* (Subramanian *et al.*, 1995) outlined below.

4.2.7.2.1 Caesium chloride separation of DNA for ICE assay

The sarkosyl lysates were thawed and overlaid onto caesium chloride buffer (CsCl) density gradients containing four different density steps. To prepare the gradients, a stock solution of CsCl (CsCl 120 g dissolved in 70 ml TE buffer, density = 1.86 g/ml) was diluted with TE buffer (1 mM EDTA) [EDTA 0.3722 g, Trizma 1.576 g per litre at

pH 7.5] to give four solutions of 1.37, 1.50, 1.72, and 1.82 g/ml. The step gradient was formed by under laying 2 ml aliquots of successively less dense solutions into a polyallomer tube.

Zero point eight two five millilitres of TE* buffer (10 mM EDTA 3.722 g per litre of Trizma at pH 7.5), 1.55 ml of caesium chloride buffer and 0.125 ml 20 % sarkosyl were added to 2 ml of lysed blood and mixed completely by vortexing at 1500 *g* for 15 minutes at room temperature. The blood lysate/caesium chloride mix was overlaid the caesium chloride gradient. The tube was then topped up with TE buffer to stop tube collapse and centrifuged for at least 18 hours at 31,000 rpm (Beckmann SW40 T1 rotor) at 25 °C to avoid the caesium chloride precipitating.

4.2.7.2.2 Isolation of DNA from gradient

Fractions of approximately 450 µl (15 drops) were collected from the bottom of the gradients until all the caesium chloride was fractionated (approximately 18 tubes). The samples were then assayed for DNA and topoisomerase I content using fluorometry immunoblotting.

4.2.7.2.3 DNA assay

One microlitre of each sample was taken and placed into a 96 well plate. Picogreen buffer was diluted to 1:20 using sterile water. The picogreen was diluted in buffer 1:400 (i.e. 50 µl in 20 ml) and 200 µl of picogreen reagent was added to each well. This was repeated for each of the samples. The plate was run on the Ascent plate reader (program PICO.SEF). The concentration of DNA was then calculated using a standard DNA concentration curve.

4.2.7.2.4 Slot blot analysis of Topoisomerase I

Analysis of the 8 samples surrounding the DNA peak at each time-point for each of the patients alongside a series of standard samples was performed using a 48 well slot blotter.

An Immobilon membrane cut to size was soaked for 30 minutes at room temperature in 25 mM sodium phosphate buffer pH 6.5. The slot blotter was assembled after washing the silicon gasket and top part of the blotter in sterile water. Two hundred microlitre fractions from the caesium chloride gradient were diluted with 550 µl of sodium phosphate buffer and left at room temperature prior to blotting. The diluted fractions were applied to the assembled blotter and a vacuum of 8-10 inches of mercury was applied to draw the samples through. The vacuum was allowed to run for 5 minutes after the samples had been drawn through the membrane.

The membrane was then removed and rinsed in 30 ml of 25 mM sodium phosphate buffer pH 6.5. The membrane was transferred into 30 ml of 0.1 % PBS-Tween (900 ml PBS and 900 µl neat Tween 20) for 10 minutes with gentle shaking on a Gyro rocker (Stuart scientific). It was then incubated in 50 ml of neat Blotto (2.5 g Marvel dried skimmed milk powder and 50 ml PBS-Tween) for 1.5 hours with gentle shaking. The membrane was then washed with PBS-Tween (30 ml per wash), with gentle shaking for 5 minutes. This was repeated twice more.

The human topoisomerase I primary antibody (#1021, Topogen link kit) was diluted to 1·400 in Blotto (50 µl antibody in 20 ml Blotto). This was incubated at room temperature for 1.5 hours with gentle shaking. The membrane was again washed three times for 5 minutes each time with PBS-Tween (30 ml per wash), with gentle shaking.

The secondary goat anti-rabbit antibody (A0545, Sigma) was diluted at 1:250 in Blotto (80 µl of antibody in 20 ml). The membrane was incubated at room temperature for 1.5 hours with gentle shaking with the diluted secondary antibody. It was washed a further three times as above and finally washed with PBS for 5 minutes with gentle shaking only.

4.2.7.2.5 Antibody visualisation with enhanced chemiluminescence

Lumiglow solution (54-61-01, Insight) was prepared by mixing equal volumes of the two Lumiglow reagents. The membrane was soaked in Lumiglow solution for 5 minutes with gentle shaking. The excess was drained off and the membrane was exposed on a gel scanner and the chemiluminescence quantified.

4.2.8 Pharmacokinetics

Five millilitres of venous blood was drawn into lithium heparin vacutainers for pharmacokinetic analysis of lomeguatrib activity on the morning of day 4 immediately prior to the start of the irinotecan infusion (approximately 12 hours after the day 3 dose of lomeguatrib). Similarly, venous blood samples were collected prior to the start and at the end of the irinotecan infusion, and at 30 minutes, 1, 2, 4, between 6 and 8 and 24 hours after the end of the irinotecan infusion (i.e. equivalent to pre-dose, 1.5, 2, 2.5, 5.5, between 7.5 and 9.5 and 25.5 hours after the start of the irinotecan infusion) for pharmacokinetic analysis of irinotecan activity. Samples were centrifuged at 3700 *g* for 5 minutes at 4 °C. Zero point five millilitres of serum was transferred into each of four 2 m^l Fppendorf tubes and immediately frozen at -70 °C. Plasma concentrations of lomeguatrib and its principle active metabolite, 8-hydroxy-lomeguatrib and irinotecan and its major circulating metabolite, SN-38 were determined by high performance

liquid chromatography with tandem mass spectrometric detection (HPLC-MS-MS) by Bioanalytical Systems Ltd (BAS), Stoneleigh, UK and HFL Ltd, Fordham, UK respectively, using non-compartmental pharmacokinetic analysis with WinNonLin Enterprise version 4.1 (Pharsight Corporation, California, USA).

4.3 Results

4.3.1 Patients

A total of 25 patients were enrolled in the study across 2 study centres with 1 screening failure: 1 patient, enrolled into cohort 1, withdrew their consent prior to receiving treatment and was excluded from the analysis. The demographic characteristics of the 24 patients included in the study are summarized in Table 7. All 24 patients were evaluable for toxicity following treatment with lomeguatrib and irinotecan. All patients had measurable disease and were evaluable for tumour response. The dose levels of lomeguatrib and irinotecan studied are shown in Figure 11.

4.3.2 Dose escalation and Extent of exposure

Twenty four patients received combination treatment with lomeguatrib and irinotecan (Figure 11). Two patients treated in cohort 1 with irinotecan 350 mg/m^2 with lomeguatrib 10 mg experienced severe grade 3 or 4 diarrhoea and 1 of these patients subsequently developed neutropenic sepsis and died. All further patients were treated with irinotecan 250 mg/m^2 (cohorts 2, 3, 4, and 5) and 300 mg/m^2 (cohort 6), in combination with varying doses of lomeguatrib. A dose-limiting toxicity was observed in 1 of the 3 patients in cohort 4 (grade 4 neutropenia for longer than 5 days) treated with lomeguatrib 40 mg and irinotecan 250 mg/m^2, resulting in cohort expansion to 6 patients with no additional DLTs.

Table 7. Patient demographics

Variable			Total (N=24)
Gender	Male		15
	Female		9
Age (years)	Median (range)		62.5 (40-75)
ECOG PS	0		21
	1		3
Stage at presentation	Stage II		2
	Stage III		6
	Stage IV		16
Current disease status	No. organs involved	1	4
		2	9
		≥3	11
Sites of metastases	Lung		19
	Liver		20
	Nodal		8
	Other*		15
Previous treatments	Surgery		20
	Radiotherapy***		3
	Chemotherapy		24
Prior chemotherapy	5-FU/capecitabine		10
	Oxaliplatin&5-FU/capecitabine		21
	Irinotecan&5-FU/capecitabine		8
	Other**		4
Median no. prior regimens (range)			2 (1-6)

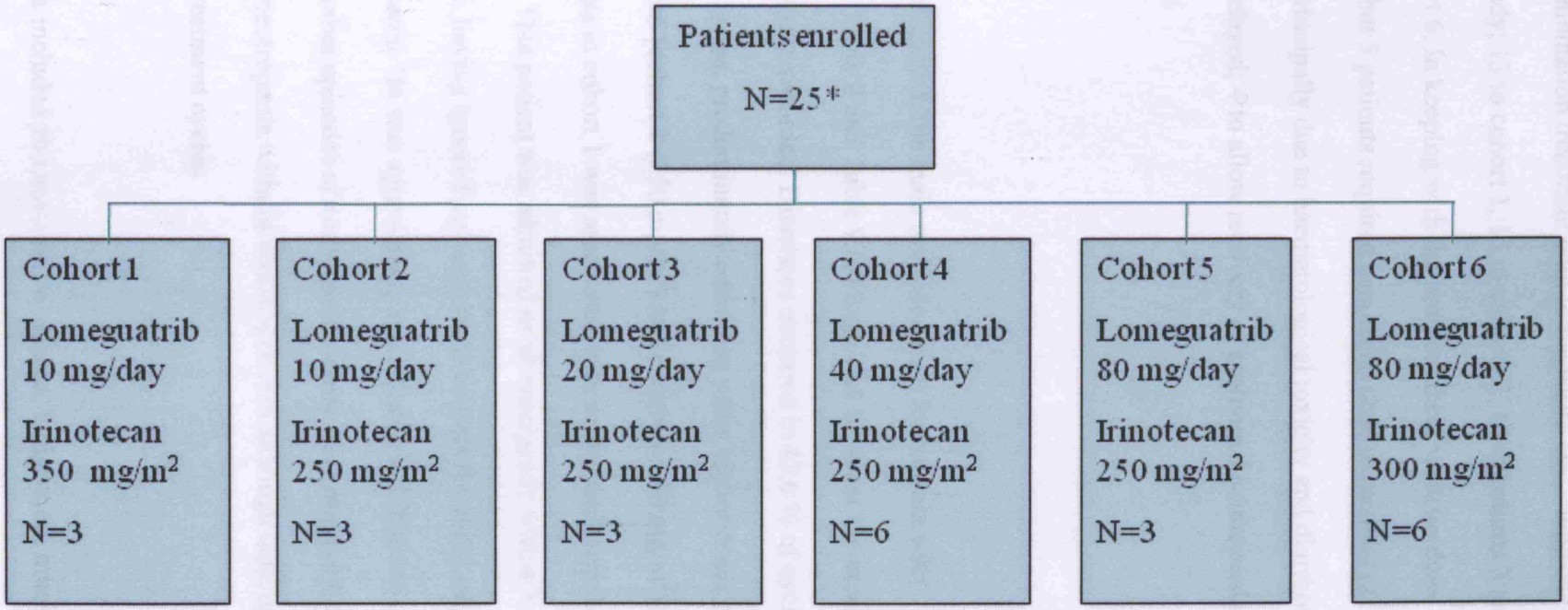
*Other-splenic, pelvic, omental, peritoneal, mesenteric, parotid, abdominal wall metastases

**Other- irinotecan, irinotecan/cetuximab, xaliproben/placebo

***adjuvant radiotherapy for rectal cancer, palliative radiotherapy for parotid and scalp and spine and shoulder metastases

Figure 11. Study design

* 1 patient failed screening



A total of 104 cycles of combination treatment with lomeguatrib and irinotecan were administered across the study; 13 to cohort 1, 15 to cohorts 2, 12 to cohorts 3 and 5, 25 to cohort 4 and 27 to cohort 6. In keeping with the protocol there were no dose reductions of lomeguatrib but 5 patients required irinotecan dose reductions (2 reductions in 2 patients), principally due to haematological toxicity and diarrhoea. Five cycles of treatment were delayed; 4 to allow recovery of prolonged neutropenia, and 1 to allow for a bank holiday.

4.3.3 Adverse Events

Adverse events were as anticipated: the main toxicities were leukopenia with neutropenia and diarrhoea. Table 8 and Table 9 list these and the other the most common/serious adverse events recorded. Diarrhoea occurred in 45.6 % of cycles and was grade 3/4 in 4.9 % of cycles, predominantly occurring when higher doses of irinotecan were administered [cohort 1 (350 mg/m^2) and cohort 6 (300 mg/m^2)]. One episode of grade 3 diarrhoea in cohort 1 was associated with neutropenic sepsis and led to the death of the patient. This patient was admitted as an emergency with a 3 day history of severe diarrhoea, having ignored instructions to contact the study centre in the event, and grade 4 neutropenia. He was aggressively resuscitated but failed to respond to treatment and died. No other episodes of neutropenic sepsis were observed. Only 1 episode of grade 3 febrile neutropenia without sepsis was seen although neutropenia was recorded in 33 % of treatment cycles.

Other common side effects included abdominal pain, nausea, indigestion, mucositis, alopecia and anorexia but none of these was severe (grade 3/4). Fatigue was also commonly reported and this was severe in 2 % of cycles (2 cases). One patient had a

pulmonary embolus whilst on treatment that may have been related to treatment, although they had sustained a pathological fracture to their hip at that time.

Table 8. Haematological toxicity

Lomeguatrib/irinotecan dose (mg/mg/m^2)	10/350		10/250		20/250		40/250		80/250		80/300		
	All AEs n (%)	Gr 3-4 n (%)	All AEs n (%)	Gr 3-4 n (%)	All AEs n (%)	Gr 3-4 n (%)	All AEs n (%)	Gr 3-4 n (%)	All AEs n (%)	Gr 3-4 n (%)	All AEs n (%)	Gr 3-4 n (%)	Total AEs n (%)
Anaemia	4 (31)	0 (0)	2 (13)	1 (7)	2 (17)	0 (0)	2 (8)	0 (0)	1 (8)	0 (0)	1 (4)	0 (0)	12 (12)
Leukopenia	4 (31)	4 (31)	2 (13)	0 (0)	2 (17)	0 (0)	12 (48)	1 (4)	1 (8)	0 (0)	1 (4)	0 (0)	22 (22)
Neutropenia*	4 (31)	3 (23)	4 (27)*	2 (13)*	1 (8)	0 (0)	14 (56)	5 (20)	4 (33)	2 (17)	7 (26)	1 (4)	34 (33)
Neutropenic Sepsis	1 (8)	1 (8)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (1)
Lymphopenia	0 (0)	0 (0)	3 (20)	0 (0)	3 (25)	0 (0)	5 (20)	0 (0)	4 (33)	0 (0)	2 (7)	0 (0)	17 (17)
Monocytopenia	0 (0)	0 (0)	0 (0)	0 (0)	2 (17)	0 (0)	7 (28)	0 (0)	2 (17)	0 (0)	0 (0)	0 (0)	11 (11)
Basopenia	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	6 (24)	0 (0)	2 (17)	0 (0)	2 (7)	0 (0)	10 (10)
Thrombocytopenia	1 (8)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	9 (36)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	10 (10)

AE: adverse event; Gr 3-4: CTC v.2 grades 3 and 4; * includes 1 episode of febrile neutropenia; n=total number of events; (%) =% of cycles per cohort

Table 9. Non-haematological toxicities with increasing irinotecan dose

Irinotecan dose (mg/m^2)	250		300		350		
	All AEs	Gr 3-4	All AEs	Gr 3-4	All AEs	Gr 3-4	**Total AEs**
	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
Hypokalaemia	12 (19)	1 (2)	1 (4)	0 (0)	5 (38)	1 (8)	18 (17.5)
Hypophosphataemia	11 (17)	4 (6)	0 (0)	0 (0)	4 (31)	1 (8)	15 (14.6)
Hyper GGT	4 (6)	0 (0)	0 (0)	0 (0)	4 (31)	0 (0)	8 (7.8)
Hyperglycaemia	14 (22)	0 (0)	1 (4)	0 (0)	1 (8)	1 (8)	16 (15.5)
Raised LDH	10 (16)	0 (0)	0 (0)	0 (0)	4 (31)	0 (0)	14 (13.6)
Raised urea	7 (11)	0 (0)	0 (0)	0 (0)	1 (8)	1 (8)	8 (7.8)
Abdominal pain	11 (17)	0 (0)	0 (0)	0 (0)	1 (8)	0 (0)	12 (11.7)
Diarrhoea	32 (50)	1 (2)	9 (33)	2 (7)	6 (46)	2 (15)	47 (45.6)
Nausea	10 (16)	0 (0)	1 (4)	0 (0)	4 (31)	0 (0)	15 (14.6)
Indigestion	7 (11)	0 (0)	0 (0)	0 (0)	1 (8)	0 (0)	8 (7.8)
Mucositis	12 (19)	0 (0)	1 (4)	0 (0)	0 (0)	0 (0)	13 (12.6)
Vomiting	8 (13)	2 (3)	4 (15)	1 (4)	3 (23)	0 (0)	15 (14.6)
Alopecia	12 (19)	0 (0)	5 (19)	0 (0)	2 (15)	0 (0)	19 (18.4)
Anorexia	10 (16)	0 (0)	3 (11)	0 (0)	2 (15)	0 (0)	15 (14.6)
Fatigue	17 (27)	1 (2)	3 (11)	0 (0)	6 (46)	1 (8)	26 (25.2)
Pulmonary Embolus	1 (2)	1 (2)	0 (0)	0 (0)	0 (0)	0 (0)	1 (1)

AE: adverse event; Gr 3-4: CTC v.2 grades 3 and 4; n=total number of events; (%) =% of cycles per dose level of irinotecan

4.3.4 Clinical Response and Efficacy results

Of the 24 patients treated, 13 patients completed 6 cycles of treatment; 7 patients were withdrawn early due to disease progression, 3 patients experienced unacceptable toxicity including 1 episode of neutropenic sepsis and death and 1 patient was withdrawn due to the investigator's decision. Overall tumour responses as measured on spiral CT using RECIST are shown in Table 10. Median time to progression was 4.0 months (range 0.4 to 16.2 months). Median overall survival was 5.4 months (range 0.4 to 16.2 months).

