

# **THIOGUANINE TOXICITY**

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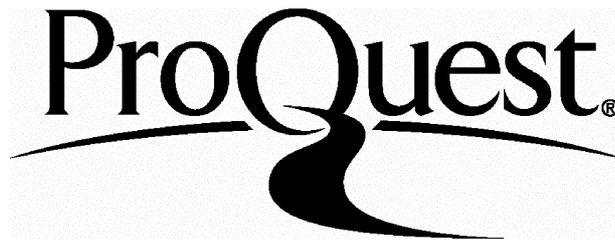
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**This work is dedicated to my mother  
and to the memory of my father**

## ABSTRACT

The toxic action of the anticancer drug thioguanine is believed to involve incorporation into DNA, methylation by S-adenosylmethionine followed by binding of the resulting S<sup>6</sup>-methylthioguanine residues by proteins of the post-replicative mismatch repair pathway. S-adenosylmethionine is a very weak methylating agent and methylates only a fraction of the thioguanine residues in DNA. If the proposed mechanism of toxicity were correct, a more powerful methylating agent would be expected to increase the cytotoxicity of thioguanine. The work in the first part of this thesis aimed to determine whether cytotoxic synergy between thioguanine and methylating agents could be demonstrated.

Using nude mouse xenografts models for glioblastoma and for melanoma, synergy between thioguanine and the clinically used methylating agent temozolomide was demonstrated. This synergy between thioguanine and temozolomide could not be reproduced in tissue culture, however there was synergy between thioguanine and the methylating agent methyl methanesulphonate. Investigation suggested that the difference between the cytotoxicity of these two methylating agents when given after thioguanine is a reflection of differences in their chemistry. It was found that methyl methanesulphonate which acts by an S<sub>N</sub>2 mechanism reacts much more with the 6-S of thioguanine residues in DNA than do methylating agents which, like temozolomide react by an S<sub>N</sub>1 mechanism.

It is known that treatment of cells with compounds giving O<sup>6</sup>-methylguanine in DNA can produce cells that are deficient in post-replicative mismatch repair therefore it is possible that thioguanine could also produce deficient cells. Production of MMR deficient cells might contribute to the high incidence of skin cancer and leukaemia associated with thioguanine. Cells in tissue culture were treated with non toxic concentrations of thioguanine followed by selection of post-replicative mismatch repair deficient cells. Some of the deficient cells were found to lack the hMLH1 protein of the post-replicative mismatch repair system. The absence was not due to a mutation in the gene but because of silencing of the promoter region, a common feature of sporadic colorectal cancers.

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## ABBREVIATIONS

|                    |   |
|--------------------|---|
| DTT                | Dithiothreitol  |
| EDTA               | Ethylaminediaminotetraacetic acid   |
| IDL                | Insertion/deletion loop   |
| HGPRT              | Hypoxanthine guanine phosphoribosyl transferase   |
| MMS                | Methyl Methanesulphonate  |
| MNU                | N-methyl-N-nitrosourea  |
| MTS                | 5-(3-methyltriazen-1-yl)imidazole-4-carboxamide   |
| MTS                | 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfopheyl)-2H-tetrazolium, inner salt |
| O <sup>6</sup> meG | O <sup>6</sup> methylguanine  |
| PCR                | Polymerase Chain Reaction   |
| RT-PCR             | Reverse Transcription followed by PCR   |
| TAE                | Tris Acetate EDTA buffer  |
| TE                 | Tris-EDTA buffer  |
| TEMED              | N, N, N', N'-Tetramethylethylenediamine   |
| TG                 | 6-thioguanine   |
| TWEEN 20           | Polyoxyethylenesorbitan monolaurate   |

# **Chapter 1**

## **General Introduction**

## Chapter 1 Introduction

### Overview of thesis

The work in this thesis arose from a proposal from our laboratory that the toxicity of the anti-leukemic drug thioguanine involves the chemical methylation of thioguanine residues in DNA by S-adenosylmethionine followed by recognition of the resultant S<sup>6</sup>-methylthioguanosine by proteins of the post-replicative mismatch repair system (Swann *et al.*, 1996). S-adenosylmethionine is a weak methylating agent reviewed in (Chiang *et al.*, 1996) and methylates only a small proportion of the 6-thioguanine residues in DNA. A more powerful methylating agent would be expected to methylate many more of these thioguanine residues and if the proposed mechanism for the cytotoxicity of thioguanine is correct, would be synergistic with thioguanine in cytotoxicity. If synergism between methylating agents and thioguanine could be demonstrated it would provide support for the proposal and might also be of clinical significance by showing the clinically used methylating agents such as procarbazine, dacarbazine, streptozotocin and temozolomide would be more effective when given with thioguanine.

Before this laboratory began work on thioguanine toxicity, it had been shown that some cells in tissue culture are resistant to both thioguanine and methylating N-nitroso compounds such as N-methyl-N-nitrosourea (Roberts *et al.*, 1971). It was known that N-methyl-N-nitrosourea is cytotoxic because it methylates the 6-oxygen of guanine residues in DNA. The discovery that the 6-sulphur of thioguanine residues in DNA are methylated by S-adenosylmethionine established a formal similarity between the mechanism of the two chemicals (reviewed in Karran and Bignami, 1996). The cells resistant to N-methyl-N-nitrosourea lack one of the proteins of the post-replicative mismatch repair system (Kat *et al.*, 1993). It

was first shown by Karran (Karran and Bignami, 1992), and subsequently confirmed by Brown (Brown *et al.*, 1993) that treatment with compounds giving O<sup>6</sup>-methylguanine in DNA produce, or select for, cells that lack proteins of the post-replicative mismatch system. There is now a consensus that cancer is caused by the accumulation of mutations in a single cell in a number of genes (Fearon and Vogelstein, 1993) and that those mutations have to be specific, for example the mutations which convert ras to its oncogenic form are almost all in codons 12, 13 and 61. Loeb (Loeb, 1991) has suggested that for this number of mutations to happen the cell must develop a 'mutator phenotype'. When Karran found that exposure to N-methyl-N-nitrosourea would produce mismatch repair deficient cells (Karran and Bignami, 1992) he made the interesting proposal that the small but continuous exposure to the N-nitrosamine which all humans have might contribute to cancer by producing *in vivo*, as it does *in vitro*, mismatch repair defective cells which would have this mutator phenotype. This hypothesis cannot be tested in humans because it is impossible to find a control group with no nitrosamine exposure. The similarity between the proposed mechanism of thioguanine toxicity and the mechanism of nitrosamine toxicity suggests that treatment with thioguanine may also lead to the production of mismatch repair defective cells. Treatment with thioguanine is known to be associated with skin cancer and leukemia. If it could be shown that thioguanine treatment produced or selected for mismatch repair deficient cells and that these cells played some role in the aetiology of the thioguanine associated cancer then it should show that the Karran hypothesis is at least plausible. Thus the work in this thesis attempts to answer two questions: is there cytotoxic synergy between thioguanine and methylating agents; and does prolonged treatment with thioguanine lead to the production of cancer through the production of mismatch repair deficient cells.

## Historic perspective; O<sup>6</sup>-methylguanine, mutation and cytotoxicity

The research on the cytotoxic mechanism of thioguanine in our laboratory originated from interest in the carcinogenic and cytotoxic nitrosamines. The nitrosamines first became the subject of much research after the discovery of their carcinogenic action by Magee and Barnes in 1956. They showed that administration of *N*-nitrosodimethylamine to rats induced liver tumours. This work was closely followed by Druckrey *et al* who showed that a great number of *N*-nitroso compounds are carcinogenic and that organ susceptibility to particular nitrosamines is dependent on the dose and structure of the nitrosamine (reviewed in Druckrey *et al.*, 1967). The carcinogenicity of the nitrosamines was first linked to alkylation of organ macromolecules by Magee when he showed that administration of <sup>14</sup>C-labelled *N*-nitrosodimethylamine to rats resulted in methylation of nucleic acids (Magee and Farber, 1962) and proteins (Magee and Hutchins 1962). This was followed by work that showed that administration of other *N*-nitroso compounds also results in the alkylation of nucleic acids (Magee and Lee, 1963). The main product of nucleic acid methylation is 7-methylguanine and it was at first believed that alkylation at this position was the cause of the carcinogenicity of *N*-nitrosodimethylamine. However it was shown that there was no correlation with the level of 7-methylguanine and tumour incidence (Swann and Magee, 1968). Instead, it was realised that the relatively minor DNA lesion, O<sup>6</sup>-alkylguanine correlated strongly with carcinogenesis (Loveless, 1969).

Alkylating agents react through mechanisms which are described as S<sub>N</sub>1 and S<sub>N</sub>2 which alkylate nucleic acid via an alkylcarbonium ion. The nitrosamines are S<sub>N</sub>1-type alkylating agents. The alkylation of nucleic acid occurs via a methylcarbonium ion (Wade *et al.*, 1987). Such agents will react about 100x more

readily with the O<sup>6</sup> atom of guanine than the S<sub>N</sub>2-type compounds (Beranek, 1990). Methyl methanesulphonate (MMS) is an example of a methylator which reacts predominantly through an S<sub>N</sub>2 mechanism. Table 1.1 shows the pattern of methylation of DNA by N-methyl-N-nitrosourea and by methyl methanesulphonate. The table shows that while the methylation of the 6-O of guanine by N-methyl-N-nitrosourea is significant (about 6-8% of the total methylation) the methylation of this position by methyl methanesulphonate is insignificant (about 0.3% of the total).