4.3.5 Pharmacokinetics of lomeguatrib

Lomeguatrib plasma concentration data 12 hours after dosing on day 3 was received from BAS Ltd. for 21 of the 24 patients dosed in the study. This demonstrated that lomeguatrib plasma levels were usually undetectable 12 hours post dosing as has previously been shown (Ranson et al., 2006a). Since the pharmacokinetic sampling for lomeguatrib was confined to a single sample, pharmacokinetic parameters could not be determined and as sampling occurred prior to administration of irinotecan it was not possible to determine whether the addition of irinotecan altered exposure to lomeguatrib and its metabolite in any way.

Table 10. Overall Tumour response

Time of assessment	Tumour response	No. of Patients (N=24)
Post cycle 2	Stable disease	17
	Progressive disease	5
	Not evaluable	2*
End of treatment evaluation	Partial response	1
	Stable disease	6
	Progressive disease	7
Overall Best Response	Partial response	1
	Stable disease	16
	Progressive disease	5
	Not evaluable	2*

*1 patient died during cycle 1 and 1 had clinically progressive disease and was taken off study prior to completing cycle 1

4.3.6 Pharmacokinetics of irinotecan and SN38

Irinotecan and SN-38 plasma concentration data was obtained for 22 of the patients dosed in the study (Table 11 and Figure 12). Maximum plasma concentrations of irinotecan and SN-38 were seen at the end of infusion in the majority of patients as expected; thereafter concentrations declined in a biphasic fashion. The terminal half-life of irinotecan was 5 to 7 hours but it was not possible to derive the terminal half life for SN38 since sampling was not continued out for long enough after dosing to adequately define the terminal phase.

4.3.7 Pharmacodynamics

4.3.7.1 Determination of the MGMT depleting activity of lomeguatrib

All 24 patients had PBMC samples collected but in some cases time points were missing or samples were insufficient to quantitate MGMT activity. Tumour samples from one patient were available for MGMT analysis [(Watson & Margison, 2000); Chapter 2]. MGMT activity was detectable in the 22 available pre-treatment PBMC samples; mean activity 11.7 fm/μg DNA (range = 6.2-22.9 fm/μg DNA) but not detectable in any of the 17 pre-irinotecan (day 4) or 13 end of treatment (day 6) samples. Similarly, MGMT activity was detected in the 1 pre-treatment tumour biopsy (6.9 fm/μg DNA) but not in the end of treatment biopsy.

4.3.7.2 Topoisomerase I cleavage products assay (ICE assay)

Blood samples were collected from all 24 patients however, despite multiple attempts the assay as described by Subramanian *et al* (Subramanian *et al.*, 1995) failed to work. Using the assay, DNA was isolated but bound topoisomerase I could not be identified.

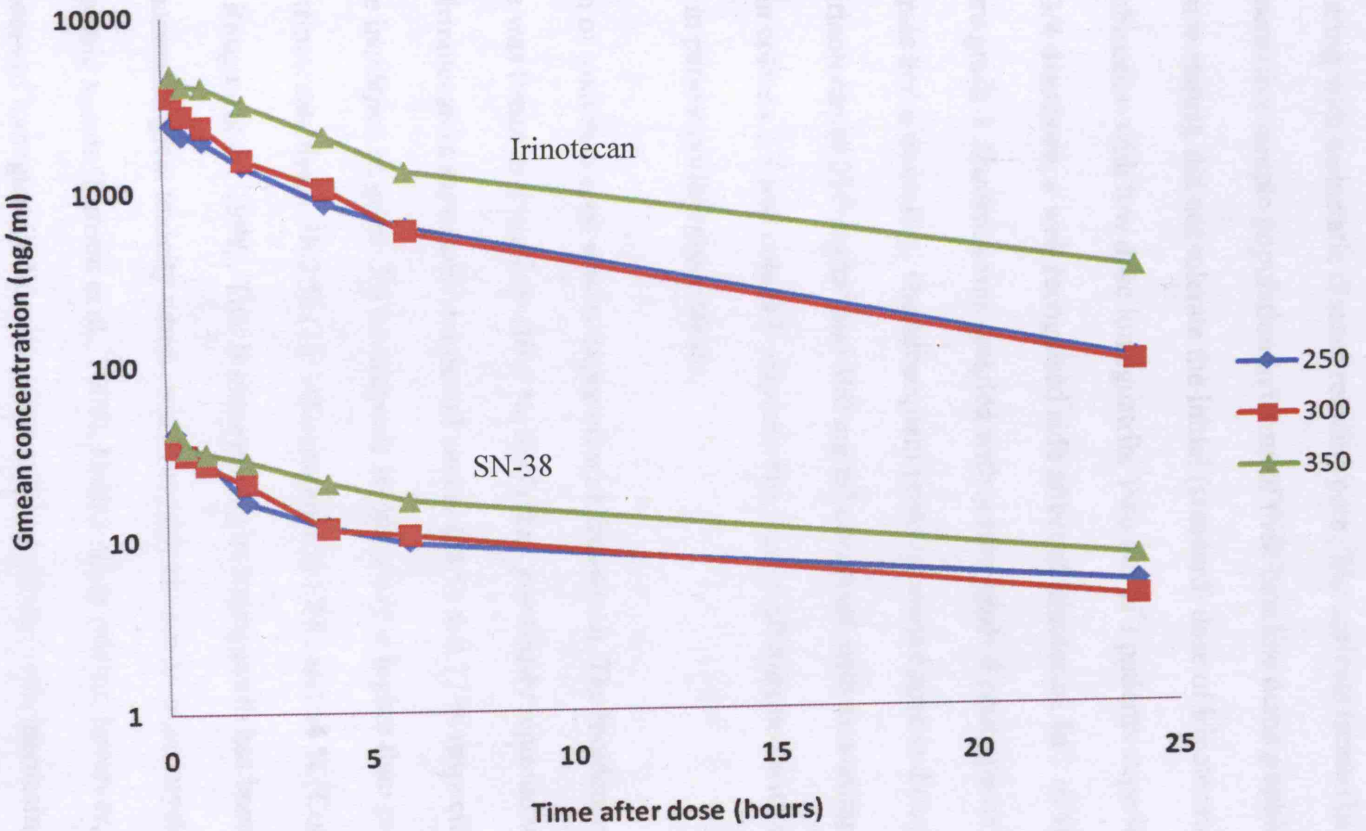
Several modifications of the methodology were made including: changing the dilution of the blood by adding more EDTA, adding protease inhibitor cocktails or chemical inhibitors of proteases to the blood prior to analysis to avoid possible lysis of topoisomerase I from DNA, modifying the caesium chloride gradient to separate DNA,using topoisomerase antibodies from different sources,. The assay was found to work using isolated lymphocytes or with cells from cell culture but not when whole blood was added. The basis for this remains unclear.

Table 11. Derived pharmacokinetic parameters for Irinotecan and SN-38

Parameter	Summary statistic	Irinotecan dose					
		250 mg/m^2 (n = 14)		300 mg/m^2 (n = 5)		350 mg/m^2 (n = 3)	
		Irinotecan	SN-38	Irinotecan	SN-38	Irinotecan	SN-38
Cmax (ng/ml)	Geometric mean (CV%)	2530 (28)	40.9 (53)	3510 (32)	35.7 (60)	4190 (34)	36.7 (38)
tmax (h)	Median (range)	1.72 (1.53 – 2.65)	1.72 (1.53 – 2. 58)	1.68 (1.65 – 2.33)	1.76 (1.67 – 2.67)	2.37 (1.8 – 2.38)	1.87 (1.8 – 1.9)
AUC0-t (ng.h/ml)	Geometric mean (CV%)	14.9 (32)	260.9 (62)	17.4 (12)	303.5 (62)	33.5 (52)	395.3 (38)

Key: Cmax, peak plasma concentration; AUC, area under plasma concentration-time curve; CV, coefficient of variation

Figure 12. Geometric mean plasma concentration time profiles for irinotecan and SN-38 following irinotecan dosing at 250, 300 and 350 mg/m^2



4.4 Discussion

Despite significant advances in the treatment of colorectal cancer, the prognosis for patients presenting with metastatic disease remains poor. The patients treated in this study, a representative sample population in terms of their baseline demographics (age and performance status), did not tolerate the initial (standard) dose of irinotecan 350 mg/m^2 in combination with low dose lomeguatrib. Two out of 3 patients experienced severe grade 3/4 diarrhoea, a well recognised side effect of irinotecan. In 1 of these patients, severe grade 3 diarrhoea was coupled with severe grade 4 neutropenia and resulted in sepsis and a mortality. The subsequent protocol amendment and dose reduction in irinotecan to 250 mg/m^2 and 300 mg/m^2 combined with escalating doses of lomeguatrib in cohorts 2-5 and cohort 6 respectively, were well tolerated with no deterioration in patient performance status.

The spectrum of toxicities seen was as expected with irinotecan. The incidence of grade 3/4 diarrhoea was lower in this study (10.6 %; 5/47) than previously reported with single agent irinotecan in metastatic colorectal cancer (21 % and 22 % respectively). However, the incidence of grade 3/4 neutropenia in this study is higher than previously reported for irinotecan alone, 38.2 % (13/34) compared to 22 % and 14 % (Cunningham et al., 1998; Rougier et al., 1998). This is unsurprising as lomeguatrib has been reported to increase haematological toxicity when used in combination with other myelotoxic chemotherapeutic agents (Ranson et al., 2007). Unlike other studies, however, increasing doses of lomeguatrib did not appear to be associated with increasing levels of myelotoxicity, even when combined with a higher dose of irinotecan (300 mg/m^2). In general haematological and non-haematological side-effects were readily managed and despite these toxicities, compliance with treatment in this study was good. No new lomeguatrib-specific toxicity was encountered.

The efficacy of combination treatment with irinotecan and lomeguatrib was disappointing with only 1 partial and no complete responses seen. However, the patients had all been pre-treated with between 1 and 6 previous lines of chemotherapy. Eleven of the patients had had irinotecan-based treatment previously. In this setting of effectively third-line treatment, a response rate of 4-6 % would be expected (Rowland et al., 2005) i.e. 1 responder in a study of this size. Therefore although not the primary end point of the study, it may be surmised that lomeguatrib does not appear to dramatically increase the efficacy of irinotecan.

The pharmacokinetic data collected from this study indicates the maximum concentrations and timing of peak concentrations of irinotecan and SN-38 are consistent with previous studies (Pitot *et al.*, 2000). However, the concentrations of both irinotecan and SN-38 over time, as measured by AUC, were lower than would be expected. This is most probably as a consequence of the reduced duration of sampling in this study as has been previously documented (Mathijssen *et al.*, 2002). Thus, there did not appear to be any interaction between lomeguatrib and irinotecan.

MGMT activity was detectable in all 22 pre-treatment PBMC samples (range = 6.2-22.9 fm/μg DNA) and the mean activity, 11.7 fm/μg DNA of the pre dose samples was similar to that previously reported (Ranson et al., 2007). Following lomeguatrib administration, inactivation of MGMT was complete and unaffected by combination with irinotecan in all samples including the only tumour biopsy taken.

The maximum doses of combination treatment with irinotecan (300 mg) and lomeguatrib (80 mg) administrated in this study were tolerable. However, the maximum dose of lomeguatrib used in this combination was defined by the protocol and may infact not be the MTD. It may be possible to escalate the dose of lomeguatrib used in combination with irinotecan further, ideally to 120 mg which has been shown in the previous chapter to consistently deplete MGMT in colorectal cancer.

At first sight, treatment with this couplet does not appear to have higher efficacy than irinotecan alone in heavily pre-treated patients. However further work is needed to establish the response rate of this combination in second-line treatment for metastatic colorectal patients. Adding a methylating agent, such as temozolomide to combination treatment with lomeguatrib and irinotecan should also be explored. This is based on the observation from pre-clinical data that treatment with an O^6-alkylating agent prior to irinotecan increases the efficacy of the latter drug (Pourquier *et al.*, 2001). A number of subsequent clinical studies that failed to administer the alkylating agent prior to irinotecan have had disappointing results with no improvement in efficacy of combination treatment over irinotecan alone (Quinn et al., 2004; Reardon et al., 2004; Friedman et al., 1999). More encouraging results have been seen when drug scheduling is optimised in accordance with preclinical data and the alkylating agent is administered prior to irinotecan. In a phase I study in recurrent malignant glioma in which patients were given temozolomide (days 1-5) and irinotecan (day 6), 31 % (10/32) patients achieved a complete or partial response (Gruber & Buster, 2004).

The effect of MGMT on this synergistic interaction has not yet been fully elucidated, but *in vitro* studies have shown adding O^6-BG to combination treatment with

temozolomide and irinotecan, results in a significant reduction in growth compared with either temozolomide or irinotecan alone or together (Friedman *et al.*, 2002). Methylation of guanine at the O6 position (e.g. by temozolomide) may trap topoisomerase I and stabilize the topoisomerase-DNA complex, thus potentially enhancing the probability of inducing irinotecan-mediated damage. In combination therapy, lomeguatrib would deplete levels of MGMT thereby increasing the levels of O^6-methylguanine and hence improving the therapeutic index of temozolomide and the cytotoxic effect of irinotecan.

Although responses to high dose therapy with chlorethylating agent BCNU have been reported in patients with metastatic colorectal cancer (Phillips *et al.*, 1983), combination treatment with lomeguatrib and temozolomide was found to have no efficacy in this patient group (Khan *et al.*, 2008). Temozolomide may however still have a role in colorectal cancer chemotherapy through the effects of the O^6-MG lesions on topoisomerase I activity and the efficacy of irinotecan as described above. However, given the overlapping toxicity profiles for temozolomide and irinotecan, in particular haematological toxicity, any future studies will have to proceed with significant caution.

In conclusion, this phase I study successfully identified suitable doses of lomeguatrib and irinotecan for use in combination therapy in patients with metastatic colorectal cancer. However it is unlikely that future studies will be conducted expanding on this data in the near future as the focus of therapy has shifted to the integration of targeted treatments in existing regimens.

5. Phase I trial of decitabine in combination with ECF chemotherapy in locally advanced or metastatic gastro-oesophageal cancer

5.1 Introduction

Gastric and oesophageal cancers are amongst the ten commonest cancers worldwide leading to significant morbidity and mortality. In 2004 in the UK, 8200 new patients were diagnosed with gastric cancer and there were 5600 deaths from gastric cancer; 7,600 new patients were diagnosed with oesophageal cancer and there were 7,400 deaths from this malignancy. The prognosis of both of these malignancies is poor with 1-year survival of 28-35 % and 5 year survival of 8-15 % (http://info.cancerresearchuk.org/cancerstats/types). In Western countries, the incidence of gastric cancer has fallen over the past few decades whereas oesophageal cancers, particularly adenocarcinomas of the gastro-oesophageal junction and lower oesophagus, are becoming more common. Approximately 30 % of patients with oesophageal cancers are considered suitable for potentially curative surgery at the time of diagnosis, however the five-year survival for those operated on is only 20-30 %; the majority of patients present with locally advanced or metastatic disease. Similarly most patients with gastric cancer will develop metastases at some point in the course of their disease. Advanced, incurable disease is present in approximately 50 % of patients at the time of initial presentation, and those who undergo potentially curative resections have a high rate of both local and distant recurrence. Local palliation may be achieved with palliative resection or bypass operations, radiotherapy, laser therapy or stenting, but the mainstay

of palliative treatment for locally advanced or metastatic gastro-oesophageal disease is chemotherapy.

Several large randomised trials have been published to evaluate the efficacy of combination chemotherapy schedules in metastatic oesophageal, gastric and gastro-oesophageal cancers. In the UK, epirubicin, cisplatin and continuous infusional 5-FU (ECF) has been regarded as the standard treatment against which new drug combinations are evaluated (Bamias *et al.*, 1996; Ross et al., 2002; Waters *et al.*, 1999; Webb et al., 1997). ECF is an intensive schedule and also requires the insertion of a central venous catheter. It is associated with significant grade 3 and 4 toxicities including neutropenia (32 %), lethargy (18 %), nausea and vomiting (11 %), anaemia (9 %), mucositis (5 %) and palmar plantar erythema (1 %) (Ross et al., 2002). Whilst response rates of 42.4 - 46.0 % are observed with ECF in randomised trials of patients with gastro-oesophageal cancer, median survival remains poor at 8.7 - 9.4 months with only approximately 40 % of patients surviving for one year (Ross et al., 2002; Waters et al., 1999; Webb et al., 1997).

As previously discussed, there has been considerable interest recently in the role of CpG-island hypermethylation at gene promoters as one mechanism whereby tumour cells inactivate genes essential for normal cellular control (epigenetic silencing), contributing to resistance to cytotoxic chemotherapy (Rountree *et al.*, 2001). A variety of different genes involved in cell homeostasis are inactivated by promoter hypermethylation in gastro-oesophageal cancers, some of which are described in Table 12 (Bian *et al.*, 2002; Carvalho et al., 2003; Corn et al., 2001; Eads *et al.*, 2000; Esteller *et al.*, 2001a; Fang et al., 2005b; Kang et al., 2001; Oue *et al.*, 2001).

Table 12. Frequency of gene promoter hypermethylation in gastric and oesophageal cancers

Gene	Mechanism of action	Oesophageal cancer (%)*	Gastric cancer (%)*
p14^{ARF}	Cell cycle/*p53*	8-22	26
p16^{INK4A}	Cell cycle/*pRb*	33-82	36-65
hMLH1	Mismatch repair	Not done	20-37
CDH-1	Cell adhesion / metastasis	66-84	40-81
MGMT	DNA repair	20-72	16-61

* The frequency of events varies widely between series mainly because of tumour variation and different techniques used for the detection of methylation.

The role of decitabine as a DNA hypomethylating agent has already been discussed. Very few studies have been conducted with low-dose decitabine in solid tumours and none previously in gastro-oesophageal cancer. It is hypothesised that reversal of hypermethylation by low dose decitabine will allow the reactivation of the genes involved in cellular homeostasis and transcription of these genes may circumvent cancer cell drug resistance leading to greater activity of chemotherapy regimens. The aim of the study discussed in this chapter and the next is to establish the maximum tolerated dose of low dose decitabine in combination with standard ECF chemotherapy in advanced gastro-oesophageal cancer. In this chapter the clinical outcomes of the phase I study of decitabine in combination with ECF in patients with advanced gastro-oesophageal cancer are presented.

5.2 Patients and Methods

5.2.1 Eligibility criteria

Individuals over the age of 18 with histologically confirmed locally advanced or metastatic gastric or oesophageal cancer (adenocarcinoma, squamous cell cancer or undifferentiated carcinoma) considered suitable for ECF chemotherapy were eligible for the study. Patients were required to have WHO performance status 2 or better and adequate bone marrow (haemoglobin $\geq$ 9.0 g/dl, neutrophils $\geq$ 1.5 x 10^9/l, platelets $\geq$ 100 x 10^9/l), renal (creatinine clearance $\geq$ 50 ml/min) and hepatic function (bilirubin $\leq$ 1.5 x upper limit of normal [ULN], aspartate transaminase $\leq$ 2.5 x ULN or $\leq$ 5 x ULN in presence of liver metastases, alkaline phosphatase $\leq$ 2.5 x ULN or $\leq$ 5 x ULN in presence of liver or bone metastases). No previous chemotherapy for oesophageal or

gastric cancer was permitted except neo-adjuvant chemotherapy. No previous radiotherapy to the primary tumour site was allowed. All patients enrolled in the study gave written informed consent. The study was approved by the Oxfordshire Research Ethics Committee and Oxford Research and Development Committee. The study was conducted in accordance with the Principles of the International Conference on Harmonisation of Good Clinical Practice Guidelines and the Declaration of Helsinki.

5.2.2 Treatment

All patients had a Hickman or PICC line inserted. Decitabine, supplied initially by SuperGen and subsequently by MGI Pharma, was administered intravenously on day 1 at a starting dose of 7.5 mg/m^2, as a 6-hour infusion. Patients were readmitted on day 11/12 of cycle 1 to commence ECF chemotherapy and attended weekly thereafter to change their 5-FU infusions. Decitabine was repeated every 21 days with the next ECF given 10-12 days later. Up to 6 cycles of treatment were given.

5.2.3 Toxicity and response evaluation

Pre-study evaluations were completed within 2 weeks of receiving decitabine including history and physical examination (including WHO performance status, vital signs, height and weight), electrocardiogram, staging CT chest, abdomen and pelvis (within 4 weeks of commencing treatment), full blood count, serum biochemistry, coagulation screen and calculated creatinine clearance. In addition, patients underwent physical examination, full blood count, measurement of serum biochemistry and calculated creatinine clearance prior to each treatment cycle.

Clinical responses were determined according to RECIST. Response duration and overall survival were calculated from the start of chemotherapy to the date of first observation of progressive disease and date of death, respectively. Formal tumour evaluation according to RECIST was repeated after 3 cycles of chemotherapy and patients with stable or responding disease were treated with a maximum of 6 cycles of chemotherapy, if progression or unacceptable toxicity did not occur. The severity of adverse events was assessed according to the National Cancer Institute Common Toxicity Criteria grading system (CTC v.2). Patients were followed up for 28 days following completion of the study.

5.2.4 Dose escalation and modification

Five dose levels of decitabine (7.5, 10.0, 12.5, 15.0 and 20.0 mg/m^2) in combination with full dose ECF chemotherapy were investigated. Three patients were treated at each dose level for a minimum of 1 cycle before dose escalation to the next cohort. Treatment was delayed by a week or until recovery of counts if on the day ECF treatment was due, any of the following were present: neutrophil count $\leq 1.5 \times 10^9/l$; platelet count $\leq 100 \times 10^9/l$; non-haematological toxicity grade 2 or above, except alopecia. If there was more than a week's delay, the treatment dose was modified as outlined below.

Dose limiting toxicity (DLT) was defined as one or more of the following in cycle 1 of combination chemotherapy: grade 4 neutropenia $\geq$ 5 days; neutropenic sepsis (pyrexia $\geq$ 38 °C with grade 3/4 neutropenia); platelets $\leq 25 \times 10^9/l$; grade 3/4 non-haematological toxicity (except nausea, vomiting or alopecia); drug related death; treatment delay in

ECF administration of ≥ 2 weeks; If 1 of the 3 patients experienced a DLT, then the cohort was expanded to 6 patients. If 2 patients within the expanded cohort experienced a DLT, then there was no further dose escalation and the previous dose level was considered the maximum tolerated dose (MTD) of decitabine and a further 3 patients were recruited at this dose level.

Any patient experiencing a DLT during cycle 1 of treatment had their decitabine dose reduced to previous dose level of decitabine for all subsequent cycles. If they had further toxicity, decitabine was discontinued. For haematological toxicity occurring in all subsequent cycles (including grade 4 neutropenia lasting ≥ 5 days, neutropenic sepsis (pyrexia ≥ 38 °C and low neutrophils grade 3/4), platelet count ≤ 25 x 10^9/l at any time in cycle and treatment delays ≥ 1 week due to haematological toxicity), epirubicin was reduced to 75 %, then to 50 %. No dose modifications were made to decitabine for non-haematological toxicities. Dose modifications for cisplatin and 5-FU related toxicity were made as per standard unit protocol (Table 13).

Table 13. Criteria for dose modifications for ECF chemotherapy

A. Cisplatin related toxicity

Renal

Calculated creatinine clearance (ml/min)*	**Cisplatin dose modification**
> 50	full dose
30-50	reduced dose according to COIN guidelines; EDTA clearance requested
< 30	omit

*Calculated creatinine clearance (Cockcroft-Gault formula)

$$= \frac{(140 - \text{age}) \times \text{weight (kg)} \times \text{constant}^{\#}}{\text{serum creatinine } (\mu\text{mol/l})}$$

#constant is 1.23 for men and 1.04 for women

Neuro-/Ototoxicity

Patients with ≥ grade 2 CTC v.2 neurotoxicity or new functional deterioration in hearing, new tinnitus or significant high frequency hearing loss on audiogram should have cisplatin discontinued. Cisplatin may be substituted with carboplatin AUC 5.

B. 5-FU related toxicity

	Grade 1	Grade 2	Grade 3	Grade 4
Neutropenia	Continue	Continue	Stop infusion until ≤ grade 2. Re-start infusion at full dose.	Stop infusion until ≤ grade 2. Re-start infusion at full dose.
Thrombocytopenia	Continue	Stop infusion until ≤ grade 1. Re-start infusion at full dose.	Stop infusion until ≤ grade 1. Re-start infusion at full dose.	Stop infusion until ≤ grade 1. Re-start infusion at full dose.
Stomatitis	Mouthwash as appropriate	Stop infusion. Restart with 50 mg/m^2 dose reduction	Stop infusion. Restart with 100 mg/m^2 dose reduction	Stop infusion. Restart with 150 mg/m^2 dose reduction
Palmar-plantar syndrome	Commence pyridoxine 50 mg tds po	Stop infusion. Restart with 50 mg/m^2 dose reduction	Stop infusion. Restart with 100 mg/m^2 dose reduction	Stop infusion. Restart with 150 mg/m^2 dose reduction
Diarrhoea	Commence codeine phosphate 30-60 mg po qds	Stop infusion. Restart with 50 mg/m^2 dose reduction	Stop infusion. Restart with 100 mg/m^2 dose reduction	Stop infusion. Restart with 150 mg/m^2 dose reduction

Note: Any patient with > grade 1 toxicity should be prescribed the therapeutic option for grade 1 toxicity as well as the 5-FU dose modification.

5.3 Results

5.3.1 Patients

Twenty three patients have been recruited into the study so far (Table 14).

5.3.2 Dose escalation and extent of exposure

Twenty two patients have received treatment with decitabine and ECF chemotherapy, 1 patient received only decitabine because of rapid clinical deterioration. A dose limiting toxicity (neutropenic sepsis) was observed in 1 of 3 patients treated with ECF/decitabine 10 mg/m^2, resulting in cohort expansion to 6 patients with no additional DLTs. Two patients treated within this cohort failed to complete the first cycle of treatment due to rapidly progressive disease and treatment associated complications (a small bowel anastomotic leak) and were therefore replaced. No DLTs were observed in cohorts 3 (ECF/decitabine 12.5 mg/m^2) and 4 (ECF/decitabine 15 mg/m^2) but 1 patient in each of these cohorts failed to complete their first cycle of treatment (both due to rapidly progressive disease) and were therefore replaced. Three out of 3 patients treated with ECF/decitabine 20 mg/m^2 developed DLTs (neutropenic sepsis) and therefore the maximum tolerated dose (MTD) of decitabine with ECF has been established as 15 mg/m^2. Expansion of cohort 4 (decitabine 15 mg/m^2) to 6 patients is ongoing.

Table 14. Patient demographics

Characteristic		No. of patients (N=23)
Median age (range) years		62 (40-69)
Female (%)		4 (17%)
WHO PS	0	12
	1	8
	2	3
Primary site	Oesophageal	16
	Gastric	2
	GOJ	5
Stage at start of trial	III	6
	IV	17
Previous surgery ± chemotherapy		4

PS: performance status; GOJ: Gastro-oesophageal junction

5.3.3 Toxicity

Toxicity data was available for all 20 patients in the first 4 cohorts. Only haematological toxicity and serious adverse events data for cycle 1 was available for the 3 patients in cohort 5 as they were still on treatment. Adverse events were predictable; the main toxicity was haematological (Table 15). The most common and severe i.e. all grade 3/4 adverse events occurring during cycle 1 of treatment for the patients treated at or below the MTD of decitabine (cohorts 1 to 4) are listed in Table 16. Highlighted was the high frequency of haematological toxicities with 80 % or more of patients experiencing anaemia or leukopenia during cycle 1. The incidence of grade 3/4 anaemia was low (10 %) but by contrast 50 % of patients had grade 3/4 leukopenia, mainly neutropenia. Despite this, only 1 patient developed febrile neutropenia. Twenty five percent of patients were thrombocytopenic, but none had grade 3/4 toxicity. On analysis of the data including patients from cohort 5 treated with decitabine 20 mg/m^2 with ECF, the established non-tolerated dose, there is an increase in all grade 3/4 haematological toxicities and neutropenic sepsis as seen in Table 17.

In general, few severe (grade 3/4) non-haematological toxicities were observed in cycle 1 (Table 16). However, 3 patients developed pulmonary emboli and 1 patient developed a grade 4 small bowel anastomotic leak requiring emergency surgery. Other adverse events recorded in cycle 1 occurring less commonly included: abdominal pain, anorexia, indigestion and mucositis.

A summary of the overall frequencies of the common adverse events of patients treated at or below the MTD of decitabine (15 mg/m^2) with ECF is listed in Table 18 and additional analysis of the haematological toxicities for all cycles including patients treated with ECF/decitabine 20 mg/m^2 is shown in Table 19. For comparison, the adverse events from the Cunningham et al. study for ECF alone are also included in Table 18 (Cunningham *et al.*, 2008).