**Table 1.1** The methylation patterns of DNA produced by the  $S_N2$  methylator methyl methane sulphonate (MMS) and the  $S_N1$  methylator N-methyl-N-nitrosourea (MNU) expressed as percent of total alkylation taken from (Beranek, 1990)

\*Note that only MNU methylates the oxygens of the bases and the oxygen of the linking phosphate groups

|                        | Proportion of total alkylation |            |
|------------------------|--------------------------------|------------|
| Site of alkylation     | MMS                            | MNU        |
| <b>Adenine</b>         |                                |            |
| N1                     | 1.9-3.8                        | 0.7-1.3    |
| N3                     | 10.4-11.3                      | 8.0-9.0    |
| N6                     | 0                              | 0          |
| N7                     | 1.8                            | 0.8-2.0    |
| <b>Cytosine</b>        |                                |            |
| O2                     | 0                              | 0.1        |
| N3                     | <1.0                           | 0.06-0.6   |
| <b>Guanine</b>         |                                |            |
| N1                     | 0                              | 0          |
| N3                     | 0.6                            | 0.6-1.9    |
| O6                     | 0.3                            | 5.9-8.2*   |
| N7                     | 81.0-83.0                      | 65.0-70.0  |
| <b>Thymidine</b>       |                                |            |
| O2                     | 0                              | 0.1-0.3*   |
| N3                     | 0.1                            | 0.1-0.3    |
| O4                     | 0                              | 0.1-0.7*   |
| Total phosphotriesters | 0.8                            | 12.0-17.0* |

O<sup>6</sup>-MeG is promutagenic because it is able to miscode during replication, a property shared by O<sup>4</sup>-alkylthymine (O<sup>4</sup>-MeT). O<sup>6</sup>-MeG causes G to A and O<sup>4</sup>-MeT causes T to C transition mutations. Initially, the general assumption was that this miscoding arises from the formation of stable base pairs between O<sup>6</sup>-MeG and T and O<sup>4</sup>-MeT and G (Leonard *et al.*, 1990). But other studies contradicted this view, indicating that O<sup>6</sup>alkyl-G·T and O<sup>4</sup>alkyl-T·G pairs are in fact less stable than their alkyl-G·C or alkyl-T·A non-mispaired counterparts (Swann, 1990). Kinetic analysis of the coding properties of O<sup>6</sup>-meG in DNA revealed that both T and C are incorporated opposite O<sup>6</sup>-MeG much slower than nucleotides forming regular A·T or G·C base pairs. The preferential incorporation of T was found to be due to discrimination at the formation of the phosphodiester bond between the 3'OH of the primer DNA and the  $\alpha$  phosphorous of the incoming dTTP or dCTP. The formation of the bond to the incoming dCTP was particularly slow (Tan *et al.*, 1994). Conversely other adducts, for example, 3-methyladenine do not cause mutations because they are able to block polymerases during replication (Boiteux *et al.*, 1984; Larson *et al.*, 1985).

The N-nitroso compounds are toxic as well as being mutagenic and carcinogenic. First evidence that the toxicity was caused by formation of O<sup>6</sup>-methylguanine in DNA came from studies in *E. coli*. In 1977 Samson and Cairns (Samson and Cairns, 1977) described a new repair pathway in *E. coli* that provides bacteria with protection against both the mutagenic and cytotoxic effects of MNNG. This they called the 'adaptive response' because it is strongly induced by prior administration of alkylating agent. The adaptive response involves the upregulation of several proteins, the most important of which is O<sup>6</sup>-alkylguanine-DNA-alkyltransferase. This is an inducible pathway controlling the *E. coli ada* gene and in response to

methylation agent treatment there is a several thousand-fold increase in the levels of a specific enzyme which demethylates O<sup>6</sup>meG residues. The alkyltransferase is the product of the *ada* gene itself and the C-terminal domain of the protein contains an activated cysteine residue. The alkyl groups are removed from O<sup>6</sup>G by the alkyltransferase and become covalently linked to the sulphur of this cysteine. The dealkylation reaction is irreversible and has a 1:1 stoichiometry. The rapid induction of the Ada protein during the adaptive response gives complete protection against the mutagenicity of methylating agents in *E. coli*. The N-terminal sequences of the Ada protein contain another activated cysteine that performs an additional function in which it receives a methyl group from an DNA methylphosphotriester. This methylation of the cysteine of the N-terminal domain converts the Ada protein into a transcriptional activator of its own gene and the other genes involved in the adaptive response. One of these genes, *AlkA*, encodes a DNA glycosylase which removes 3-methyladenine, 3-methylguanine and O<sup>2</sup>-methylated pyrimidines from DNA.

Similar methyltransferase activity was found in mammalian cells in 1981 (Bogden *et al.*, 1981). The liver has a greater concentration of O<sup>6</sup>-methylguanine-DNA methyltransferase (MGMT) than any other organ. The MGMT in the liver could be increased by chronic exposure to low concentrations of dimethylnitrosamine (Montesano *et al.*, 1980). However, the increase in liver MGMT was only about 3 fold compared with 1000 fold in bacteria (Cooper *et al.*, 1982). Subsequently, the mammalian MGMTs were found to be homologues of the C-terminal domain of the Ada protein: They lack any counterpart to the N-terminal domain of the *E. coli* MGMT and therefore have no activity towards methylphosphotriesters (Tano *et al.*, 1990) nor is a co-ordinated inducible adaptive response to DNA methylation damage a feature of mammalian cells.

Advances in understanding the role of O<sup>6</sup>-meG in the cytotoxicity of the methylating N-nitroso compounds also came from a completely different direction involving two independent studies. Day and Ziolkowski (1979) described a subset of brain tumour-derived cell lines in which a virus that had been treated with a methylating agent was unable to grow even though the same cells were able to support the growth of the undamaged virus or virus treated with UV irradiation. They called these cells Mer<sup>-</sup>. Sklar *et al* (Sklar and Strauss, 1981) independently described a human lymphoblastoid cell line in which removal of O<sup>6</sup>-MeG from DNA was defective and were consequently hypersensitive to killing by MNNG. They called these cells Mex<sup>-</sup>. It subsequently became apparent that the Mer<sup>-</sup> and Mex<sup>-</sup> cells were the same. The characteristic phenotype of these cells is a reduced, often undetectable level of MGMT expression and hypersensitivity to both the cytotoxic and mutagenic effects of methylating agents. This clear association between sensitivity towards methylating agents and the absence of MGMT activity suggested that O<sup>6</sup>meG could be a lethal DNA lesion in human cells. Unequivocal evidence that O<sup>6</sup>meG is responsible for the cytotoxicity of methylating agents came from several studies published around the same time showing that methylating agent sensitivity of Mer<sup>-</sup> cells could be reversed by expression of an intact *E. coli* Ada protein (Brennand and Margison, 1986a; 1986b; Kataoka *et al.*, 1986; Samson *et al.*, 1986).

In 1989 it was found that some Mer<sup>-</sup> cells are unexpectedly resistant to MNU (Aquilina *et al.*, 1989) and the resistance is not due to lack of methylation of DNA since such cells have the same level of methylation damage as the methylation sensitive parental line and O<sup>6</sup>-MeG is equally persistent in the DNA. This indicated that the presence of O<sup>6</sup>-MeG in DNA, although necessary, is not enough for cytotoxicity. Some other cellular process must be involved. Interestingly, these

cells were also found to be cross resistant to thioguanine. A previous observation suggesting a similarity in the cytotoxic mechanisms of thioguanine and methylating agents was made by Roberts *et al* (1971) who showed the cells treated with N-methyl-N-nitrosourea or with thioguanine die after the second round of replication. Clues as to the nature of the mechanism of cytotoxic resistance came in 1993 (Branch *et al.*, 1993) when it was discovered that Mer<sup>r</sup> MNU resistant cells were analogous to cells from patients with the disease hereditary non-polyposis coli colorectal cancer (HNPCC). These cells lack one of the proteins of the post-replicative mismatch repair pathway, usually hMSH2 or hMLH1. Cells from HNPCC patients have an increased level of mutation throughout the genome (referred to as a 'mutator phenotype') and instability in the length of the microsatellite repeats caused by a defect in the post-replicative mismatch repair machinery. Two of the methylation tolerant cell lines isolated had similar mutator phenotype and microsatellite instability. Further evidence of the analogy to HNPCC came from a third methylation tolerant cell line which was found to be defective in mismatch repair in an *in vitro* assay (Kat *et al.*, 1993). These resistant cells, analogues of HNPCC cells, lack some component of the post-replicative mismatch repair system. There is one very important difference between HNPCC cells and the resistant cells produced by exposure to low doses of N-methyl-N-nitrosourea. The HNPCC cells and the analogous cell lines for example LoVo and HCT116 which are resistant to N-methyl-N-nitrosourea have mutations in one of the genes coding for the proteins of the postreplicative mismatch repair system. But the acquired resistance that can be produced by exposing cells repeatedly to N-methyl-N-nitrosourea (Karran and Bignami, 1992) and cisplatin (Aebi *et al.*, 1996) is mainly caused by down-regulation of one of the repair genes, commonly *mlh1*.

The first explanation for the similarity between the cytotoxicity of methylating agents and thioguanine was advanced by Stephenson *et al* who showed that the proteins of the mismatch repair system bind to thioG·T in synthetic oligomers *in vitro* (Stephenson and Karran, 1989). The authors supposed that thioG·T mismatches would be produced by the misincorporation of T during DNA synthesis. However, it was shown that this explanation is unlikely to be correct since thioguanine does not appreciably miscode (Swann *et al.*, 1996). Rather, it was proposed that after incorporation into DNA, thioguanine is methylated by S-adenosylmethionine. The resultant S<sup>6</sup>-methylthioguanosine residues are able to pair with both cytosine and thymine. Base pairs containing S<sup>6</sup>-methylthioguanine residues in DNA were shown to be bound by the proteins of the post-replicative mismatch repair pathway (Swann *et al.*, 1996).

## **Hereditary non-polyposis colorectal cancer and post-replicative mismatch repair**

Because of the importance of the post-replicative mismatch repair system in the toxicity of thioguanine and in the experiments described in this thesis, it is necessary to describe the system in some detail. Much of what is understood about post-replicative mismatch repair in humans has come from studies on *E. coli* (reviewed by Modrich, 1987) and from the study of cells from patients with the disease hereditary non-polyposis colorectal cancer (HNPCC) (Kinzler and Vogelstein, 1996). There are two known inherited conditions that give a predisposition to colon cancer. One of these, familial adenomatous polyposis (FAP), is easily recognised because the affected individuals have hundreds or thousands of polyps, benign tumours in the large intestine. This condition accounting for about 1% of all colorectal cancer involves the APC gene (Altered in Polyposis Coli Colorectal Cancer). In the second condition, HNPCC there are no visible signs that can allow unequivocal diagnosis. HNPCC is usually indicated where the affected individual comes from a family in which at least three relatives in two generations have had colorectal cancer, with one of the individuals being diagnosed when less than 50 years old (Lynch *et al.*, 1993). This imprecise diagnostic criterion means that the exact number of people with this condition is still unknown, but HNPCC has been estimated to account for 4 to 13 % of colorectal cancer in industrial nations (Peltomaki *et al.*, 1993). In addition to the colon, HNPCC patients commonly have tumours in the endometrium, stomach, biliopancreatic system and urinary tract (Vogelstein, 1988). The existence of a familial gene for colon cancer was proved by the study of two large HNPCC families. Using microsatellite markers, the whole genome was searched and the HNPCC gene was mapped to 2p 15-16 by its close association with the marker D2S123 (Peltomaki *et al.*, 1993).