Table 15. Cumulative Haematological Toxicity (all cycles): Worst grade per patient

	Total complete cycles administered		**Toxicity grade 3/4 (No. of patients)**			
Decitabine dose	N	Range	Leukopenia	Neutropenia	Anaemia	Thrombocytopenia
7.5 mg/m^2 (N=3)	17	5-6	0/1	2/1	1/0	0/1
10 mg/m^2 (N=8)	28	0-6	3/0	2/1	0/0	0/0
12.5 mg/m^2 (N=5)	14	0-5	4/0	2/1	0/1	0/0
15 mg/m^2 (N=4)	10	0-6	2/1	0/3	0/0	0/0
20 mg/m^2 (N=3)	3	1	1/1	1/2	2/0	1/0

Table 16. Most frequent and all Grade 3/4 adverse events for Cycle 1 for patients treated with ECF/decitabine ≤ 15 mg/m^2

Cycle 1 AEs	Total events (%)	G3/4 events (%)
Haematological (n=20 patients)		
Anaemia	18 (90)	2 (10)
Leukopenia	16 (80)	10 (50)
Neutropenia	17 (85)	12 (60)
Lymphopenia	18 (90)	3 (15)
Thrombocytopenia	5 (25)	0
Non-Haematological (n=20 patients)		
Alopecia	7 (35)	0
Constipation	7 (35)	1 (5)
Diarrhoea	4 (20)	1 (5)
Fatigue	5 (25)	0
Hypergammaglutyltransaminasemia	6 (30)	2 (10)
Hypokalaemia	6 (30)	2 (10)
Hyponatraemia	6 (30)	1 (5)
Hypophosphataemia	6 (30)	2 (10)
Nausea	10 (50)	1 (5)
Neutropenic sepsis	1 (5)	1 (5)
Pulmonary embolus	3 (15)	3 (15)
SI anastomotic leak	1 (5)	1 (5)
Vomiting	5 (25)	1 (5)

SI: small intestinal

Table 17. Haematological adverse events for cycle 1 for patients treated with ECF/decitabine ≤20 mg/m^2

Cycle 1 AEs	Total events (%)	G3/4 events (%)
Haematological (n=23 patients)		
Anaemia	20 (87)	4 (17)
Leukopenia	19 (83)	13 (57)
Neutropenia	20 (87)	15 (65)
Lymphopenia	19 (83)	3 (13)
Thrombocytopenia	6 (26)	1 (4)
Neutropenic sepsis	4 (17)	4 (17)

Table 18. Overall (all cycles) most common adverse events with ECF/decitabine (≤ 15 mg/m^2) and ECF alone (Cunningham *et al.*, 2008)

	ECF/decitabine n=20 patients		**ECF alone (Cunningham et al.)**	
	All grades (%)	**G3/4 (%)**	**All grades (%)**	**G3/4 (%)**
Anaemia	19 (95)	2 (10)	(78.4)	(13.1)
Neutropenia	17 (85)	9 (45)	(73.6)	(41.7)
Neutropenic sepsis	2 (10)	2 (10)	(13.2)	(9.3)
Thrombocytopenia	10 (50)	1 (5)	(14.5)	(4.7)
Alopecia	11 (55)	5 (25)*	(81.5)	(44.2)*
Constipation	10 (50)	1 (5)	NR	NR
Diarrhoea	8 (40)	2 (10)	(39.3)	(2.6)
Fatigue	15 (75)	1 (5)	(89.7)	(16.6)
Nausea	13 (65)	1 (5)	(79.1)	(10.2)
Vomiting	8 (40)	2 (10)		
Mucositis	8 (40)	0	(50.9)	(1.3)
PPE	5 (25)	0	(29.8)	(4.3)
Peripheral neuropathy	4 (20)	0	(30.0)	(0.4)
Thromboembolism	3 (15)	3 (15)	(16.9)	(16.9)

PPE: palmar plantar erythema

*maximum grade of alopecia is grade 2, which is listed in grade 3/4 column

NR: not recorded

Table 19. Haematological adverse events for all cycles for patients treated with ECF/decitabine $\leq$20 mg/m^2

	ECF/decitabine (n=23 patients)	
	All grades (%)	**G3/4 (%)**
Anaemia	22 (96)	4 (17)
Neutropenia	20 (87)	17 (74)
Neutropenic sepsis	5 (22)	5 (22)
Thrombocytopenia	11 (48)	2 (8)

5.3.4 Treatment delays and dose reductions

There were 32 delays in delivering either ECF or decitabine during the 69 complete cycles of treatment administered. The delays were predominantly due to prolonged neutropenia (23 episodes), but other causes for treatment delays included: neutropenic sepsis, mucositis, Hickman line infection, pulmonary embolus, administrative problems and bank holidays. Prolonged neutropenia resulted in dose reductions in epirubicin for 4 of these patients. Four patients had dose reductions of their 5-FU due to diarrhoea and/or mucositis. There was no increase in the frequency of treatment delays or dose reductions with increasing decitabine dose.

5.3.5 Anti-tumour activity

Fifteen patients were assessable for response; of the remaining 5 patients all came off study early with clinically progressive disease including 1 patient that required urgent radiotherapy for haematemesis and did not have re-imaging performed. All of these patients had just received 1 full cycle of treatment or less and 1 had received decitabine only. Best overall responses for the assessable patients were stable disease for 2 patients, partial response for 9 patients and progressive disease for 4 patients. The 2 evaluable patients treated at the MTD both had partial responses as their best responses. The median progression free survival for all 20 patients was 4.43 months (range 0.23 to 16.1 months) and median overall survival was 6.43 months (range 0.62 to 27.8 months).

5.4 Discussion

This is the first study to report the feasibility of delivering a DNA methyltransferase inhibitor (decitabine) in combination with standard ECF chemotherapy. From our results to date, decitabine at 15 mg/m^2 administered as a 6-hour IV infusion can be combined safely with full dose ECF administered on day 11/12 of a 21-day cycle. The main toxicities observed were predictable, mainly myelosuppression, as previously reported with ECF alone (Cunningham *et al.*, 2008; Ross et al., 2002) and manageable at the recommended doses of combined therapy.

The adverse events data for the patients treated with ECF and decitabine at or below the MTD of decitabine (15 mg/m^2) show the overall incidence of anaemia, neutropenia and thrombocytopenia in our study were higher than those reported by Cunningham et al. in patients treated with ECF alone (Cunningham et al., 2008) as shown in Table 17 (page 167), (95 vs. 78.4 %; 85 vs. 73.6 %; 50 vs. 14.5 % respectively) but the incidence of grade 3/4 toxicities were comparable (10 vs.13.1 %; 45 vs. 41.7 %; 5 vs. 4.7 % respectively). Despite the higher incidence of neutropenia in this study, the incidence of neutropenic sepsis was comparable to the Cunningham study (overall 10 vs. 13.2 %; grade 3/4 10 vs. 9.3 %). Combination therapy with decitabine 20 mg/m^2 and ECF was not tolerated due to the high level of neutropenia (74 % grade 3/4) and associated neutropenic sepsis (22 % grade 3/4).

In patients treated with ECF/decitabine $\leq$ 15 mg/m^2, lower frequencies of grade 3/4 lethargy (5 vs. 16.6 %), mucositis (0 vs.1.3 %), palmar plantar erythema (0 vs. 4.3 %) and peripheral neuropathy (0 vs. 0.4 %) were seen compared with the data reported by Cunningham et al. but higher levels of nausea and vomiting and diarrhoea were seen (15

vs. 10.2 % and 10 vs. 2.6 % respectively). Interestingly, similar levels of thromboembolic disease were reported (15 vs. 16.9 %) probably as a consequence of a combination of factors including an indwelling central venous line and malignancy.

Combination treatment with decitabine and ECF resulted in a good response rate (47 %), however both the median overall survival and time to progression (6.43 and 4.43 months respectively) were lower than previously reported with ECF alone (9.9 and 6.2 months respectively) (Cunningham et al., 2008). This is possibly due to the patient population as a number of patients, despite fulfilling the eligibility criteria, deteriorated rapidly and failed to complete their first cycle of therapy.

The effect of this dose of decitabine on DNA methylation both in PBMCs, tumour and normal mucosa will be discussed in the following chapter.

Recently published data has recommended a dose of 90 mg/m^2 of decitabine to be safely combined with carboplatin (AUC 5 or 6) on day 8 of a 28-day cycle. At this dose level, a 35 % global reduction in 5-methyl-2'-deoxycytidine levels in the DNA of PBCs up to 10 days following treatment was seen. Also, demethylation of the CpG islands of the *MAGE1A* gene was seen in both PBCs and tumour, 8 to 12 days after treatment with decitabine (Appleton et al., 2007). In view of these results, it is possible that a dose of decitabine higher than the MTD established in this study in combination with ECF will be required to consistently achieve genomic demethylation. In order to achieve this, the next phase of this study will involve dose escalation of decitabine in combination with CF chemotherapy on the premise that most of the myelosuppression observed with ECF is due to epirubicin.

The recently reported REAL-2 study, mentioned above, has demonstrated that 5-FU in ECF may be exchanged for capecitabine (ECX) with comparable efficacy (response rate 40.7 % ECF vs. 40.8 % ECX; median overall survival 9.9 months with both regimens). However, replacing cisplatin and 5-FU in ECF with oxaliplatin and capecitabine (EOX) has been shown to be superior to ECF; hazard ratio for death 0.80 (95 % confidence interval 0.66-0.97; p=0.02). The toxicity of capecitabine was comparable to that of 5-FU and replacing cisplatin with oxaliplatin resulted in less grade 3/4 neutropenia, alopecia, renal toxicity and thromboembolic disease but more diarrhoea and neuropathy but no difference in quality of life assessment. In view of this and other corroborative studies (Al-Batran *et al.*, 2006; Kang *et al.*, 2006) it is possible that the treatment for gastro-oesophageal cancer may change in the near future and further translational and clinical work will be required to determine the role of decitabine in combination with these newer agents.

6. Effect of low dose decitabine in combination with ECF chemotherapy on DNA methylation in PBMCs, tumour and adjacent mucosa in advanced gastric and oesophageal cancer patients

6.1 Introduction

A variety of genes are transcriptionally silenced by aberrant promoter hypermethylation in gastro-oesophageal cancer development (Toyota & Issa, 2005). These genes, which play a role in tumorigenesis, may also influence cellular response to chemotherapy and non-expression may lead to failure of cells to engage in normal cell cycle control or apoptosis after chemotherapy induced DNA damage (Egger et al., 2004; Esteller, 2003b).

DNA methyltransferases (DNMTs) catalyse the addition of a methyl group to cytosine residues at CpG dinucleotides and are therefore key targets for potential DNA demethylating agents (Lyko & Brown, 2005). Pre-dosing with low, non-cytotoxic doses of decitabine, a DNMT inhibitor and hypomethylating agent, prior to standard chemotherapy may allow a short period of gene reactivation and transcription circumventing cancer cell drug resistance (Plumb *et al.*, 2000).

This chapter will examine the biological effects on peripheral blood cells (PBCs), tumour and oesophageal/gastric tissue of escalating doses of decitabine in advanced gastro-oesophageal cancer. The aim of this study was to determine the effect of escalating low doses of decitabine on CpG island demethylation of the *MAGE1A* gene

promoter in oesophago-gastric tumours, adjacent oesophagus/gastric tissue and PBCs. The *MAGE1A* gene promoter is bi-allelically methylated in most normal tissues in adults and is transcriptionally silent (De Smet *et al.*, 1996; De Smet *et al.*, 2004). Pyrosequencing was used to assess demethylation of the CpG islands of the *MAGE1A* gene promoter, which is used as a surrogate for other gene loci. Gene expression of a variety of candidate genes was assessed by measuring protein products including $p14^{ARF}$, $p16^{INK4}$, MGMT, hMLH1 and e-cadherin by immunohistochemistry.

6.2 Patients and Methods

Patients were required to fulfil the eligibility criteria as presented in the previous chapter. All patients enrolled in the study gave written informed consent. The study was approved by the Oxfordshire Research Ethics Committee and Oxford Research and Development Committee. Chemotherapy (decitabine and ECF) was administered as outlined in the previous chapter. The dose of decitabine was escalated in combination with standard dose ECF as described in chapter 5.

6.2.1 Blood sample collection

Prior to the start of chemotherapy, a baseline 10 ml blood sample was taken in EDTA-containing vacutainers for methylation analysis. Blood samples for methylation analysis were also taken on days 1, 8, 11, 15, 22 and 25 in cycle 1 and on days 1, 8 and 15 for all subsequently cycles. PBCs were isolated from whole blood using density centrifugation. The samples were centrifuged at 1200 *g* for 10 minutes at 4 °C, within an hour of being taken. The plasma (supernatant) from each tube was transferred to a fresh 2 ml universal container using a sterile plastic pipette. The remaining material containing PBCs were

left in their vacutainers and all tubes were transferred immediately to a freezer at -70 °C.

6.2.2 Tumour and oesophageal/gastric biopsies

Prior to commencing chemotherapy, all patients had a baseline endoscopy under sedation with midazolam (non-proprietary; 1-10 mls) or local throat anaesthesia using 2-3 sprays of Xylocaine spray (lidocaine 1 %). Up to six biopsies were taken from the primary tumour and six biopsies from adjacent mucosa. A second endoscopy with repeat biopsies from the primary tumour and adjacent mucosa was performed 10 to 12 days after decitabine administration in cycle 1 for the first two cohorts of patients and cycle 2 for patients from cohort 3 onwards. This change in protocol was made following new data suggesting a more representative assessment of methylation changes following two doses of decitabine (Appleton *et al.*, 2007).

Four cancer and four adjacent normal mucosa biopsies were placed individually in screw top Eppendorfs and immediately frozen on dry ice before storage at -70 °C for methylation analysis. The remaining two cancer and two normal mucosa biopsies were immediately placed in individually labelled universal containers containing 20 mls formalin (formal saline 10 %, formaldehyde 3.8-4 % w/v) for fixing and storage prior to immunohistochemical analysis.

6.2.3 Methylation analysis

Subsequent processing of both blood and tissue samples for methylation analysis was conducted by Professor Robert Brown and his team at the Analytical Services Unit, Centre for Oncology & Applied Pharmacology, University of Glasgow (Glasgow, UK).

6.2.3.1 DNA extraction

In summary, human genomic DNA extraction from blood was performed using a BACC2 Nucleon DNA extraction kit (GE Healthcare Life Sciences Ltd, Buckinghamshire, UK). After thawing the samples at room temperature, the red cells were lysed by twice mixing 1:4 parts with a solution containing 10 mM Tris-hydrochloric acid (HCl), 320 M sucrose, 5 mM magnesium chloride (MgCl) and 1 % Triton X-100, pH 8. White blood cells were lysed by adding reagent B (supplied with the kit). Seven hundred and fifty nanograms RNase (Qiagen) was added to this solution and incubated for 30 minutes at 37 °C. Deproteinisation was performed using 5 M sodium perchlorate (supplied with the kit) followed by genomic DNA extraction with cold chloroform and Nucleon^{TM} resin (supplied with the kit). DNA recovery and washing was performed using cold ethanol. DNA was stored at -70 °C.

For tissue DNA extraction, the frozen tissue was ground into a fine powder using a Mikro-Dismembranator U (B Biotech International) and added to reagent B (supplied with the kit). Subsequent steps were as outlined above.

6.2.3.2 Bisulfite modification

One microgram of DNA was modified according to the manufacturer's protocol in the CpG DNA Modification kit (Millipore, Billerica, MA). Bisulfite modification of DNA changes any unmethylated cytosines into uracils then to thymines and leaves methylated cytosine unchanged. In summary, 1 μg of DNA was denatured using mild heat at an alkaline pH. The DNA was then incubated at 50 °C overnight with Reagent I (supplied with kit) containing a sodium salt of bisulphite ion. Reagent III, a micro-particulate carrier, was then bound to the DNA in the presence of Reagent II, a salt. The bound DNA was desalted by repeat washes in 70 % ethanol. Alkaline desulfonation and desalting were repeated with 90 % ethanol. The DNA was eluted from the carrier by heating in TE buffer and the modified DNA sample was stored at -20 °C.

6.2.3.3 DNA Pyrosequencing

After bisulfite treatment of DNA, PCR was carried out using primers that bracket the CpG island of the *MAGE1A* gene promoter: forward PCR, 5'-TTTTTATTTTTATTTAGGTAGGAT-3'; reverse PCR, biotin-5'-TCTAAAAACAACCCAAACTAAAAC-3' but do not contain CpG sites and therefore do not distinguish between methylated and unmethylated . Forty microlitres of the PCR product was immobilized to streptavidin-coated Sepharose beads and single-stranded templates were prepared using the Vacuum Prep Workstation. Sixteen picomoles of the sequencing primer 5'-TGTTGTTAGTTTTGGTTTAT-3' were annealed to the template before analysis in the PSQ96MA pyrosequencing system (Biotage, Uppsala, Sweden). The DNA template and primer complex were incubated with the enzymes DNA polymerase, ATP sulfurylase, luciferase and apyrase and the substrates adenosine 5' phosphosulphate (APS), luciferin and single stranded DNA binding protein.

Dinucleotide triphosphates were added to the reaction. If complementary to the template strand, then the incorporation of the dinucleotide was accompanied by the release of a pyrophosphate (PPi). The PPi was converted to ATP in the presence of APS, which drives the conversion of luciferin to oxyluciferin, generating visible light detected by the pyrosequencing equipment software. The degree of methylation at individual CpG sites was then analyzed with the AQ software (PSQ96MA, version 2.1; Biotage).

6.2.4 Immunohistochemistry

Immunohistochemistry was performed at the Department of Cellular Pathology at the John Radcliffe Hospital, Oxford. Oesophageal biopsies were embedded in paraffin blocks and subsequently cut into 5 μm sections and collected on slides. Five antibodies at the following concentrations were used: MGMT (Neomarker) 1:100, $p14^{ARF}$ (Neomarker) 1:100, $p16^{INK4A}$ (BD Pharmingen) 1:200, e-cadherin (Novacastra) 1:50 and hMLH1 (BD Pharmingen) 1:100, after dilution titration and optimisation.

All reagents used on the Bond Maxx staining machines were supplied by Vision Biosystems. All equipment and other reagents were supplied by the Department of Cellular Pathology, John Radcliffe Hospital. The following tissues were used as positive controls for each of the antibodies: MGMT-liver, $p14^{ARF}$-tonsil, $p16^{INK4A}$–thyroid adenocarcinoma, e-cadherin-breast and hMLH1-colon, and were included in each run. Separate negative controls were not required as internal negative controls could be seen in gastro-oesophageal tissue structures such as blood vessels.

Sections were stained using the Vision Biosystem Bond Maxx staining machine. This staining machine included steps to dewax, pretreat and stain slides. A chain polymer-conjugated system was used for the immunohistochemical staining using a primary

antibody that attaches to the tissue antigens. A polymer-conjugated chain with attached secondary antibodies and peroxidase was then applied and attached to the primary antibody. DAB (Diaminobenzidine) was then added and acted as a substrate for the peroxidase; this reaction created a brown colouration that was insoluble in water. Endogenous peroxidase in tissue was blocked prior to the addition of antibodies to prevent non-specific reactions.

On completion of staining, the slides were evaluated using light microscopy. First, morphological features were assessed using an H&E slide. Then for each tissue sample stained with each of the panel of antibodies, specified features were assessed including: intensity (0 = no staining, 1 = weak staining, 2 = moderate staining, 3 = strong staining), proportion (focal or diffuse) and localisation (nuclear, cytoplasmic or membranous). Results were scored under the supervision of a consultant histopathologist and senior pathology registrar at separate times and under blinded conditions.

6.3 Results

6.3.1 DNA methylation

6.3.1.1 Oesophageal/gastric tissue

Preliminary results from DNA methylation analysis are available for 7 patients at this time, 5 from patients treated with decitabine 10 mg/m^2 and 2 from patients treated with 12.5 mg/m^2. Mean methylation levels of 3 different CpG islands of the *MAGE1A* promoter evaluated by pyrosequencing demonstrated 91 % (SD 5.5) methylation prior to any therapy in normal oesophageal and gastric mucosa. Methylation levels were minimally changed in repeat biopsies taken either 10-12 days post-decitabine mean 91

% (SD 4.4) (Figure 13). There was a slight increase in mean methylation in 4 samples, a decrease in 2 samples and no change in the remaining sample. Calculation of the ratio of methylation after treatment versus baseline for each of the 3 CpG sites is shown in Figure 14.

6.3.1.2 PBCs

In PBCs, *MAGE1A* methylation levels were comparable to those seen in normal tissue at baseline (mean 92 % SD 4.0), however after decitabine treatment there was a reduction in mean methylation of all 3 CpG islands; in 5 patient samples this was modest but more marked in 2 patient samples in which decitabine treatment resulted in a 10 % decrease in methylation (Figure 15). This effect did not appear to be dose related.

6.3.1.3 Tumour

In tumour samples, *MAGE1A* promoter methylation analysis revealed a very different situation (Figure 16). Baseline methylation levels were very variable between tumours with mean methylation of the 3 sites of the *MAGE1A* promoter of 76 % (SD 13.2). The effect of decitabine was also more disparate. In 3 patient samples, mean methylation of the *MAGE1A* gene locus fell by 37 %, in 2 other biopsies the decrease in mean methylation was minor, only 3 %, the remaining 2 patient biopsies had an increase in mean methylation levels of 22 %.

Figure 13. Mean methylation of *MAGE1A* gene in oesophageal/gastric tissue before and after decitabine treatment

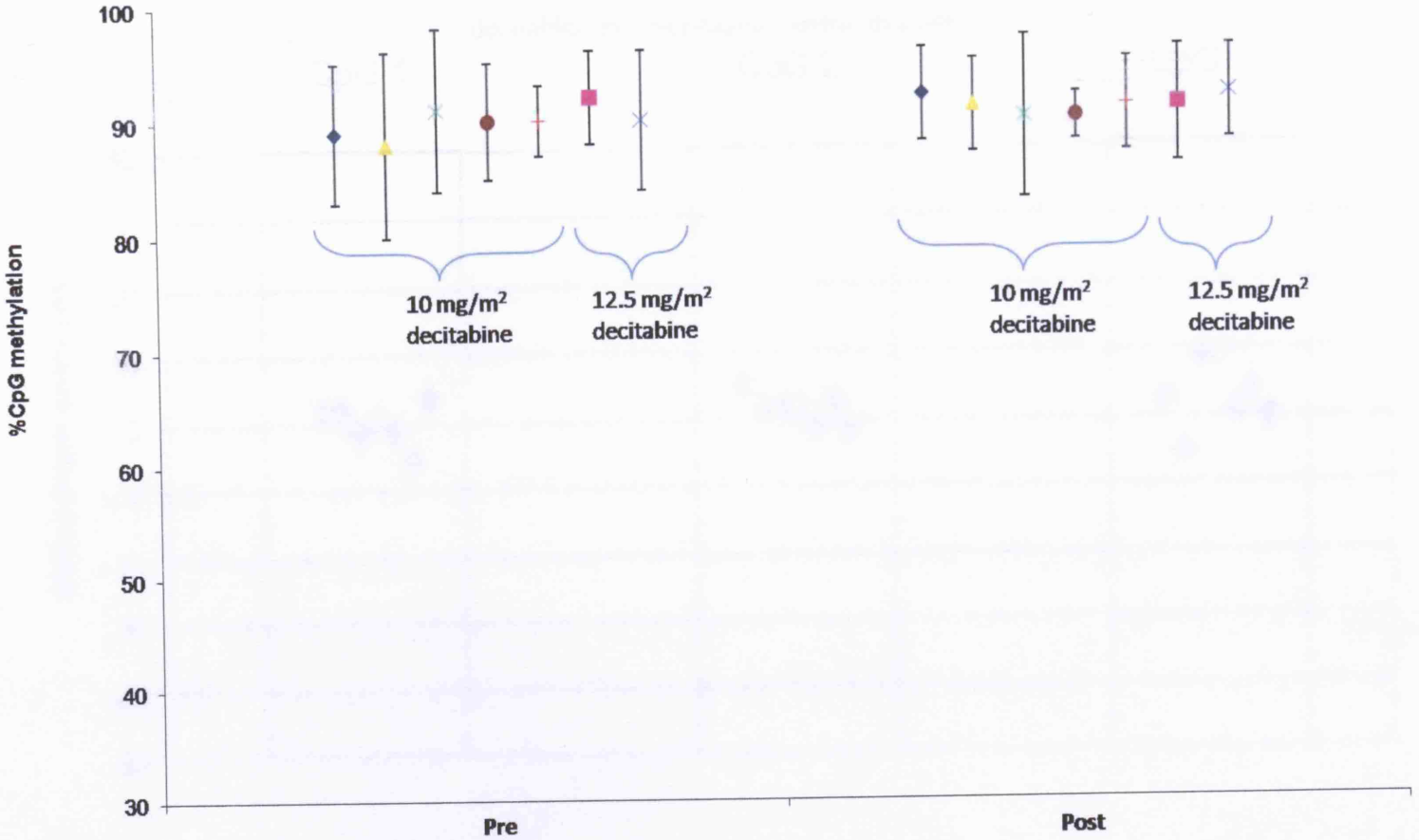


Figure 15. Mean methylation of *MAGE1A* gene in PBCs before and after decitabine treatment

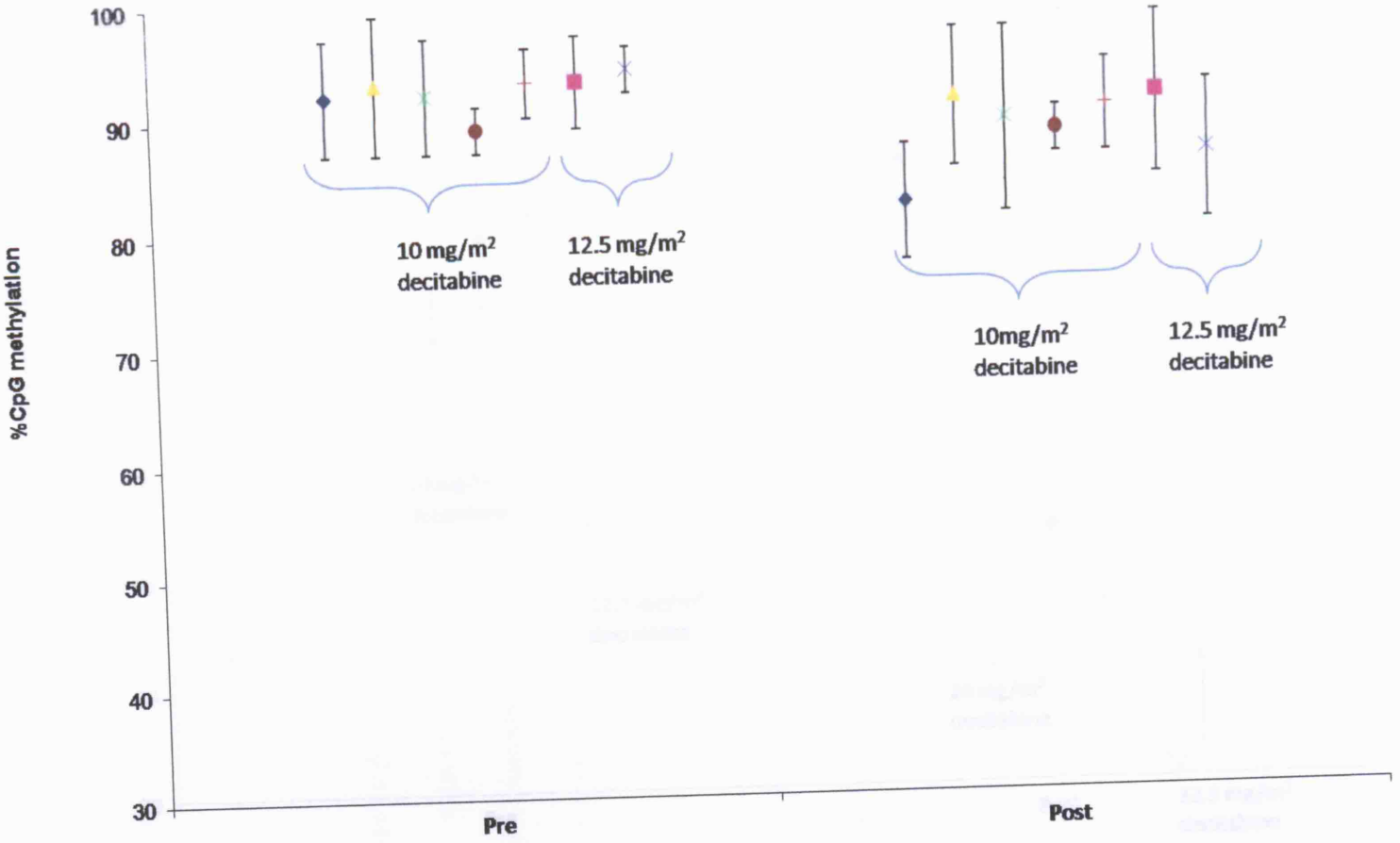
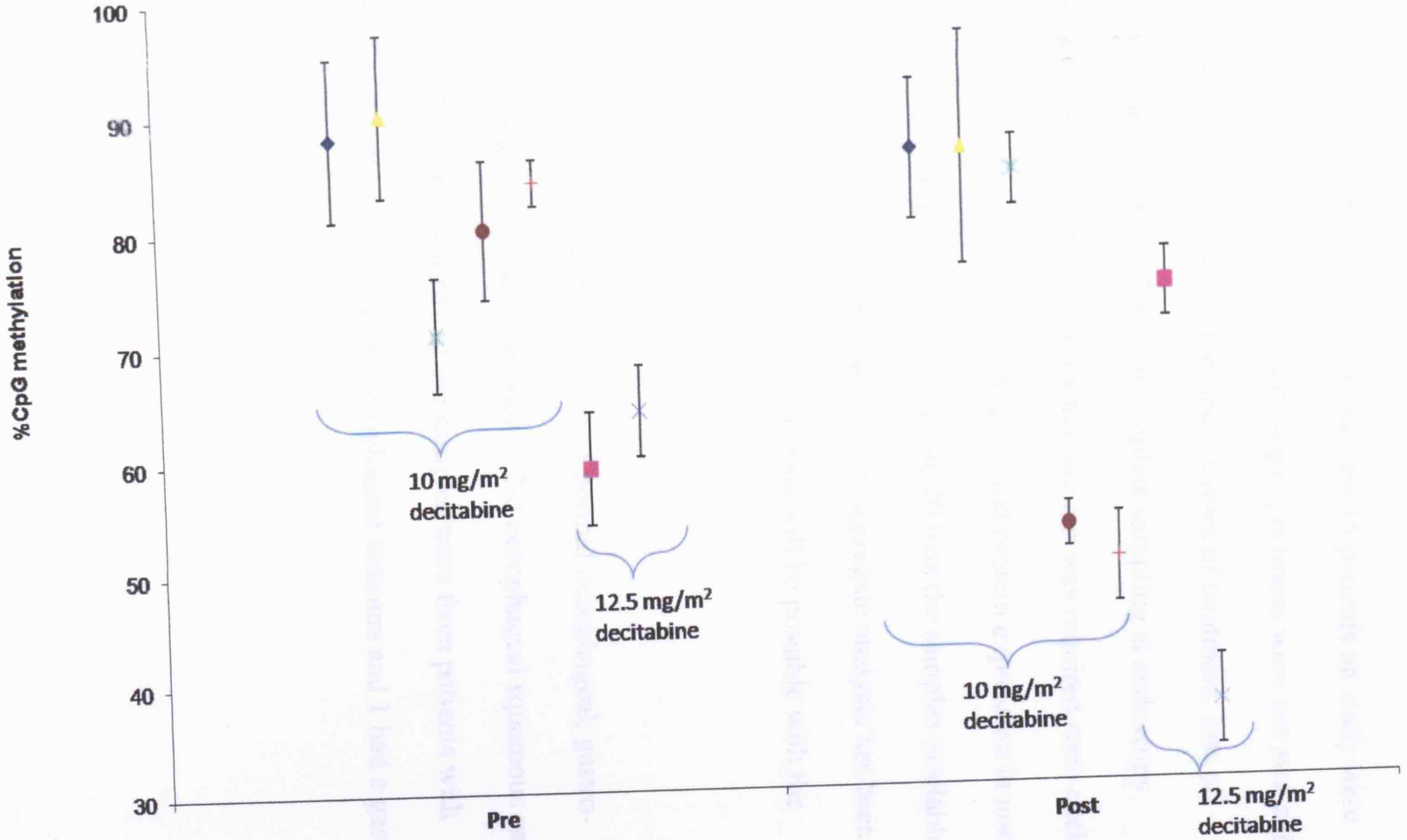


Figure 16. Mean methylation of *MAGE1A* gene in oesophageal/oesophago-gastric tumour before and after decitabine treatment



6.3.2 Immunohistochemistry

Preliminary immunohistochemistry results from the first 15 patients on study were available but unfortunately a number of histopathology specimens were not available for analysis due to patients not completing the first 2 cycles of treatment, loss of samples with changes in laboratory staff and incomplete sampling at endoscopy. Samples taken of oesophageal/gastric mucosa when tumour was required were excluded from analysis due to the documented changes in gene and protein expression in normal tissue adjacent to tumours (Zhang *et al.*, 2003). Table 20 lists the samples available for review. Due to the limitations of the sample set mainly aggregate analysis has been performed however it is anticipated that paired analysis will be possible with the samples currently being gathered.