At the time that the HNPCC gene was mapped, the biochemical role of the gene product was completely unknown, however further genetic studies revealed its role. A number of cancer predisposition syndromes involving tumour suppressor genes are characterised by loss in the tumour of the normal allele of the gene, termed loss of heterozygosity (LOH). Examples are p53 (Bhattacharyya *et al.*, 1994) and the retinoblastoma gene (Knudson *et al.*, 1977). To see whether allelic loss had occurred in the HNPCC tumours, the linked marker D2S123 from the patients tumours was compared with matched normal DNA from the same individual (Aaltonen *et al.*, 1993). D2S123 which contains a (CA)<sub>n</sub> sequence is one of many microsatellite markers used for genetic analysis. (Watson, 1994; Weissenbach, 1992). The locus was not deleted in any of the 14 HNPCC tumours studied and was deleted in only one of the 46 sporadic tumours examined. However, there had been a change in the electrophoretic mobility of (CA)<sub>n</sub> repeats in the majority of tumours due to either an increase or decrease in the number of CA repeats. This suggested that replication errors had occurred in these sequences as the cells divided during tumour development. A further 6 other markers were also examined and similar shift in electrophoretic mobility was additionally observed in these markers. A later independent study showed microsatellite instability in 25 out of 90 randomly chosen colorectal tumours (Thibodeau *et al.*, 1993). Microsatellites are an abundant class of repetitive DNA sequences. There are 100,000 (CA)<sub>n</sub> repeats scattered throughout the genome and many exhibit length polymorphism (Thibodeau *et al.*, 1993). Tumours that exhibit microsatellite instability frequently contain alterations in the lengths of repetitive sequences within a variety of cancer-associated genes including, *APC*, *IGF*, *TGF-β*, *hMSH3* and *hMSH6* (Rampino *et al.*, 1997; Markowitz *et al.*, 1995). Mutations in HNPCC are not localised to repetitive sequences but also occur in non-repetitive gene sequences and colorectal carcinoma cell lines with mismatch repair deficiency have mutation

rates that are several hundred fold greater than normal cells (Bhattacharyya *et al.*, 1994).

Due to the genome wide nature of the mutations, it was suggested that predisposition to HNPCC is associated with a susceptibility to replication errors. The gene associated with HNPCC was therefore predicted to code for a protein involved in DNA replication or repair.

### **The post-replicative DNA mismatch repair system**

The post-replicative mismatch repair system repairs two kinds of replication errors. The first consists of paired non-complementary bases and is generally referred to as base-base mispairing or mismatching. Base-base mispairing can occur in duplex DNA in the form of purine-purine (G·G, A·A, G·A), purine-pyrimidine (G·T, T·G, A·C) or pyrimidine-pyrimidine (C·C, T·T, T·C) mismatches. With the exception of C·C, all are subject to correction by the post-replicative mismatch repair system (Jiricny, 1998b).

A second sort of mispairing can occur because of misalignment of two complementary single strands in double stranded DNA. Depending of the extent of misalignment, single stranded loops can form of one or more unpaired bases in the DNA duplex. These are called insertion/deletion loops (IDLs) and are also efficiently detected and corrected by the mismatch repair system (Jiricny, 1998a). The source of base-base mismatches is by misincorporation of a non-complementary base in the newly replicated strand. The main source of IDL formation is the slippage of one DNA strand along the other during replication.

A clean distinction must be made between post-replicative mismatch repair and the repair of mismatches arising from the damage to the genome after replication. For example the deamination of cytosine or 5-methylcytosine forms uracil or thymine respectively. Mismatches arising from replication errors are repaired by the postreplicative mismatch repair system, while those arising from deamination of cytosine or of 5-methylcytosine are repaired by a base-excision repair pathway.

### ***The Postreplicative Mismatch Repair System in Escherichia coli***

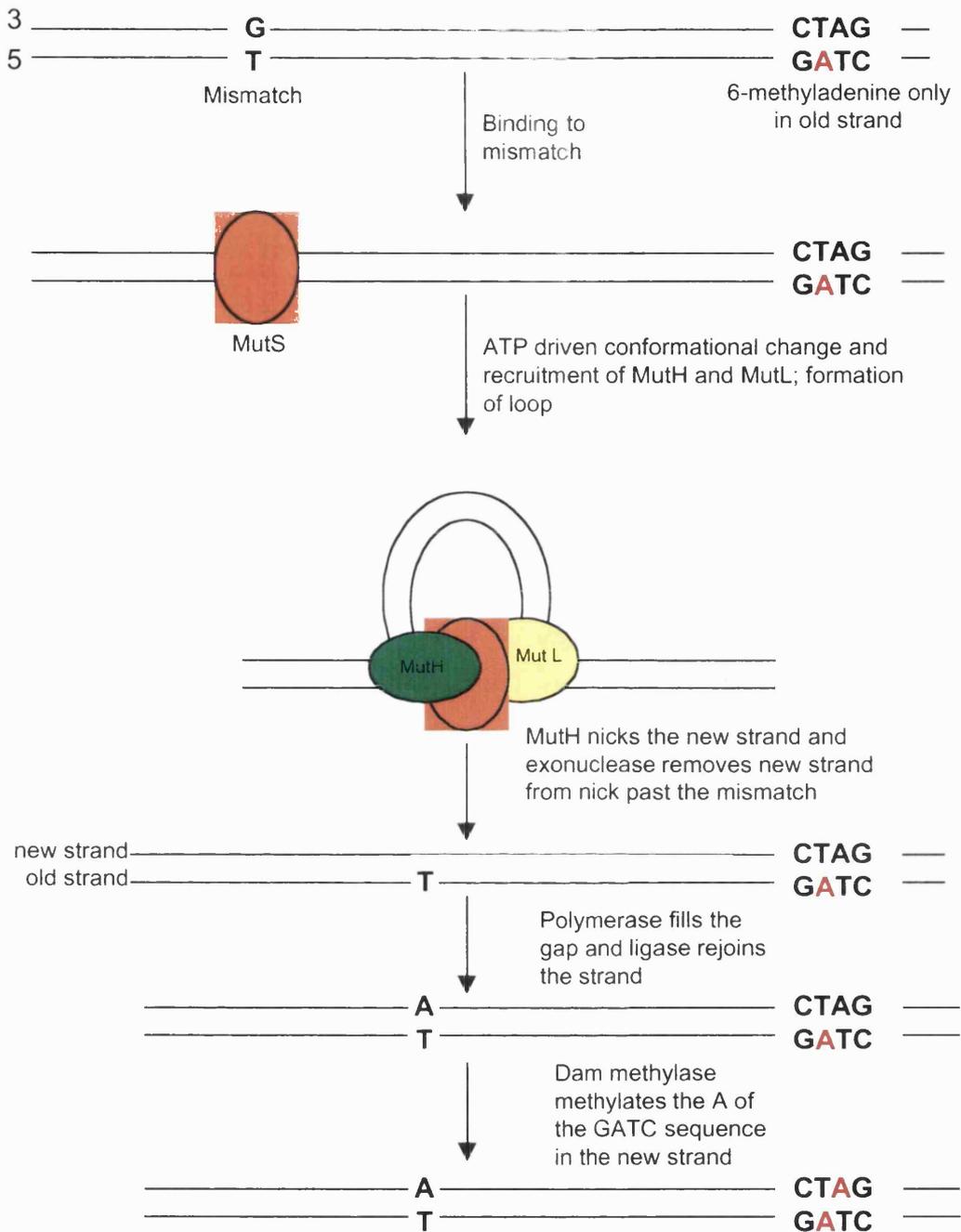
The methyl directed mismatch repair system in *E.coli* has been well characterised for over 20 years and represents the best characterised system to date (Modrich, 1991; Modrich and Lahue, 1996). Known as the DNA adenine methylase (Dam)-instructed MutHLS mismatch repair pathway, it is a general DNA repair pathway that recognises and repairs all single base mispairs (Modrich and Lahue, 1996). It also repairs small (less than 4 base) insertion/deletion mispairs.

The repair reactions of the methyl directed mismatch repair system have been characterised *in vivo*, allowing the development of an *in vitro* assay for methyl-directed mismatch repair. This led to the identification of the eight proteins required for mismatch repair (Dohet *et al.*, 1985; Jones *et al.*, 1987). In *E.coli* the adenine in GATC sequences is methylated by a post-synthetic process. Thus for a short while, the new DNA strand is unmethylated. This allows the bacterium to distinguish between the new and the parent DNA strand. Repair is initiated by binding of MutS to the mismatch, with an ATP dependent conformational change of MutS initiating a cascade of events. ATP hydrolysis drives the movement of the DNA through the bound MutS as well as the assembly of a multiprotein complex containing the MutS and MutL homodimers. This complex activates MutH, an endonuclease that makes a nick at a GATC site. In cases where the unmethylated

GATC was situated 5' from the mispair the cleaved strand is degraded from the nick up to and slightly past the mismatch site either by *ExoVII* or *RecJ*. When the nick was situated 3' from the mispair, the cleaved strand is degraded by *ExoI*. The single-stranded region generated is protected by the single stranded binding protein, Ssb. The gap is filled in by DNA polymerase III and DNA ligase repairs the nick. The process is completed by methylation of the GATC site by Dam methylase. This is summarised in figure 1.1.

The role of MutL protein in this pathway is the least understood. It has been thought of as a 'master co-ordinator' or 'molecular matchmaker' of methyl-directed mismatch repair in its ability to interact with the other mismatch repair proteins to stimulate their activities (Hall and Matson, 1999), and indeed direct physical interaction between MutL and MutH has recently been shown (Hall and Matson, 1999).