H&E staining of the slides identified the presence of normal oesophageal, gastro-oesophageal or stomach mucosa or adenocarcinoma. No oesophageal squamous cell carcinomas were identified. The majority of the samples were from patients with oesophageal primaries, 2 patients had gastro-oesophageal tumours and 1 had a gastric primary tumour (Table 20).

Table 20. Summary of samples available for immunohistochemical analysis (oesophageal unless otherwise indicated)

Decitabine dose (mg/m^2)	**Normal tissue pre-treatment**	**Normal tissue post treatment**	**Tumour pre-treatment**	**Tumour post-treatment**
7.5	3*	2*	1	3*
10	4	2	4	1
12.5	3**	2**	3**	1

*includes 1 gastro-oesophageal and 1 gastric biopsy

**includes 1 gastro-oesophageal biopsy

Evaluation of the primary oesophageal cancer and adjacent normal mucosa slides stained with each of the 5 antibodies: e-cadherin, MGMT, $p14^{ARF}$, $p16^{INK4A}$ and hMLH1 are summarised in Figure 17 - Figure 26.

The cellular distribution of each of the antibodies was variable: e-cadherin localised to cellular membranes, MGMT was nuclear and cytoplasmic, $p14^{ARF}$ and $p16^{INK4A}$ were primarily cytoplasmic and hMLH1 was nuclear.

Baseline normal oesophageal biopsies demonstrate low intensity and localised expression of $p16^{INK4A}$, hMLH1 and $p14^{ARF}$ and higher levels of expression of MGMT and e-cadherin. Higher expression of all of the antibodies, except e-cadherin was seen in the oesophageal tumour biopsies compared to normal oesophageal mucosa at baseline.

Figure 17. Intensity and distribution of e-cadherin staining in oesophageal mucosa before and after decitabine treatment

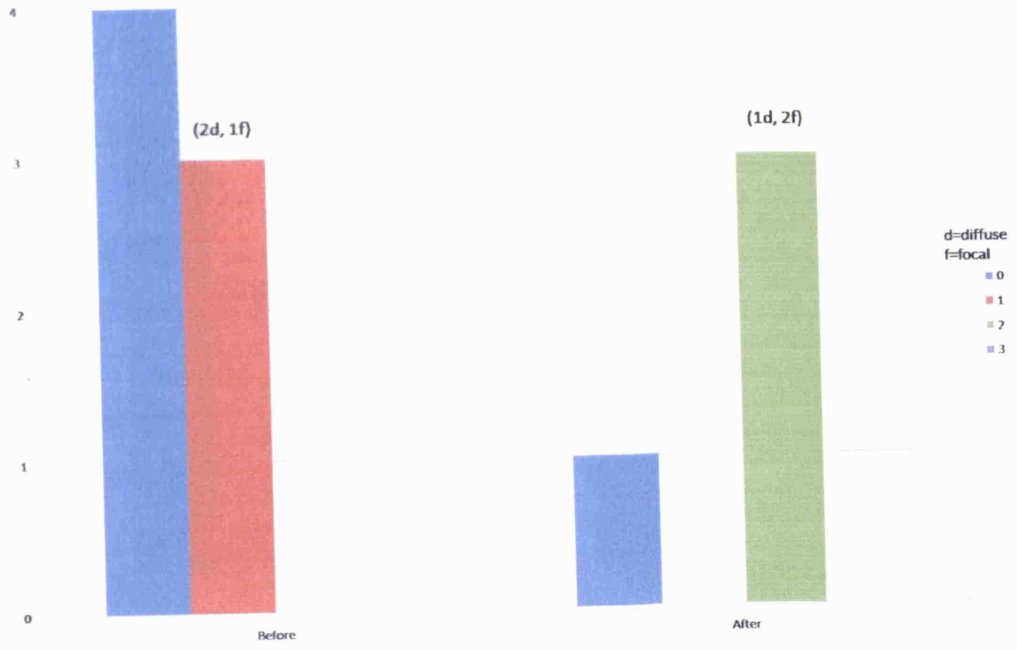


Figure 18. Intensity and distribution of e-cadherin staining in oesophageal adenocarcinoma before and after decitabine treatment

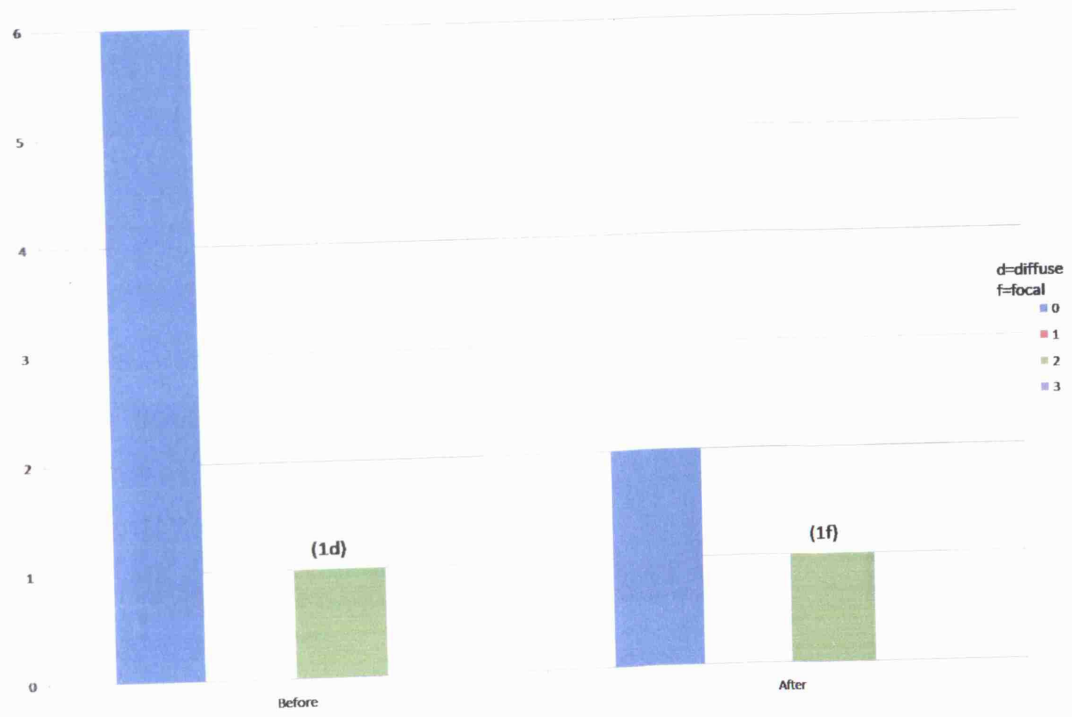


Figure 19. Intensity and distribution of MGMT staining in oesophageal mucosa before and after decitabine treatment

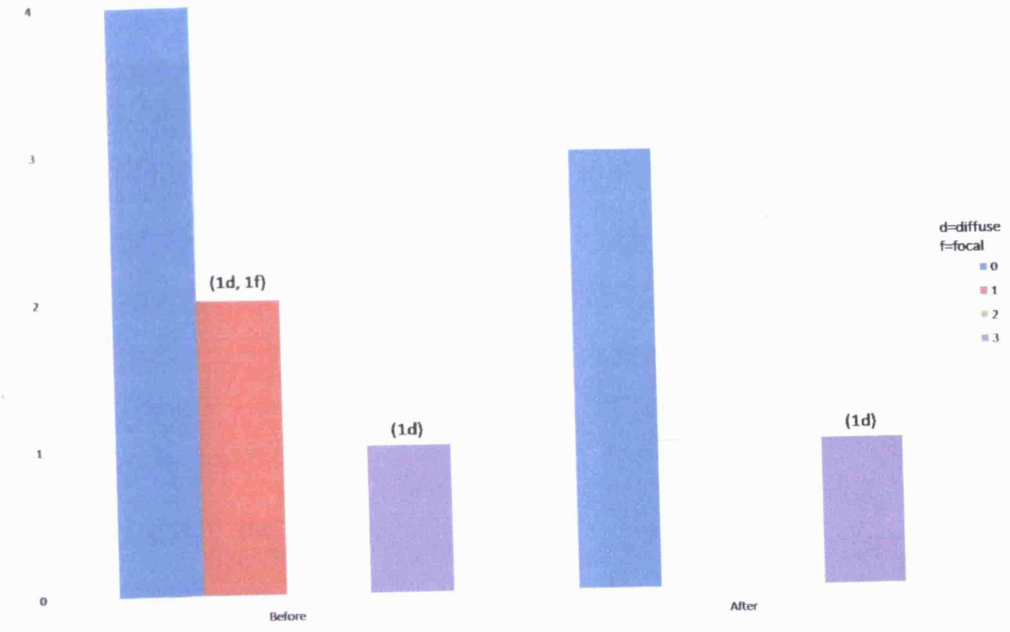


Figure 20. Intensity and distribution of MGMT staining in oesophageal adenocarcinoma before and after decitabine treatment

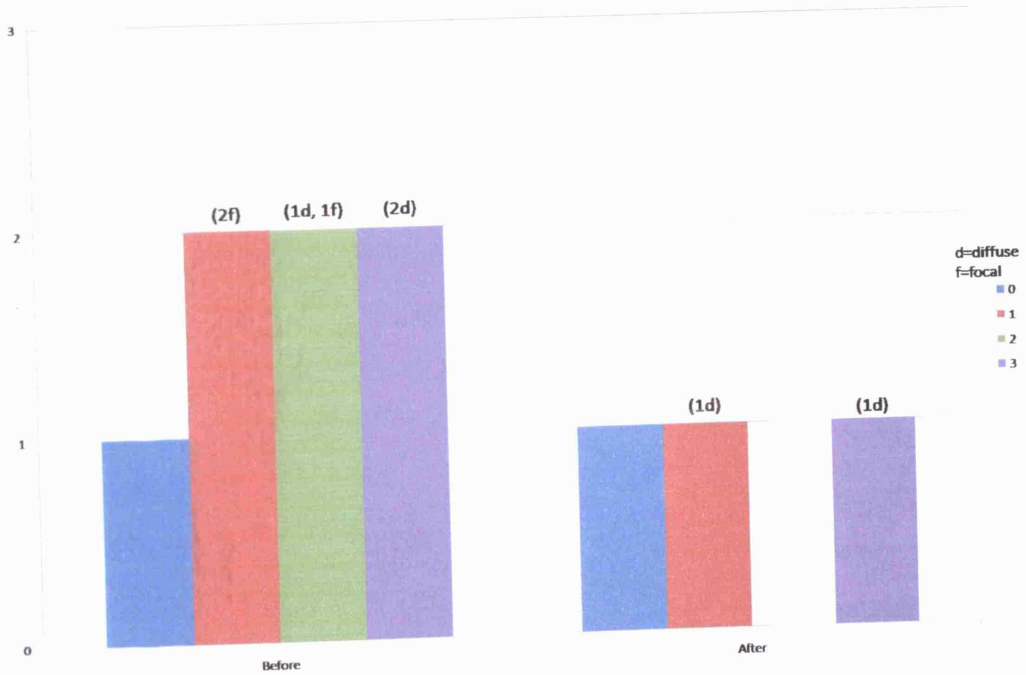


Figure 21. Intensity and distribution of $p14^{ARF}$ staining in oesophageal mucosa before and after decitabine treatment

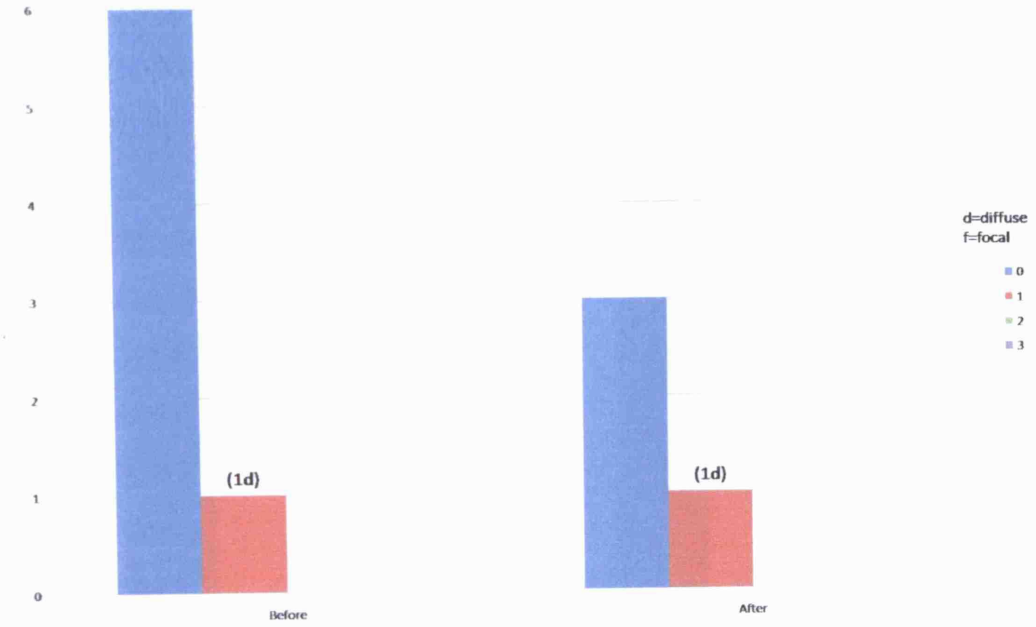


Figure 22. Intensity and distribution of $p14^{ARF}$ staining in oesophageal adenocarcinoma before and after decitabine treatment

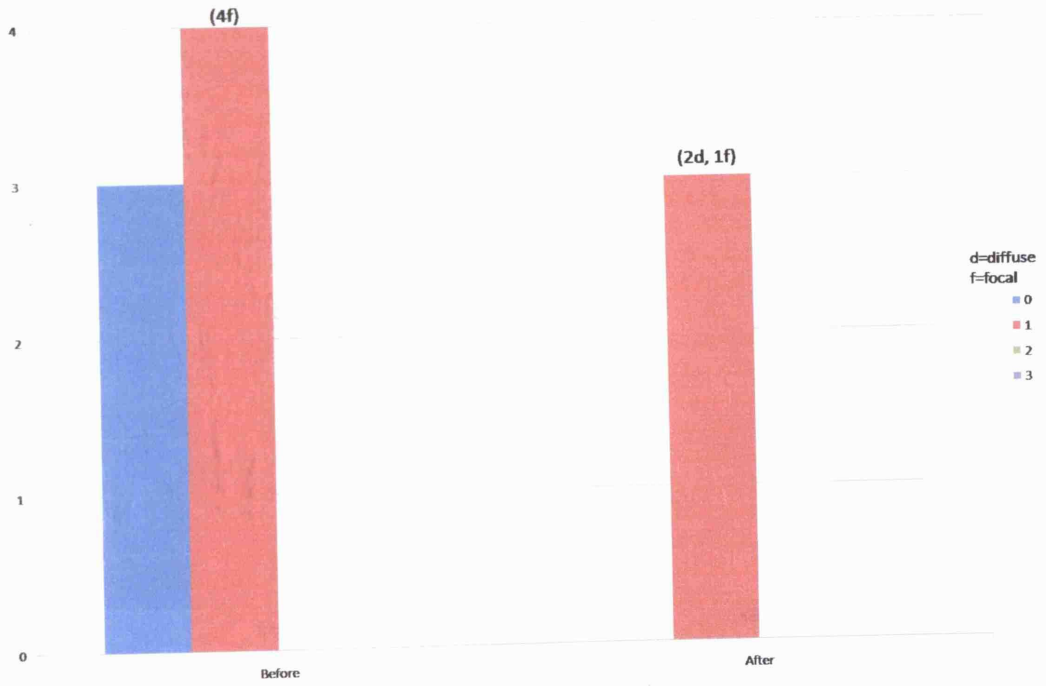


Figure 23. Intensity and distribution of $p16^{INK4A}$ staining in oesophageal mucosa before and after decitabine treatment