The MutHLS pathway recognises different mispaired bases with varying efficiency. As we might expect, the highly distorted purine:purine or pyrimidine:pyrimidine mismatches are more efficiently repaired than purine:pyrimidine mismatches. However, the sequence context of a mismatch can greatly affect its repair efficiency (Kramer *et al.*, 1984).



**Figure 1.1 Overview of the methyl directed mismatch repair pathway in *E.coli*.**

A detailed description can be found in the text. Adenines denoted with a red A represent adenines methylated at the 6-position by *E.coli* Dam methylase.

### **The Mismatch Repair System in Eukaryotes**

The identification of MutS and MutL homologues in eukaryotes from yeast to multicellular organisms, including mammals suggests that the key components of the bacterial mismatch correction system have been conserved throughout evolution

Knowledge of mismatch repair in lower eukaryotes came initially from studies with the yeast *Saccharomyces cerevisiae* (reviewed in Fishel and Kolodner, 1995). Mutants defective in the loci, *PMS1*, *PMS2* and *PMS3* were found to be defective in the repair of heteroduplex DNA. Specifically, the *pms1* and *pms2* mutants were found to be deficient in the repair of base pair mismatches and a single extrahelical base (Kramer *et al.*, 1989).

The *PMS1* locus was cloned and sequenced and found to be a homologue of the *E.coli mutL* gene (Kramer *et al* 1989b; Mankovich *et al.*, 1989). Another *mutL* homologue, *mlh1* was found in *S.cerevisiae*, unlike *E.coli* where there is only one MutL. *mlh1* mutants have the same phenotype as *pms1* mutants and furthermore, the mutator phenotypes of *pms1 mlh1* double mutants are the same as those of the *pms1* and *mlh1* single mutants. Together, these results provided strong evidence that the *mlh1* gene product also plays a role in mismatch repair that is related to the role of the MutL protein. The requirement for both proteins was explained when it was found that the MLh1 and PMS1 proteins physically associate to form a heterodimer (Prolla *et al.*, 1994).

A degenerate PCR approach was then used to identify MutS homologues in *S. cerevisiae*. There are in fact at least six *S. cerevisiae* proteins, MSH1-MSH6 that show a high degree of amino acid similarity with the bacterial proteins. The

phenotype of *msh2* mutants was found to be very similar to that of *pms1* mutants. Both have a high spontaneous mutation rate (Reenan and Kolodner, 1992a; Reenan and Kolodner, 1992b). Also, the *msh2* mutant was similar to the *pms1* mutant in being defective in the repair of all base pair mismatches as well as 1, 2, and 4 IDLs (Alani *et al.*, 1994). These properties suggested that the *msh2* gene product also participates in a mismatch repair pathway that is similar to the *E. coli* methyl-directed (long patch) mismatch repair pathway. The function of the remaining MutS proteins is unknown.

### **The Human Mismatch Repair System**

Mismatch binding was first described in human cells in 1988 when it was shown that there was an abundant factor with high affinity to oligonucleotide substrates containing a G·T mismatch (Jiricny *et al.*, 1988). This was later shown to be the heterodimer MutS $\alpha$ . There are six known MutS homologues in human cells, three of which have been shown to be involved in pairwise interactions. MutS $\alpha$  is a heterodimer of hMSH2 and hMSH6 and MutS $\beta$  is a heterodimer of hMSH2 and hMSH3. The other MutS proteins, hMSH1, hMSH4 and hMSH5 are not involved in the recognition of base-base mismatches or insertion-deletion loops in nuclear DNA. (Marra and Schar, 1999).

Human cells with defects in hMSH2, hMSH3 or hMSH6 display different mutator phenotypes similar those observed with the corresponding yeast mutants. Some are mainly affected in the recognition and repair of base-base mispairs, others in the recognition and repair of IDLs and others in the repair of both. The extent of genome instability is related to the specific MutS homologue that has been mutated, thus reflecting the different contributions of hMSH2, hMSH3 and hMSH6 to the recognition and repair of specific base pairing errors. Although hMSH2 has been

shown to bind to G·T and IDL heteroduplex DNA substrates, it appears that mismatch recognition in human cells is mainly carried out the heterodimeric complexes, MutS $\alpha$  and MutS $\beta$  (Fishel *et al.*, 1994a; Fishel *et al.*, 1994b).

The existence of the MutS $\alpha$  complex was demonstrated in two ways. Using protein extracts from HeLa cells, hMSH2 and hMSH6 were shown to form a stable heterodimer (Drummond *et al.*, 1995). The production of hMSH2 and hMSH6 by *in vitro* translation or in the baculovirus system has demonstrated that both proteins are required for efficient binding to a G·T heteroduplex (Gradia *et al.*, 1997; Palombo *et al.*, 1996). IDLs of one nucleotide are also bound by MutS $\alpha$  (Hughes and Jiricny, 1992). IDLs greater than one extrahelical nucleotide are also recognised but with sequence contexts being important (Palombo *et al.*, 1996; Genschel *et al.*, 1998). The affinity of MutS $\alpha$  for other types of base-base mismatches as measured by gel shift assays is very low.

The existence of the MutS $\beta$  complex was demonstrated in a similar way. Co-immunoprecipitation and co-purification of the hMSH2 and hMSH3 proteins was achieved in insect cells infected with both hMSH2 and hMSH3-encoding baculovirus vectors. In contrast to MutS $\alpha$ , the purified hMSH2-hMSH3 heterodimer was shown to bind to IDL heteroduplex substrates but not G·T mispairs (Acharya *et al.*, 1996; Palombo *et al.*, 1996). MutS $\beta$  compensates for the loss of hMSH6 in loop repair since cells that are mutated in *hMSH6* do not display an instability of microsatellites consisting of dinucleotide repeats and larger (Papadopoulos *et al.*, 1995).

Although all three MutS homologues are expressed in human cells, MutS $\alpha$  appears to play the predominant role in repair. Cells that over-express MSH3 and therefore

sequester MSH2 display a strong mutator phenotype. Normally there is more MutS $\alpha$  than MutS $\beta$  because the competition for hMSH2 in normal cells between hMSH6 and hMSH3 favours hMSH6. The fact that there are several MutS homologues in eukaryotes suggests that during evolution, mismatch recognition has improved to accommodate the demands of more complex genomes (Marra and Schar, 1999).

ATP has a similar role in mismatch recognition in eukaryotic cells as it does in bacterial cells. ATP binding and hydrolysis is required for the induction of conformational changes in the MutS $\alpha$ -DNA complex that lead to an association with MutL $\alpha$  and the translocation of DNA through or along the MutS $\alpha$ -MutL $\alpha$  recognition complex (Allen *et al.*, 1997). Therefore the formation of an  $\alpha$ -loop may be involved in mismatch binding in the eukaryote system. Here, the  $\alpha$ -loop would be stabilised at its base by MutS $\alpha$  or MutS $\beta$  in a complex with MutL $\alpha$ , a heterodimer of MLH1 and PMS2 in human cells and MLH1 and PMS1 in *S. cerevisiae*.

The process of strand recognition in eukaryotes appears to be distinct from the bacterial system since there is no evidence of MutH homologues in eukaryotes and strand discrimination is not dependent on methylated DNA sequences. It is plain that strand discrimination must occur but it is not yet known beyond doubt how it is done. Proliferating cell nuclear antigen (PCNA) could be involved in strand discrimination in eukaryotes and interaction between mismatch repair proteins and PCNA has been found (Umar *et al.*, 1996). It was already known that PCNA functions in both DNA replication and nucleotide excision repair. The interaction of PCNA with the mismatch repair system suggests that it may form a physical link between mismatch repair and the replication machinery. This would provide

a possible mechanism for identification of the newly replicated strand, thus eliminating the need for a eukaryote MutH-endonuclease homologue for strand discrimination. The *in vitro* models of post-replicative mismatch repair usually direct the repair by placing a nick 5' to the mismatched base in the strand which is to be repaired and Jiricny has suggested that the nicks in the lagging strand and the growing point of the leading strand may be the strand discrimination signal (Jiricny, 1998a).

## **Proposed mechanisms of cell death via the post-replicative mismatch repair system in response to O<sup>6</sup>-methylguanine**

Since the work in this thesis is largely concerned with the cytotoxicity of O<sup>6</sup>-meG, a discussion of the proposed mechanism of cell death following binding of proteins of the mismatch repair system is necessary. Although the details of the role of mismatch repair in the correction of replication errors are well characterised, the mechanism of induction of cell death by the postreplicative mismatch repair system is poorly understood. An early proposal was the 'futile cycling' theory (Karran and Hampson, 1996). The basis of this theory was that since O<sup>6</sup>-meG remains in the parental strand after mismatch repair which removes the C or T incorporated opposite the O<sup>6</sup>-meG in the daughter strand, resynthesis will again result in the formation of imperfect base pairs. The theory was that the cell attempts round after round of 'futile repair' resulting in breaks in the newly synthesised strand. These breaks could then be converted into double strand breaks during the second S phase after methylating agent exposure with resulting cell death. Although no direct evidence for this proposal has been found it is not entirely inconsistent with recent work, which suggests that upon binding of the MutS $\alpha$  and MutL $\alpha$  proteins to DNA lesions, direct signalling to the apoptotic machinery occurs with resulting death by apoptosis.

Apoptosis was initially described by its morphological characteristics, including cell shrinkage, membrane blebbing, chromatin condensation and nuclear fragmentation (Kerr *et al.*, 1994). It is now known that apoptosis is a gene directed program and defects in apoptotic pathways are thought to contribute to a number of human diseases ranging from neurodegenerative disorders to cancer. In the 1970s kinetic studies of tumour growth suggested that tumour growth rates could be less than 5% of that predicted from proliferation rates (Kerr *et al.*, 1972) and it was suggested

that a large amount of cell loss from tumours was due to apoptosis rather than necrosis.

The vast majority of currently used anticancer agents were originally developed by chemical screens that were designed to identify agents that selectively kill tumour cells. The focus was on intracellular targets and the nature of the cellular damage produced. However, in the 1970s pathologists noticed that radiation and chemotherapy could induce cell death with morphological features of apoptosis. It is now well established that anticancer agents induce apoptosis and that disruption of apoptotic programs can reduce drug treatment sensitivity (Scmitt *et al.*, 1999).