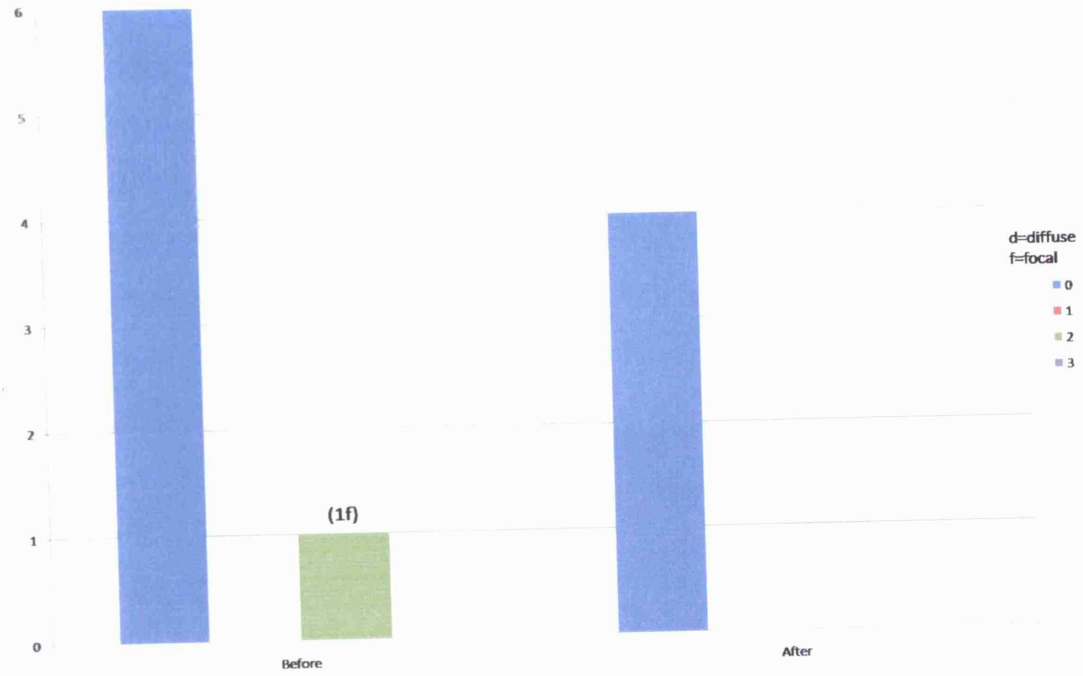


Figure 24. Intensity and distribution of $p16^{INK4A}$ staining in oesophageal adenocarcinoma before and after decitabine treatment

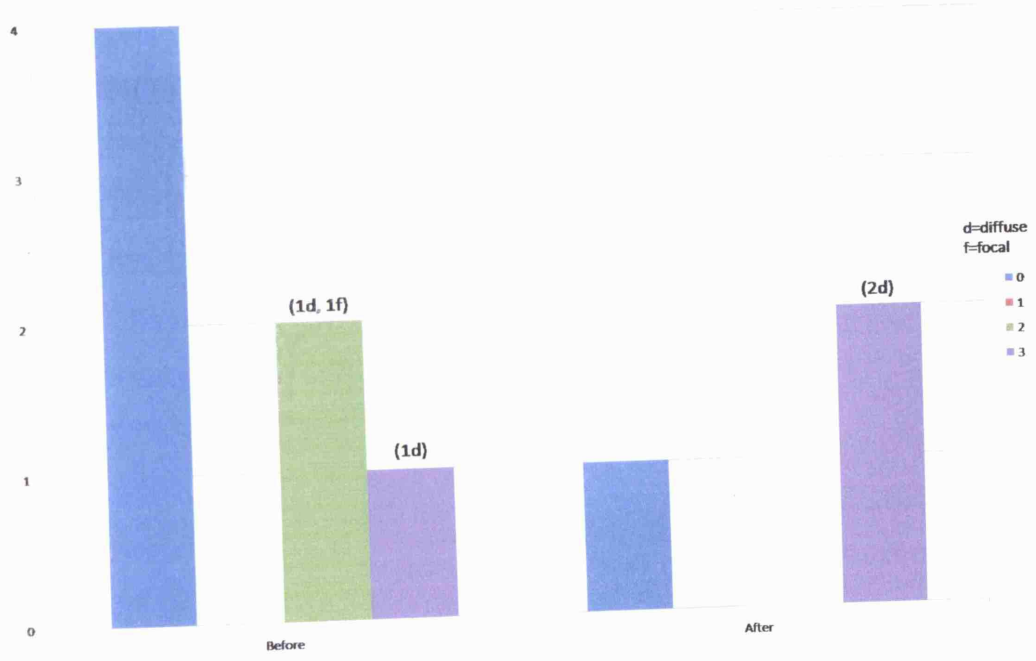


Figure 25. Intensity and distribution of hMLH1 staining in oesophageal mucosa before and after decitabine treatment

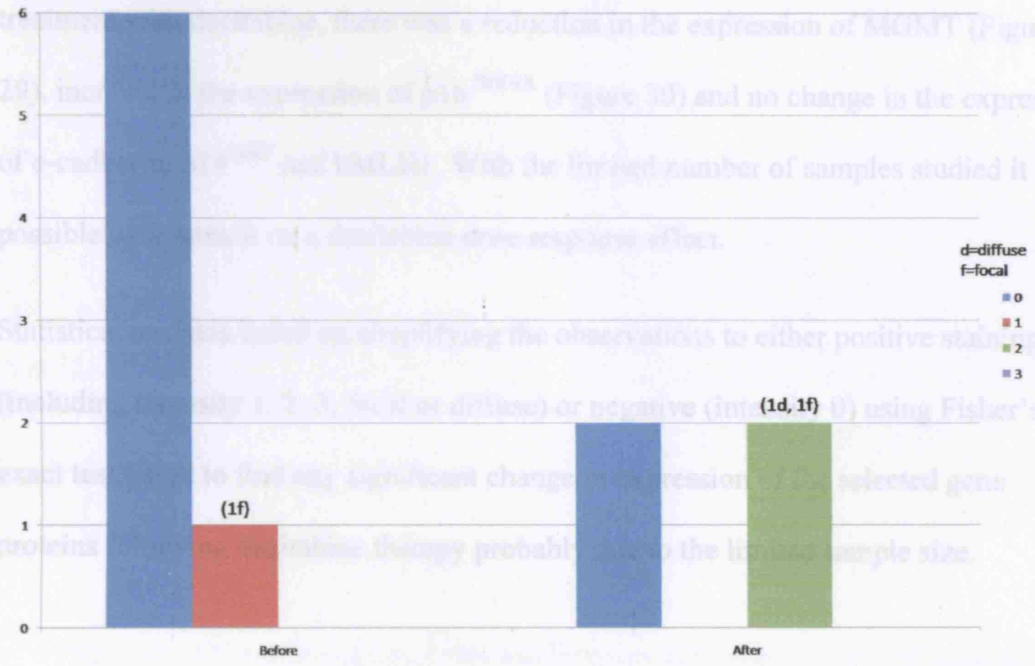
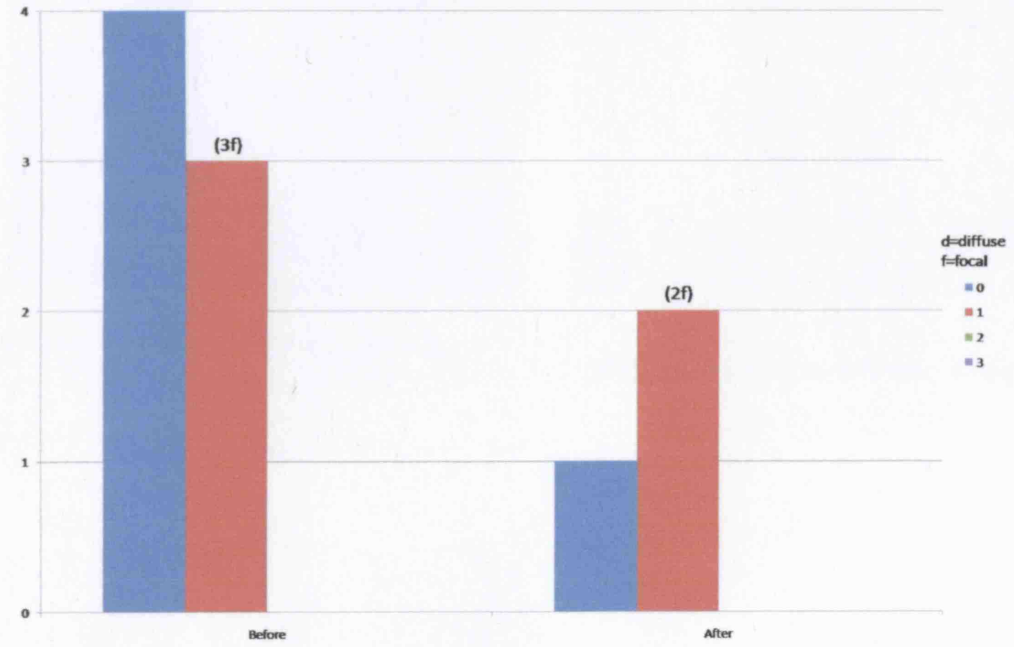


Figure 26. Intensity and distribution of hMLH1 staining in oesophageal adenocarcinoma before and after decitabine treatment



In oesophageal mucosa following treatment with decitabine there appears to be a reduction in the expression of MGMT (Figure 27), an increase in the expression of hMLH1 and e-cadherin (Figure 28) and no change in the expression of p14 ^{ARF} and p16 ^{INK4A}. On review of the oesophageal adenocarcinoma biopsies before and after treatment with decitabine, there was a reduction in the expression of MGMT (Figure 29), increase in the expression of p16 ^{INK4A} (Figure 30) and no change in the expression of e-cadherin, p14 ^{ARF} and hMLH1. With the limited number of samples studied it is not possible to comment on a decitabine dose response effect.

Statistical analysis based on simplifying the observations to either positive staining (including intensity 1, 2, 3; focal or diffuse) or negative (intensity 0) using Fisher's exact test failed to find any significant change in expression of the selected gene proteins following decitabine therapy probably due to the limited sample size.

Figure 27. Paired oesophageal mucosa biopsies before and after treatment with decitabine stained for MGMT (magnification x40)

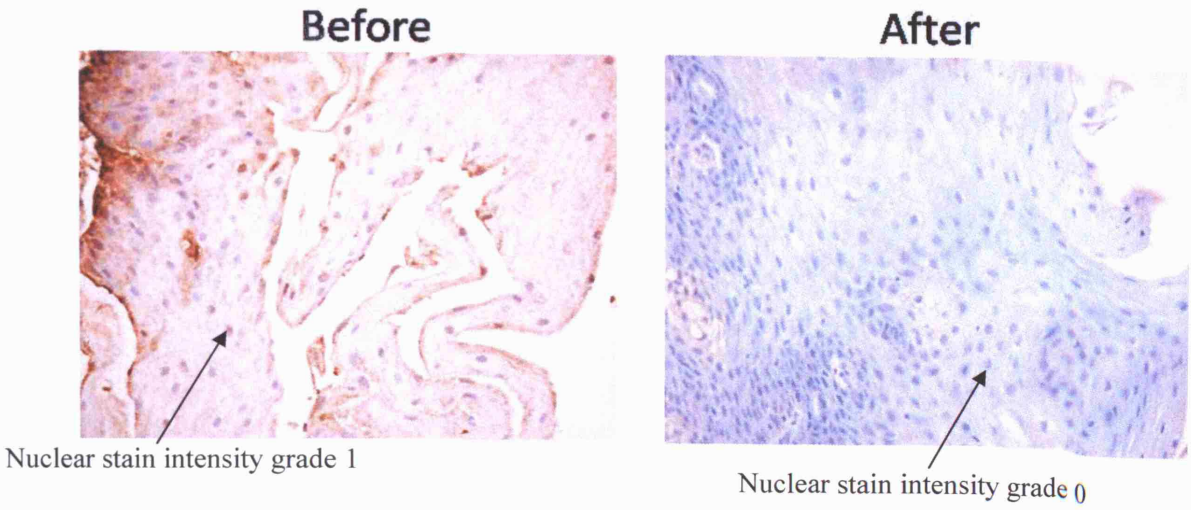


Figure 28. Paired oesophageal mucosa biopsies before and after treatment with decitabine stained for e-cadherin (magnification x40)

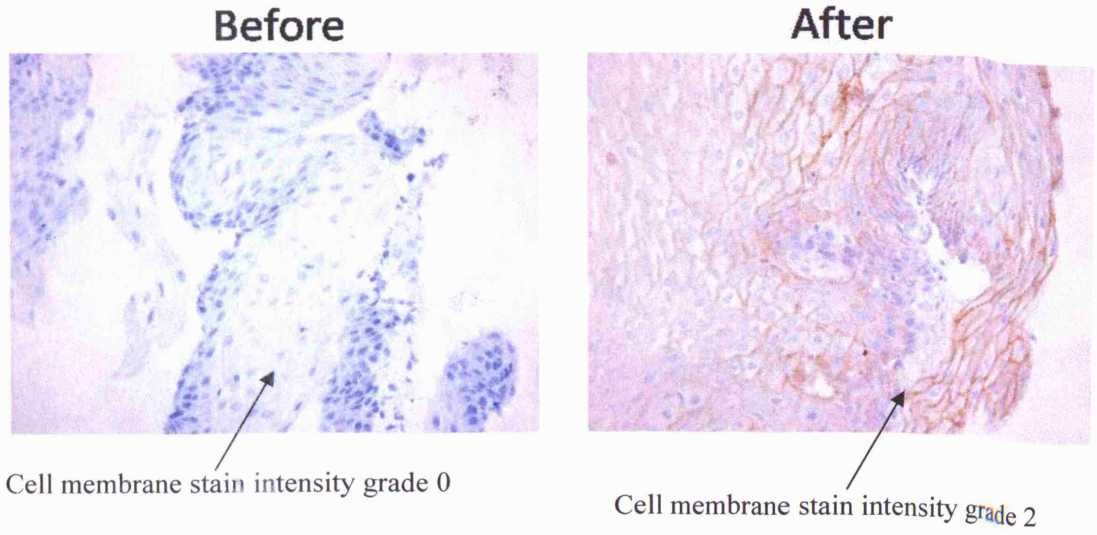


Figure 29. Paired oesophageal adenocarcinoma biopsies before and after treatment with decitabine stained for MGMT (magnification x40)