Evidence that cell death involving the mismatch repair system could also occur through apoptosis first came from studies on the cytotoxicity of cisplatin. Cisplatin is used in the treatment of several types of cancer and is particularly effective in the treatment of ovarian and testicular cancer. The main lesions formed by cisplatin are intrastrand crosslinks in DNA between closely adjacent purine bases, 90% of which are 1,2 crosslinks and 5% are 1,3 crosslinks (Fichtinger-Schepman *et al.*, 1985). The main mechanism for the removal of the 1,3 intrastrand crosslinks is nucleotide excision repair, while post-replicative mismatch repair removes the 1,2 crosslinks (Duckett *et al.*, 1996) (Yamada *et al.*, 1997). Cell death in response to cisplatin treatment was found to be by apoptotic cell death (Anthony *et al.*, 1996). Quantitation of cisplatin induced apoptosis by flow cytometry, showed that the ovarian cell line A2780 can more readily engage apoptosis after cisplatin treatment than can cisplatin resistant derivatives (Anthony *et al.*, 1996). Additionally, it was reported that the introduction of a dominant negative mutant *p53* gene into A2780 cells led to an increased resistance to cisplatin (Drummond *et al.*, 1996). *p53* is a tumour suppressor gene that plays an important role in

mutation avoidance by preventing cell cycle progression until damage is repaired, or in the case of high lesional load, removal of the damaged cell by apoptosis (Oren, 1999). Its concentration is low in normal cells but accumulates after DNA damage (Levine, 1997). The tumour suppressing properties of p53 are mainly associated with its function as a sequence specific DNA-binding protein and transcription factor that controls the expression of a number of genes (Hupp *et al.*, 2000). A reflection of its importance in tumour avoidance is the fact that approximately 50% of human cancers contain mutations in the *p53* gene, although interestingly some types of cancers never have *p53* mutations (Levine, 1997). The *bax* gene product has been identified as a mediator of p53-dependent apoptosis by antagonising the Bcl-2-dependent survival pathway (Miyashita *et al.*, 1994). The Bcl-2 family of proteins comprise a family of proteins that play an important role in the regulation of apoptosis, with some promoting apoptosis and others promoting cell survival, following cell injury (Oltvai and Korsmeyer, 1994).

In support of a role for p53 in the cytotoxic mechanism of cisplatin, several other studies have shown that disruption of p53 function resulted in drug resistance (Perego *et al.*, 1996) (Gallagher *et al.*, 1997). Branch *et al* (Branch *et al.*, 2000) recently showed that methylation tolerant variants selected from normal A2780 cells, in addition to the expected defective mismatch repair, expressed mutated p53. Defects in either p53 or mismatch repair increased cisplatin resistance but the p53 response is the major determinant of cisplatin sensitivity. However, other research has argued that p53 is not a determinant of cisplatin cytotoxicity in ovarian cells (Feudis *et al.*, 1997) and also testicular cells (Burger *et al.*, 1997). Furthermore, cell lines that do not have a predominant apoptotic response to chemotherapy, such as breast cancer were in fact sensitised to cisplatin by the inactivation of p53 (Fan *et*

*al.*, 1995) (Hawkins *et al.*, 1996). Therefore p53 is not an absolute requirement for cisplatin induced cell death.

Similarly, there is increasing evidence that apoptosis is the main mode of cell death in response to alkylating agent treatment. Alkylating agents produce a range of DNA damage but Meikrantz *et al* (1998) confirmed that O<sup>6</sup>-methylguanine is the major lesion responsible for triggering apoptosis by measuring apoptosis in cells that were able to repair the lesion with those that could not. They found that the majority of apoptosis was eliminated in cells expressing O<sup>6</sup>-methylguanine-DNA methyltransferase compared with cells that did not express O<sup>6</sup>-methylguanine-DNA methyltransferase. In the absence of repair of O<sup>6</sup>-alkylguanine by O<sup>6</sup>-methylguanine-DNA methyltransferase or in the cases of high lesional load, the mismatch repair binding to O<sup>6</sup>-alkylguanine is believed to directly signal apoptosis. Evidence for this comes from the fact that MutS $\alpha$  can recognise and bind to synthetic oligonucleotides containing O<sup>6</sup>-methylguanine or S<sup>6</sup>-methylthioguanine (Griffin *et al.*, 1994; Duckett *et al.*, 1996; Swann *et al.*, 1996; Waters and Swann, 1997).

A similar approach was used in another study using cells that were either hMutS $\alpha$  or MutL $\alpha$  negative compared with mismatch repair proficient cells (Wu *et al.*, 1999). It was found that apoptosis associated with mismatch repair binding could be mediated through both p53 and p53 independent apoptosis. Some mechanistic detail of the p53 dependent pathway has come from a study that shows that after treatment of cells with N-methyl-N-nitrosourea or N-methyl-N'-nitro-N-nitrosoguanidine phosphorylation of p53 occurs on serine residues 15 and 392 and these phosphorylation events depend on the presence of functional hMutS $\alpha$  and hMutS $\beta$  (Duckett *et al.*, 1999). Furthermore, methylation induced killing of O<sup>6</sup>-

methylguanine-DNA methyltransferase negative cells was found to be attributable to the potent induction of apoptosis 48 hours after treatment preceded by a decrease in Bcl-2 protein level, with hypophosphorylation of Bad (Ochs and Kaina, 2000). The selective phosphorylation of p53 observed in response to agents producing O<sup>6</sup>-methylguanine, O<sup>4</sup>-methylthymine or the cisplatin-d(GpG) adduct, may be part of the signal from p53 of the damaged DNA response, resulting in activation of p53 and arrest of cell cycle progression. Cells with defective hMutL $\alpha$  or hMutS $\alpha$  fail to elicit this G2 checkpoint response when they are treated with these agents. These cells are therefore tolerant of DNA lesions that are otherwise lethal to mismatch repair proficient cells.

## Scope of Thesis

### ***Can the knowledge of the mechanism of action of thioguanine be used to improve the treatment of cancer?***

The first aim of the work described in this thesis was to determine whether knowledge of the mechanism of action of 6-TG can be used to improve cancer chemotherapy. Thioguanine has been used since the 1950s for the treatment of acute leukaemia (Elion, 1989) although it is ineffective in the treatment of solid tumours. The toxic mechanism of 6-TG has long been known to involve both changes in purine metabolism and the formation of 2'-deoxy-6-thioguanosine triphosphate and its incorporation into DNA (reviewed in Elion, 1989). The first clue that the post-replicative mismatch repair system may be involved with 6-thioguanine associated cell death came from the observation that *mer<sup>r</sup>* cells that are resistant to N-methyl-N-nitrosourea are cross resistant to 6-TG (Aquilina *et al.*, 1989). Cells are usually resistant to 6-TG usually due to loss of the enzyme hypoxanthine-guanine phosphoribosyl transferase (HGPRT), an enzyme that is part of the purine salvage pathway but these *mer<sup>r</sup>* resistant cells had HGPRT.

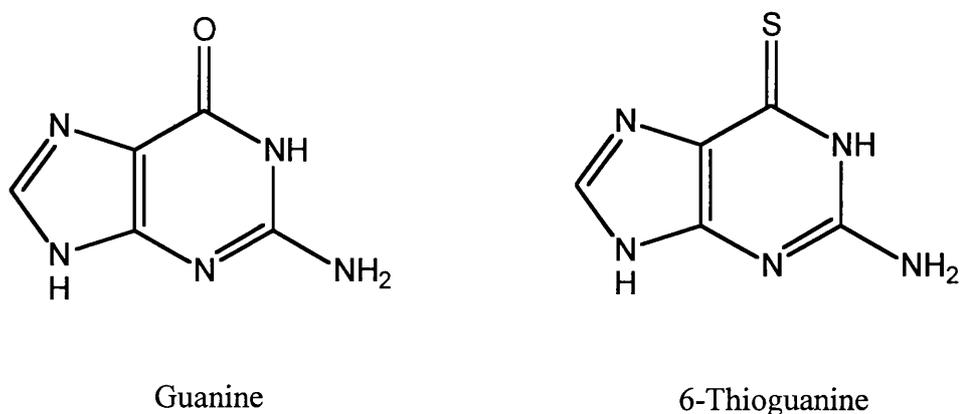


Figure 1.2 Structures guanine and 6-thioguanine

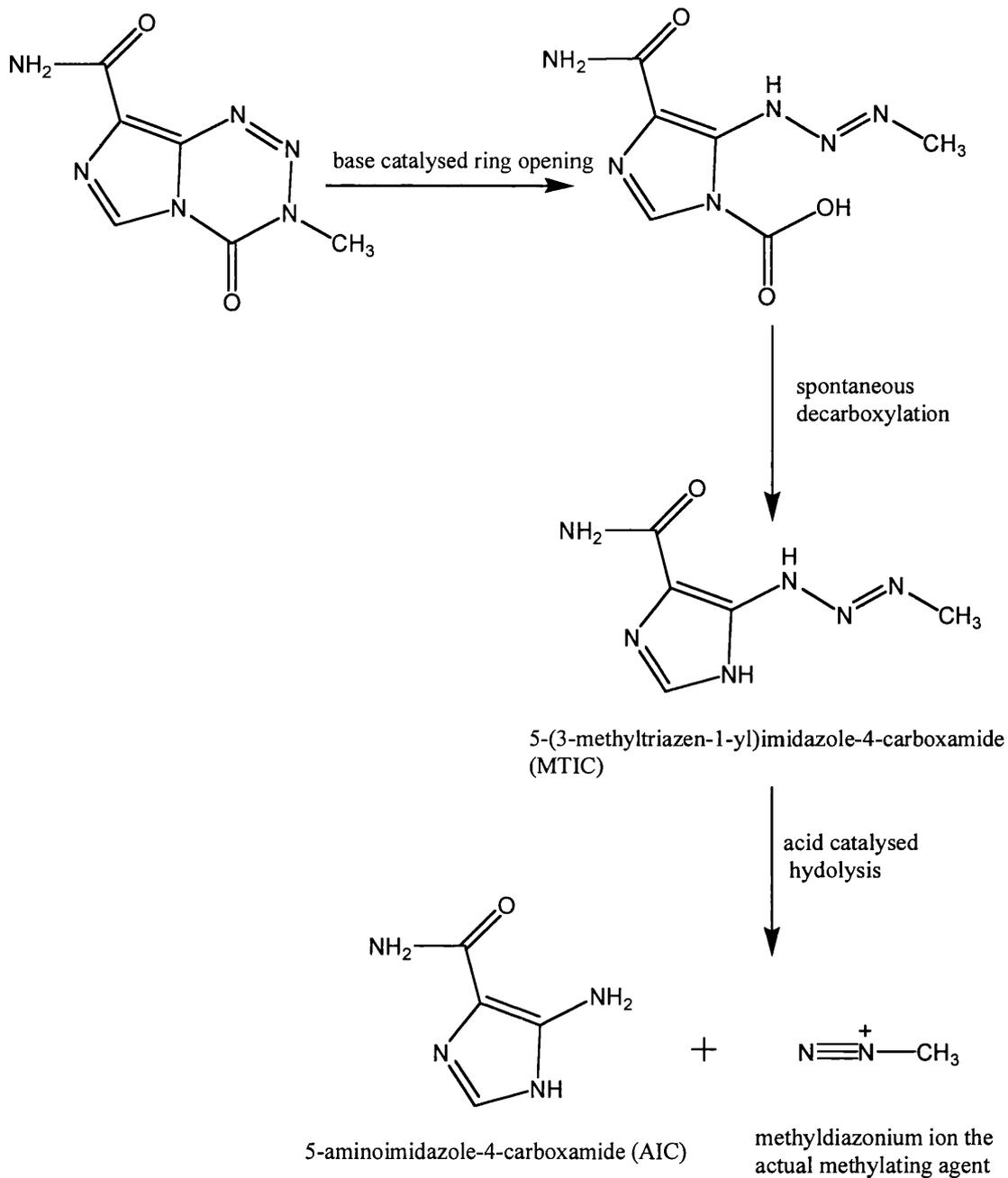
The reason why thioguanine has this similarity to N-methyl-N-nitrosourea is believed to depend on the fact that after incorporation into DNA, 6-TG is methylated by endogenous S-adenosylmethionine to form S<sup>6</sup>-methylthioguanine (Swann *et al.*, 1996). During replication, like O<sup>6</sup>-MeG, the S<sup>6</sup>-methylthioguanine (S<sup>6</sup>MeG) directs the incorporation of either thymine or cytosine into the new DNA strand and the resultant base pairs containing S<sup>6</sup>-MeG are bound by MutS $\alpha$  and cell death is signalled by an unknown mechanism (Swann *et al.*, 1996). The crucial step in this proposed mechanism of toxicity is the methylation of the thioguanine residues in the DNA. This is an inefficient step because S-adenosylmethionine is a very poor methylating agent. Thus if this proposed mechanism is correct then one would expect that methylating agents and thioguanine would be synergistic in their toxicity.

The first choice would be to use a methylating agent that is already in clinical use. The latest cytotoxic methylating drug to be given a clinical licence is temozolomide which is currently used for the treatment of glioblastoma and melanoma (Newlands *et al.*, 1992) (O'Reilly *et al.*, 1993). The chemical breakdown of temozolomide is shown in figure 1.3. The first product of aqueous hydrolysis of temozolomide is 5-(3-methyltriazene-1-yl)imidazole-4-carboxamide (MTIC), which ultimately transfers its methyl group to a nucleophile (Newlands *et al.*, 1997; Stevens and Newlands, 1993). The subsequent reactions of MTIC require acid catalysis. The triazene ring of MTIC is fragmented to form the methyldiazonium ion. The final step is the reaction of the methyldiazonium ion with a nucleophilic site on DNA, transferring a methyl group.

There is only a small pH range, close to physiological pH at which the whole process from prodrug activation to methyl group transfer can occur. This is because temozolomide is stable under acid conditions but degrades in neutral and basic pH, whereas MTIC is stable at basic pH but degrades rapidly in acid. This enables temozolomide to be administered as oral capsules because it is able to withstand the strong acidity of the stomach (Newlands *et al.*, 1997). Temozolomide shows most clinical efficacy against brain tumours than any other tumour. As with thioguanine, the toxicity of temozolomide is limited by its chemistry. Its cytotoxicity depends on the formation of O<sup>6</sup>-methylguanine in DNA, however the 6-oxygen of guanine has very poor reactivity.

The experiments in chapter 2 show that treatment of nude mice bearing glioblastoma or melanoma xenografts with thioguanine and temozolomide resulted in a synergistic delay in tumour growth. Such nude mouse experiments are difficult, long and very expensive so a great deal of time was spent trying to demonstrate a similar cytotoxic synergy in tissue culture. Contrary to the *in vivo* results, there was no synergy between temozolomide and thioguanine in tissue culture. However, there was synergy observed between thioguanine and methyl methanesulphonate. Although temozolomide and methyl methanesulphonate are both methylating agents there is a profound difference in their chemistry. The active methylating agent from temozolomide, the methyldiazonium ion, methylated by an S<sub>N</sub>1 mechanism, while methyl methanesulphonate methylates by a predominantly S<sub>N</sub>2 mechanism. Thus as it seemed possible that the differences between temozolomide and methyl methanesulphonate were a reflection of this difference in chemistry, duplex DNA containing thioguanine was reacted with N-methyl-N-nitrosourea (S<sub>N</sub>1) and methyl methanesulphonate (S<sub>N</sub>2) and compared with the reaction of these chemicals with DNA containing no thioguanine. N-

methyl-N-nitrosourea was used rather than temozolomide because it is easier to synthesize the  $^{14}\text{C}$ -labelled compound. Since N-methyl-N-nitrosourea and temozolomide produce the same methylating species, the methyl diazonium ion, N-methyl-N-nitrosourea can be used instead of temozolomide. The results show that when thioguanine is present in DNA, methylation of the sulphur group by methyl methanesulphonate is greatly favoured compared with methylation of any other nucleophilic site in DNA. By contrast N-methyl-N-nitrosourea does not prefer to methylate the sulphur.



**Figure 1.3** Decomposition of temozolomide to the methyldiazonium ion in aqueous conditions

Notice that the same methyl diazonium ion is formed during the base induced breakdown of N-nitroso-N-methylurea

***Does prolonged exposure to thioguanine lead to the development of DNA repair defective cells?***

The work in the second part of this thesis was designed to find out whether prolonged thioguanine treatment leads to the development of post-replicative repair deficient cells. The observations that defective mismatch repair is the cause of a hereditary form of cancer could have significant implications for chemically induced carcinogenesis. It is possible that any compound that can reduce the expression of a mismatch repair gene, or produce selective pressure in favour of replication of repair defective cells will produce a cancer prone phenotype. This mechanism of carcinogenesis would be distinct from the genotoxic mechanism of action, but the end result, mutation of DNA in crucial genes, would be the same.

At the present time we know that the cytotoxicity of N-methyl-N-nitrosourea (Karran *et al.*, 1993), cisplatin (Aebi *et al.*, 1996), temozolomide (Newlands *et al.*, 1997) and thioguanine (Swann *et al.*, 1996) is mediated by the post-replicative mismatch repair system. Two of these, cisplatin and N-methyl-N-nitrosourea have been shown to convert mismatch proficient cells to mismatch repair deficient cells. If thioguanine were also capable of converting mismatch repair proficient cells to mismatch repair deficient cells it might, in part, explain one of the problems associated with the use of the thioguanine prodrug azathioprine. There is an increased incidence of skin cancer in renal transplant patients who are taking the thioguanine precursor azathioprine (Bouwes-Bavinck *et al.*, 1996), which could be a result of loss of functional mismatch repair. This suggested that it might be valuable to investigate whether thioguanine would convert normal mismatch repair proficient cells to mismatch repair deficient cells and whether there was any evidence that the azathioprine associated skin tumours were mismatch repair deficient.

Chapter 3 describes the production of mismatch repair defective cells after prolonged exposure to thioguanine. Resistance to thioguanine can arise either because of defective mismatch repair or because of loss of HPGRT. To select only for mismatch repair defective cells, a toxic concentration of MNU was used to kill all cells except those with mismatch repair deficiency. RNA and protein was extracted from surviving clones. Reverse Transcription followed by polymerase chain reaction (RT-PCR) was used to measure expression of the mRNA for the mismatch repair genes and western blotting was used to determine the presence of the mismatch repair proteins. The MLH1 protein was found to be absent in several clones with accompanying loss of mRNA expression. The absence of MLH1 expression was found to be caused by methylation silencing of the promoter region of the gene rather than mutation.

## **Chapter 2**

# **Synergy between thioguanine and methylating agents**

## Chapter 2

### Synergy between thioguanine and methylating agents

#### Introduction

As detailed in chapter 1, the proposed mechanism of thioguanine toxicity suggests that a crucial step is the methylation of thioguanine residues in DNA by S-adenosylmethionine. S-adenosylmethionine is a poor methylating agent and only a small proportion of the thioguanine residues are methylated by it. Therefore one would expect that co-administration of more powerful methylating agents would increase the methylation and increase the cytotoxicity of thioguanine. This chapter describes the experiments performed to determine whether such cytotoxic synergy between thioguanine and methylating agents exists.

The nude mouse xenograft model was chosen as the *in vivo* system for testing for synergy between thioguanine and methylating agents in the treatment of cancer. This model has been used extensively for the study of anti cancer drugs. For these *in vivo* experiments, the methylating drug temozolomide was chosen, which is used clinically for the treatment of brain tumours (glioblastoma) and skin tumours (melanoma). The cell lines used to produce the xenografts were those used as models in the preclinical development of glioblastoma (U87MG) and melanoma (A375P) (Wang *et al.*, 1991; Wedge and Newlands, 1996; Wedge *et al.*, 1997).