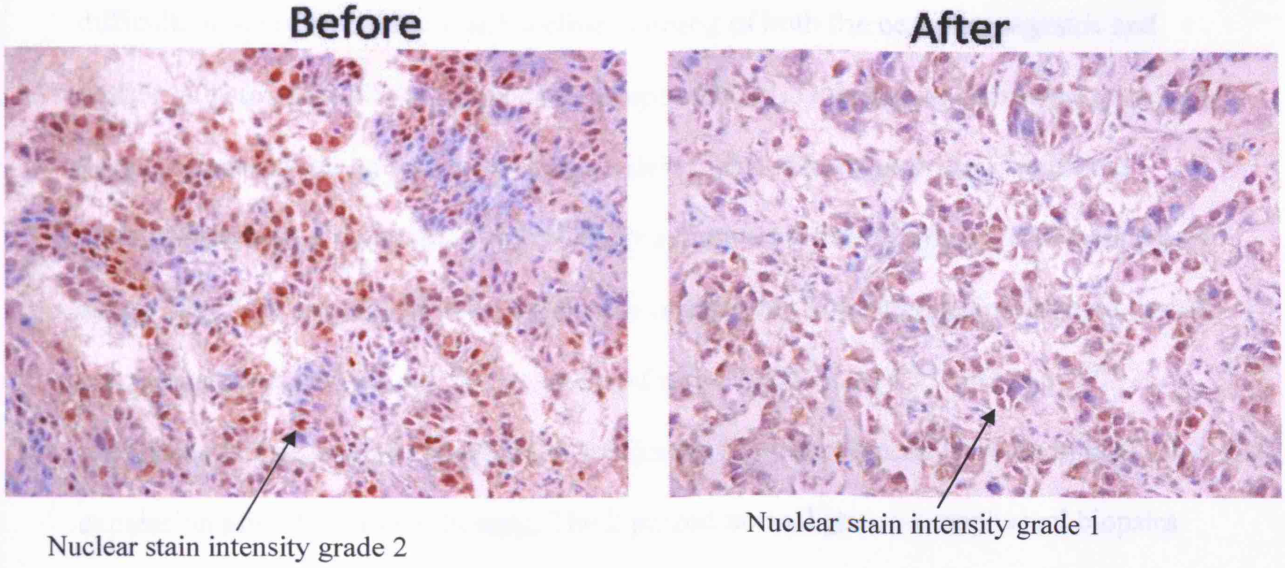
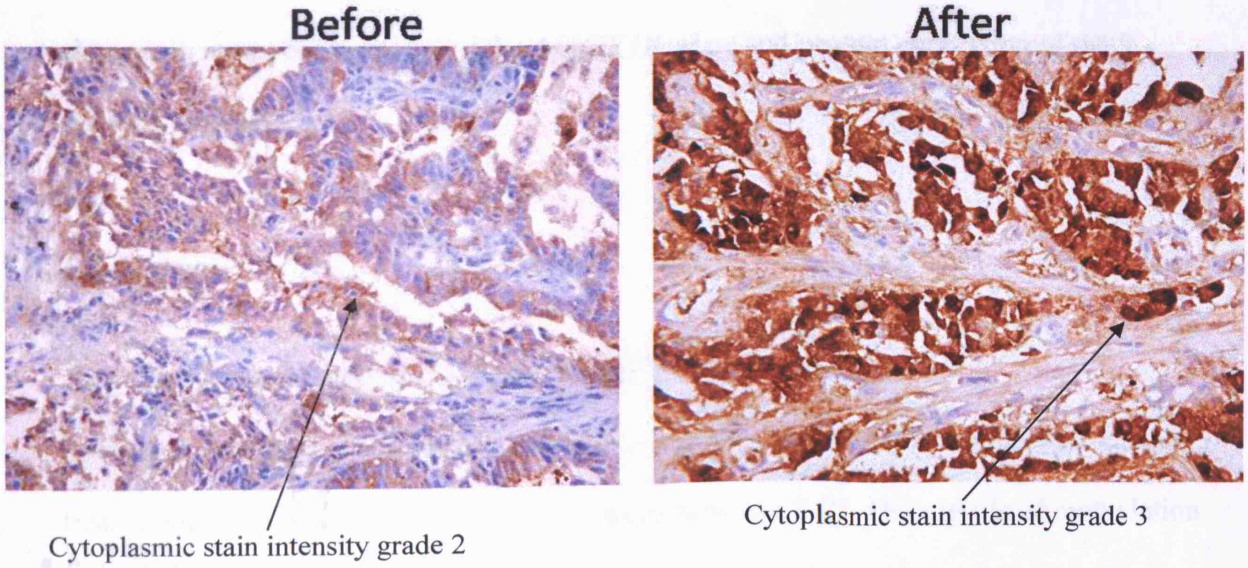


Figure 30. Paired oesophageal adenocarcinoma biopsies before and after treatment with decitabine stained for $p16^{ARF}$ (magnification x40)



There were only 2 patients with gastro-oesophageal cancers and 1 patient with a gastric cancer in this cohort with some missing samples making any analysis of results difficult. In summary, there was baseline staining of both the oesophago-gastric and gastric biopsies with all the antibodies except hMLH1. Only a post-decitabine treatment gastric adenocarcinoma specimen was available; this stained positively for all the antibodies except e-cadherin. Unfortunately no paired tumour samples were available to assess the effect on tumour before and after decitabine. The single paired normal gastric mucosa samples showed no change in e-cadherin, MGMT, $p14^{ARF}$ and $p16^{INK4A}$ expression before and after decitabine treatment, but there was an increase in hMLH1 expression after decitabine therapy. The 2 paired normal gastro-oesophageal biopsies showed no change in hMLH1, e-cadherin and $p14^{ARF}$ expression after decitabine treatment, but there was a reduction in MGMT and $p16^{INK4A}$ expression.

Three patients in the current data set had methylation analysis results as well as immunohistochemical analysis of their biopsies. No clear relationship was seen between changes in methylation status of the *MAGE1A* gene and protein expression of the 5 selected gene loci.

6.5 Discussion

This chapter presents the preliminary results for the methylation and immunohistochemical analysis of the first few patients with gastro-oesophageal cancer treated with combination treatment with decitabine and ECF. High levels of methylation of the *MAGE1A* gene promoter have been demonstrated in oesophageal and gastric mucosa and in PBCs, however methylation of the *MAGE1A* promoter in oesophago-gastric tumours was found be variable. Over 70 % of tumour biopsies (5/7 samples)

demonstrated hypomethylation of the *MAGE1A* promoter which is in keeping with previous data (De Smet et al., 2004; Honda *et al.*, 2004).

In this study, decitabine appeared to have little effect on the methylation status of *MAGE1A* in oesophagogastric mucosa and PBCs but a more marked effect on tumour samples was seen. This is in contrast to the recently reported Appleton et al study, which found lower *MAGE1A* promoter demethylation in tumour samples compared to PBCs (Appleton et al., 2007). This may be due to the rate of cellular proliferation in our tumour samples as a higher rate of cellular replication may increase the effect of decitabine. Paradoxically, hypermethylation was seen in 2 tumour samples following decitabine therapy, both with initially low levels of *MAGE1A* promoter methylation, indicating that other epigenetic mechanisms may be active influencing the overall methylation status of the tumour. Alternatively this could be an effect of sampling as methylation status may not be uniform across a tumour.

From the results to date, no dose effect on *MAGE1A* promoter demethylation has been demonstrated with the doses (10 and 12.5 mg/m^2) and schedule of decitabine used, however these doses are lower than those used in previous studies in solid tumours in which dose-dependent demethylation has been seen (Appleton et al., 2007) and it may be that this will be observed as the dose of decitabine is escalated. There did not appear to be an association between tumour *MAGE1A* promoter hypomethylation and response to treatment within our limited sample set. Interestingly, both tumour samples in which there was an increase in methylation after treatment with decitabine had a PR as best tumour response.

It is difficult to draw firm conclusions on the effect of methylation on gene expression at the five loci studied due to the limited numbers of histopathology specimens collected so far. The expression of p16 ^{INK4A}, hMLH1, e-cadherin, p14 ^{ARF} and MGMT appeared to be different in normal oesophageal mucosa compared to oesophageal adenocarcinoma as previously reported. Unexpectedly, higher levels of staining with all the antibodies except e-cadherin were seen in the oesophageal tumour biopsies compared to adjacent oesophageal mucosa at baseline. Aberrant hypermethylation has been detected in up to 55 % of oesophageal tissue adjacent to cancers that is not found in normal oesophageal tissue in cancer free individuals or in oesophageal tissue adjacent to tumours with no hypermethylation, suggesting a 'field methylation' effect consistent with field cancerisation. This may be responsible for the lower levels of protein expression seen in normal mucosa compared with tumour (Zhang et al., 2003). E-cadherin expression is reduced in the majority of oesophageal cancers (Bongiorno *et al.*, 1995).

Following decitabine therapy there was a change in protein expression in both normal oesophageal mucosa and oesophageal adenocarcinoma with an increase in the expression of some gene products, for example e-cadherin in oesophageal mucosa, and reduction in the expression of others, for example MGMT in both oesophageal mucosa and adenocarcinoma. In normal gastric mucosa and gastro-oesophageal samples before and after decitabine treatment, there was no change in protein expression for most of the gene loci examined including e-cadherin and p14 ^{ARF}, but there was an increase in hMLH1 expression after decitabine therapy in gastric mucosa and a reduction in MGMT and p16 ^{INK4A} expression in gastro-oesophageal biopsies. An increase in protein expression following therapy could be associated with a reduction in gene promoter methylation by decitabine. However, there are large inter-individual variations in the

expression of gene proteins independent of promoter methylation status, making any conclusions impossible at this stage.

In this chapter we have presented the preliminary data set of changes in methylation of the *MAGE1A* promoter gene locus and the associated effects on gene products including MGMT, p14 ^{ARF}, p16 ^{INK4A}, hMLH1 and e-cadherin assessed by immunohistochemistry following treatment with decitabine. Due to the limited results available to date it has been difficult to draw firm conclusions and no clear relationship was seen between changes in methylation status of the *MAGE1A* gene and protein expression of the 5 selected gene loci. However, methylation analysis of the *MAGE1A* gene and immunohistochemical analysis of gene product expression of the remaining samples is ongoing. Further analysis is also planned to look at the methylation status of the promoters of the 5 selected gene loci, *p16* ^{INK4A}, *p14* ^{ARF}, *hMLH1*, *MGMT* and *CDH1* (e-cadherin). Once a full set of biopsy samples are available we intend to use microarray technology to assess the methylation profiles of both tumours and adjacent tissue before and after decitabine therapy. It is hoped that with the results of the remaining specimens and this further analysis a more complete picture of the action of decitabine alongside standard chemotherapy in gastro-oesophageal cancer will emerge.

7. Future directions

The work presented in this thesis shows that a number of different approaches may be used to target DNA repair in an effort to improve the efficacy of conventional chemotherapy. Lomeguatrib was found to deplete MGMT in tumour biopsies in a variety of solid tumours with variable sensitivity to O^6-alkylating agents including prostate, primary CNS and colorectal cancers at doses of 120-160 mg.

Combination treatment with irinotecan and lomeguatrib in patients with metastatic colorectal cancer was tolerable in this group of heavily pre-treated patients. Inactivation of MGMT with lomeguatrib was achieved with no obvious interaction with irinotecan.

Epigenetic modification through the use of a DNMTI, decitabine, in combination with standard ECF chemotherapy in the treatment of gastro-oesophageal cancer was investigated and this study is still ongoing. The maximum tolerated dose of decitabine with this combination of treatment was established at 15 mg/m^2. Methylation changes in the primary tumour were confirmed using pyrosequencing following decitabine treatment, and associated changes in protein expression of a number of gene loci important in maintaining cellular integrity are under investigation.

7.1 Lomeguatrib

Based on the results from this thesis on the inactivation of MGMT following lomeguatrib administration in primary and secondary colorectal cancer, combination treatment with lomeguatrib and irinotecan and previously published work on therapy with lomeguatrib and temozolomide in advanced colorectal cancer (Khan et al., 2008), a

further study exploring the role of lomeguatrib with temozolomide and irinotecan in patients with this malignancy would be the next step. Patients with advanced colorectal cancer might receive lomeguatrib 120 mg a day for 5 days with escalating doses of temozolomide from 50-125 mg/m^2 daily followed by irinotecan 125-250 mg/m^2 also on day 5. Pharmacokinetic data would be collected to confirm no drug interactions were taking place and pharmacodynamic evaluation from tissue biopsies would be required to confirm the recruitment of topoisomerase I following alkylating agent damage, as has been shown in pre-clinical models. The maximum tolerated dose of temozolomide and irinotecan within this triplet would be determined and the toxicity and efficacy established. Given the overlapping myelosuppressive profiles of temozolomide and irinotecan special attention should be given to this aspect of patient care.

Similar studies could be considered in other malignancies in which temozolomide is known to have activity including melanoma and glioblastoma multiforme. A phase I trial of combination treatment with lomeguatrib and temozolomide in metastatic melanoma has been reported by Ranson et al., as previously discussed (Ranson et al., 2007). Although there is little data on the activity of i.v. irinotecan in melanoma, there is some phase I evidence that the oral formulation of the drug has activity in melanoma (Dumez *et al.*, 2006) and therefore therapy with the triplet combination of lomeguatrib, temozolomide and irinotecan may be considered.

Combination therapy with lomeguatrib and temozolomide in primary brain tumours may also be of interest based on data from this thesis which confirms MGMT depletion following lomeguatrib administration, the known efficacy of temozolomide in this setting and on the observation that treatment with temozolomide, in combination with radiotherapy produces no improvement in survival in patients in the absence of

methylation of the *MGMT* gene (Hegi et al., 2005). An early phase study of O^6-BG in combination with temozolomide and irinotecan is currently on going, a similar study replacing O^6-BG with lomeguatrib would also be of interest.

The high levels of MGMT expressed in prostate cancer and the inactivation of MGMT seen following lomeguatrib therapy in this thesis indicate a therapeutic window which could be exploited. Although phase II data has established that temozolomide has little activity in metastatic prostate cancer, other alkylating agents for example, estramustine, a chemotherapeutic composed of estradiol and nornitrogen mustard, are known to have activity in this patient group and therefore this may be a potential avenue to investigate.

7.2 Epigenetic therapy

Future work arising from the results from this thesis may include adding decitabine to newer combinations of treatment for example oxaliplatin, epirubicin and capecitabine in the treatment of upper gastrointestinal cancer as the standard treatment for this malignancy evolves. The evolution of second generation demethylating agents such as S110, which have improved biological properties needs assessment in the early phase setting as is ongoing, with a view to combining it with standard chemotherapy in the future. With the current exploration of the field of epigenetics, it has become apparent that a holistic approach to reversing epigenetic changes is required combining for example HDACis and DNMTIs with conventional treatment. Combination treatment appears to be schedule-dependent resulting in synergism only when the DNMTI is administered prior to the HDACi. Early phase studies looking at combination therapy are currently ongoing and preliminary results in solid tumours have been promising (Candelaria *et al.*, 2007).

7.3 Individualised chemotherapy

Small advances have been made in individualising standard chemotherapy based on an individual's tumour characteristics including: administering trastuzumab in breast cancers with high expression or amplification of the HER-2 receptor, using epidermal growth factor receptor (EGFR) inhibitors such as erlotinib and gefitinib in non-small cell lung cancers (NSCLC) with EGFR mutations or amplifications, giving adjuvant cisplatin chemotherapy in resected NSCLC tumours not over-expressing ERCC1 and using concurrent temozolomide with radiotherapy in the treatment of glioblastomas demonstrating methylation of *MGMT*. Molecular-targeted treatments are also being individualised. *K-ras* mutations have been shown to determine resistance to cetuximab and panitumumab in colorectal cancer and may also be associated with the absence of benefit with EGFR inhibitors in NSCLC.

Despite this progress, empirically administered chemotherapy alongside radiotherapy and surgery remains the mainstay of treatment for most cancers. The challenge presenting itself to us is the integration of novel targeted therapies with existing standard regimens. This can only be achieved by well conducted early phase clinical studies as presented here with biological endpoints assessing pharmacodynamic efficacy and kinetics of the drugs with a view to optimising the design of definitive randomised phase III trials.

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