*In vivo* xenograft models of glioblastoma using the U87MG cell line and of melanoma using the A375P cell line were set up to study the effect of tumour growth after treatment with thioguanine and temozolomide. The results showed that there was a synergistic delay in tumour growth after treatment with thioguanine and temozolomide in both the glioblastoma and melanoma models. Experiments with xenografts are very expensive and time consuming so to study

further the synergy tissue culture experiments were carried out. The most satisfactory way of measuring the cytotoxicity is the clonogenic assay in which a single cell suspension of cells is plated, treated with the drug and the number of colonies counted. However both U87MG and A375P cells have very weak adherence to tissue plates so were unsuitable for clonogenic assays. As an alternative, the MTT assay was used to assess the effect of treatment with thioguanine and methylating agents. Surprisingly, temozolomide had very little effect on the toxicity of thioguanine in this tissue culture system. The contrast between this result *in vitro* and the marked synergism seen *in vivo* needed explanation and for this reason a number of experiments were done first with temozolomide and then with another methylating agent methyl methanesulphonate. In sharp contrast to temozolomide, methyl methanesulphonate was synergistic with thioguanine. Since it seemed possible that this difference between the two methylating agents was the result of differences in their reactivity, their reaction with thioguanine residues in DNA was studied.

Temozolomide breaks down in aqueous solutions to produce the methyldiazonium ion, which methylates DNA (see figure 1.3). The same ion is produced by the base induced breakdown of the direct acting nitrosamides, therefore temozolomide alkylates DNA by the same mechanism as the nitrosamides, i.e. by an  $S_N1$  reaction (Beranek, 1990).  $S_N1$  reactions follow first-order kinetics. The rate of the entire reaction is dependent on the formation of an electrophilic intermediate, in this case the methyldiazonium ion. This intermediate is then rapidly trapped by the nucleophilic centres on the DNA forming covalently bound methyl adducts. Methyl methanesulphonate however reacts with DNA by a predominantly  $S_N2$  mechanism.  $S_N2$  reactions are bimolecular, and are strictly dependent on steric accessibility. This type of substitution involves a transition

state when the electrophile attacks a nucleophilic centre forming a transition complex which then forms a methylated product with release of the leaving group. The most important difference between  $S_N1$  and  $S_N2$  methylating agents is that the  $S_N1$  agents can react with the ring oxygens with around 7% of the methylation of DNA occurring at the 6-oxygen of guanine. However,  $S_N2$  agents show a preference for the most nucleophilic sites on DNA, with around 80% of total methylation occurring on the N-7 of guanine, but negligible reaction with the ring oxygens.

It was thought that this difference in the chemistry of temozolomide and methyl methanesulphonate might explain the difference in toxicity when given after thioguanine in tissue culture. If there was more favourable methylation of the 6-S of thioguanine by methyl methanesulphonate than by an  $S_N1$  methylating agent then methyl methanesulphonate would be expected to have greater cytotoxic synergy with thioguanine. Oligonucleotides containing thioguanine were reacted with  $^{14}C$ -labelled methyl methanesulphonate and the  $^{14}C$ -labelled  $S_N1$  methylator, N-methyl-N-nitrosourea. After reaction with the methylating agent the DNA was depurinated by treatment with the acid. The amount of S<sup>6</sup>-methylthioguanine could then be measured by HPLC and scintillation counting. The method also enabled the detection of N<sup>7</sup>-methylguanine, O<sup>6</sup>-methylguanine and N<sup>3</sup>-methyladenine. It was found that methylation with the  $S_N2$  methylating agent greatly favours the methylation of thioguanine residues in DNA compared with the  $S_N1$  methylator N-methyl-N-nitrosourea.

## **Materials and Methods**

### ***Drugs and Chemicals***

Temozolomide was a gift from Professor Stevens (Cancer Research Campaign Experimental Cancer Chemotherapy Research Group, Cancer Research Laboratories, Department of Pharmaceutical Sciences, University of Nottingham, Nottingham). Deoxythioguanosine was made by Dr Xu in this laboratory. Thioguanine, N-methyl-N-nitrosourea and methyl methanesulphonate were purchased from Sigma.

### ***Working Safely with Carcinogens***

Temozolomide and N-methyl-N-nitrosourea are very potent carcinogens and were handled with great care. In this Department carcinogens are stored in a locked freezer in a separate Hazard laboratory which is used only for the handling of carcinogens and radioactive materials. Dispensing of the solid material from stock was done only by Professor Swann. Gloves were worn whenever the solutions were handled. Utensils were decontaminated in alkaline aqueous solution, which very rapidly decomposes these carcinogens to innocuous products. Benches were wiped with alkaline solution at the end of each experiment. Animals do not excrete these carcinogens but, as a precaution, the animal bedding was double bagged for incineration and the cages washed before being returned to stock. Gloves were always worn when toxic but less hazardous materials such as the possible carcinogens methyl methanesulphonate and ethidium bromide and the neurotoxin acrylamide were handled and any spills were immediately cleaned up.

## **Treatment of Xenograft Bearing Nude Mice with Thioguanine and Temozolomide**

### **Production of Glioblastoma and Melanoma Xenografts in Nude Mice**

Athymic MF-1 (*nu/nu* genotype) mice were bred at the Royal Free Hospital, London, UK. The mice were housed in a barrier facility with 12 hour light and dark cycles and were provided with food and water *ad libitum*. The human glioblastoma astrocytoma cell line U87MG was obtained from the European Tissue Culture Collection, Porton Down, UK. The human malignant melanoma tumour cell line A375P was kindly supplied by Professor Ian Hart, Dimbleby Department of Cancer Research, St. Thomas's Hospital, Lambeth Palace Rd, London, UK. Both cell lines were grown as monolayers in Dulbecco's modified Eagle medium (Gibco, Paisley, UK), supplemented with 10% (v/v) heat inactivated fetal calf serum (Gibco, Paisley, UK), L-glutamine (2mM), penicillin (100 U/ml) and streptomycin (100 µg/ml). Xenografts were initially established by subcutaneous injection of  $10^7$  cells in 0.2 ml PBS into the right hind flank of the animal. The tumour was allowed to grow for 28 days before it was removed and cut into pieces measuring 2 mm on each side. The tumour pieces were transplanted subcutaneously onto new mice and allowed to grow. Tumour length, width and depth were measured using digital calipers (Mitutoyo (UK) Ltd) and tumour volumes were calculated using the formula, length x width x depth x 0.5 as previously used by Friedman *et al* (Friedman *et al.*, 1995). This process of passaging the tumour was repeated 6 times. On the 6<sup>th</sup> passage the glioblastoma xenograft took 8 days and the melanoma xenograft took 7 days to reach 100 mm<sup>3</sup>. Xenograft from the 6<sup>th</sup> passage were then established in 50 mice. The tumours were measured daily and dosing began when the mean tumour volume was 100 mm<sup>3</sup>. Xenograft sizes ranged from those that were too small to accurately measure to 400

mm<sup>3</sup>, however, only those that were between 70 and 200 mm<sup>3</sup> were included in the experiment. The animals were randomly assigned to the 4 dosing groups, control, thioguanine only, temozolomide only, thioguanine and temozolomide.

### **Treatment of Xenografts with Thioguanine and Temozolomide**

Mice with tumours between 70 and 200 mm<sup>3</sup> were randomly assigned to each group on the first day of treatment which is day 0 in fig. 2.4 and 2.5. For the glioblastoma experiment there were 7 mice per treatment group and for the melanoma experiment there were 10 mice per treatment group. Dosing solutions were prepared immediately prior to use and given at a volume of 10 µl/g of body weight by intraperitoneal injection. Thioguanine was dissolved in 0.15M NaOH followed by neutralisation with HCl. Temozolomide was dissolved in dimethylsulphoxide and then added to a solution of phosphate buffered saline at 10% (v/v) of the total volume. Thioguanine was given in 4 daily doses of 4 mg/kg body weight. The temozolomide was given as a single dose of 5 mg/kg body weight, 8 h after the final thioguanine dose.

### **Evaluation of Xenograft Response**

Tumour volume was measured at daily intervals. Tumour response was assessed by the delay in tumour growth, calculated as the difference in the mean time for tumours in the treated and control animals to reach a volume of 1000 mm<sup>3</sup>.

### **Treatment of Glioblastoma and Melanoma Cells in Culture with Thioguanine and Temozolomide**

All cell lines used for cytotoxicity assays with thioguanine and temozolomide (U87MG, A375P, HT29 and HeLa) were grown as monolayers in Dulbecco's modified Eagle medium (Gibco, Paisley, UK). This medium was supplemented with 10% (v/v) heat inactivated fetal calf serum (Gibco, Paisley, UK), L-glutamine

(2mM), penicillin (100 U/ml) and streptomycin (100 µg/ml). The cells were maintained in sub-confluent exponential growth before harvesting.

### **Clonogenic assays**

For measurement of colony formation, the cells were seeded onto 75-mm diameter tissue culture dishes at 1000 cells per plate. At least 4 plates of cells were used in the assessment of the toxicity of each dose. After allowing the cells to attach for 24 h the medium was replaced with either normal medium or thioguanine containing medium as appropriate. After a further 3 days, the medium was replaced with either normal medium or containing temozolomide. Thioguanine solutions were prepared in tissue culture medium and added directly to the cells. Temozolomide was dissolved in dimethyl sulphoxide (DMSO) and 10 µl of this solution added for each ml of medium.

The cells were then incubated for approximately 10 days or until colonies of around 50 cells had formed. The colonies were fixed in absolute methanol and stained in 1% (w/v) crystal violet in methanol. Cytotoxicity was assessed by counting the number of colonies on each plate using a colony counter.

### **3-(4,5-dimethylthiazol-2-yl)-2,5, diphenyltetrazolium bromide (MTT)-Based Cytotoxicity Assays**

MTT is a water-soluble tetrazolium dye that is reduced by live, but not dead cells to a purple formazan product that is insoluble in aqueous solutions. Cells were plated at 400 cells per well onto 12 well plates, allowed to attach for 24 h, and the medium exchanged with either normal or thioguanine containing medium. After 96 h, MMS or temozolomide containing medium was added. The MTT assay was performed 9 days after first plating by removing the medium, addition of 200 µl of MTT solution (1mg/ml) and incubation at 37°C, 4% CO<sub>2</sub> for 3 h. The MTT

solution was removed, 200 µl of propan-2-ol added and the plates gently agitated on an orbital shaker for 30 minutes. The propanol solution was removed from each well, centrifuged in order to remove cell debris and the absorbance of each sample was read at 570 nm.

A second MTT based method was also used which is suitable for use with suspension cultures since a water soluble formazan product is formed. In this case the tetrazolium substrate is (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS), together with an electron coupling reagent, phenazine methosulfate. The use of a soluble substrate enables the direct measurement of absorbance from a 96 well assay plate without removal of medium and resuspension in organic solvent. The quantity of formazan product measured by the amount of absorbance at 490 nm is proportional to the number of living cells in culture.

### ***Measurement of Mismatch Repair Gene Expression in U87MG and A375P cells by Reverse-Transcription Polymerase Chain Reaction (RT-PCR)***

#### **Extraction of Total RNA**

Total RNA was extracted using the Ultraspec RNA Isolation System (Biotecx Laboratories, Inc., Texas, USA). The cells used for RNA preparation had been harvested during exponential growth, pelleted and stored at -70°C for no more than 2 weeks. Pellets containing  $1 \times 10^7$  cells were resuspended in 1 ml of Ultraspec™ solution by repetitive pipetting. Following homogenisation, the homogenate was stored on ice for 5 minutes to allow the complete dissociation of nucleoprotein complexes. Chloroform (0.2 ml) was added and mixed vigorously for 15 seconds. The tubes were then stored on ice for 5 minutes before centrifugation at 12,000 g at 4°C for 15 minutes. After the addition of chloroform and centrifugation, the homogenate forms two phases: the lower phase is the

organic phase and the upper is the aqueous phase. DNA and protein are in the organic and interphase while RNA is in the aqueous phase. To precipitate the RNA, the aqueous phase was carefully transferred to a fresh tube while taking care not to disturb the interphase. An equal volume of propan-2-ol was added and the sample stored on ice for 10 minutes. The samples were then centrifuged at 12,000 g at 4°C for 10 minutes. The RNA precipitate was visible as a white pellet at the bottom of the tube. The supernatant was removed and the pellet washed twice in 75% (v/v) ethanol (1ml of 75% ethanol/1ml initial Ultraspec solution used). Washing was by vortexing and centrifugation for 5 minutes at 7,500 g at 4°C. The RNA pellet was dried for 5 to 10 minutes and redissolved in 50 to 100 µl of diethylpyrocarbonate (DEPC) treated water. Incubation at 60°C was occasionally required to fully resuspend the RNA pellet.

### **Measurement of Yield and Purity of Extracted Total RNA**

Appropriate dilutions of the RNA solution were made and absorbance readings at 260 and 280 nm were taken. RNA preparations that are free of DNA and proteins have  $A_{260}/A_{280}$  ratios of 1.8 to 2.0. The RNA concentration was calculated from the relation 1 OD unit = 40 µg RNA per ml. A typical yield from  $1 \times 10^7$  mammalian cells was 100 to 200 µg RNA

### **Electrophoresis of RNA**

#### **Preparation of Denaturing Agarose Gel (1.2 % Agarose, 2.2 M Formaldehyde)**

Agarose (4.8 g) was measured into a 500 ml conical flask with 20x PB and 308 ml of distilled water and melted in a microwave oven at medium power with intermittent stirring. The agarose solution was allowed to cool to about 60°C (hand hot) and formaldehyde (72ml, 37% w/v) added in a fume cupboard. The mixture was immediately poured into a 25-cm horizontal gel casting tray (Maxi Gel System,

Hybaid, UK) until the depth of the liquid was about 10-mm, the required combs were inserted and the gel was left to set for about 30 minutes.

### **Agarose Gel Electrophoresis of Total RNA**

RNA was prepared for electrophoresis by the addition of three volumes of denaturing mix [formaldehyde (24 % w/v), formamide (8.8 % w/v) in 13.3 mM phosphate buffer, pH 7.4/0.67 mM EDTA] to one volume of RNA (4mg/ml). The RNA was denatured (65°C, 30 min) then snap cooled on ice and 0.1 vol loading buffer [50% (v/v) glycerol, 0.4% (w/v) bromophenol blue in 10x PB/1 mM EDTA] added. Samples (20µl) of the mixture were electrophoresed on a 1.2% denaturing gel (4V/cm, 1 h) using 1x PB containing 0.1 µg/ml ethidium bromide as electrophoresis buffer. When the bromophenol blue had moved to within 3 cm of the edge of the gel, the gel was photographed. The appearance of ribosomal RNA bands and the absence of smearing were used as a measure of the quality of the RNA.

### **Reverse Transcription of Total RNA**

For cDNA synthesis, the isolated total RNA was reverse transcribed in 20  $\mu$ l reactions containing 0.5  $\mu$ g of random primers, 200 units of Moloney murine leukemia virus reverse transcriptase (Promega, UK), 1  $\mu$ g of total RNA, 50 mM 5x RT buffer (250 mM Tris-HCl, pH 8.3/375 mM KCL/50 mM DTT/15 mM MgCl<sub>2</sub>), 0.05 mM each dNTP, 20 units RNasin (Promega) and diethyl-pyrocabonate treated water. Each reaction was incubated at room temperature for 10 minutes, 42°C for 45 minutes, 90°C for 10 minutes and then quick chilled on ice.

Table 2.1. Primer Sequences used in the RT-PCR Reactions

| Gene           | Primer Sequence                         | Position of 5' nucleotide of primer in cDNA | PCR Product Size |
|----------------|---|---|------------------|
| $\beta$ -actin | 5'-ACACTGTGCCCATCTACGAGG-3' (sense)     | 2,147                                       | 621*             |
|                | 5'-AGGGGCCGGACTCGTCATACT-3' (antisense) | 2,954                                       |                  |
| <i>hMSH2</i>   | 5'-GTCGGCTTCGTGCGCTTCTTT-3' (sense)     | 52  | 429              |
|                | 5'-TCTCTGGCCATCAACTGCGGA-3' (antisense) | 460   |                  |
| <i>hPMS2</i>   | 5'-TGCATGCAGCGGATTTGGAAA-3' (sense)     | 389   | 338              |
|                | 5'-GAACCCCTCAGAATCCACGGA-3' (antisense) | 726   |                  |
| <i>hMSH6-1</i> | 5'-CCCTCAGCCACCAAAGAAGCA-3' (sense)     | 838   | 268              |
|                | 5'-CTGCCACCACTTCTCATCCC-3' (antisense)  | 1,105                                       |                  |
| <i>hMSH6-2</i> | 5'-AGATGCGGTGCTTTTAGGAG-3' (sense)      | 28  | 285              |
|                | 5'-CCCTCCGTTGAGGTTCTTC-3' (antisense)   | 312   |                  |
| <i>hMLH1-1</i> | 5'-GTGCTGGCAATCAAGGGACCC-3' (sense)     | 466   | 215              |
|                | 5'-CACGGTTGAGGCATTGGGTAG-3' (antisense) | 660   |                  |
| <i>hMLH1-2</i> | 5'-GTTCGTGGCAGGGGTTATT-3' (sense)       | 27  | 193              |
|                | 5'-GGTGCCATTGTCTTGGATCT-3' (antisense)  | 219   |                  |
| <i>hPMS1</i>   | 5'-GCGGCAACAGTTCGACTCCTT-3' (sense)     | 96  | 154              |
|                | 5'-AGCCTTGATSCCCTCCCGTT-3' (antisense)  | 249   |                  |

\*The  $\beta$ -actin fragment was used to monitor DNA contamination. The 621-bp fragment of  $\beta$ -actin cDNA extends from exon 3 to exon 5. Intron 3 is 95 base pairs and intron 4 is 112 base pairs so if the cDNA was contaminated with genomic DNA the PCR product would contain a 828 base pair band.

### **Sequences of Primers used for RT-PCR**

The primer sequences for RT-PCR of mismatch repair genes in human cells are shown in table 1. The cDNA sequences were obtained from the NCBI Entrez nucleotide database at <http://www.ncbi.nlm.nih.gov/Entrez>. Primer sequences except MSH6-2, and MLH1-2 were the same as those used by Wei *et al* (Wei *et al.*, 1997). Other sequences were designed using a web based primer design program [http://www.genome.wi.mit.edu/cgi-bin/primer/primer3\\_www.cgi](http://www.genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi).

### **Detection of Mismatch Repair Proteins in U87MG and A375P Cells**

#### **Preparation of Protein Extracts of U87MG and A375P Cells**

The method of protein extraction used was the same as that of Nedderman and Jiricny (Nedderman and Jiricny, 1993). All procedures were carried out in a 4°C cold room. Frozen pellets of  $1 \times 10^7$  cells were quick thawed in a 37°C water bath and allowed to swell in three cell volumes of hypotonic buffer (25mM HEPES/NaOH, pH 7.8, 1mM EDTA, 2mM dithiothreitol, 1mM phenylmethylsulfonyl fluoride, 0.5 mM spermidine and 0.1 mM spermine) at 4°C for 20 min. The cells were homogenised in a glass Dounce homogeniser with 20 strokes of a tight fitting pestle, glycerol added to give a final concentration of 20% (v/v), followed by a solution of saturated neutralized  $(\text{NH}_4)_2\text{SO}_4$  (11ml/100 ml of extract). The suspension was stored on ice for 30 minutes to allow complete mixture and the extract cleared by centrifugation in a Beckman ultracentrifuge using a Ti-70 rotor at 370,000g (60,000 rpm) for 90 min at 4°C. The resulting supernatant was stored in 20 µl aliquots at -70°C.

#### **Estimation of Protein Content of Cell Extracts**

The Bio-Rad Protein Microassay was used for protein estimation (Bio-Rad Laboratories Ltd, Hertfordshire, England). This assay is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-

250 shifts from 465 nm to 595 nm when binding to protein occurs, and is based on that of Bradford (Bradford, 1976). Bovine serum albumin was used to produce a standard curve of concentration range 1 to 25  $\mu\text{g}/\text{ml}$ . Standards (0.8 ml) and appropriately diluted samples were added to clean, dry eppendorf tubes with 0.8 ml of sample buffer used as a blank. Dye Reagent Concentrate (0.2 ml) was added to each tube and vortexed, avoiding excessive foaming. After a period of 5 min to 1 h the absorbance at 595 nm was measured versus reagent blank. A standard curve of  $A_{595}$  versus concentration of standards was then plotted to determine the protein content of the unknown samples.

### **SDS-PAGE of Protein**

This was based on the original method of Laemmli (Laemmli, 1970). To make a 7% resolving gel, 30% (w/v) acrylamide/0.8% (w/v) methylene-bisacrylamide (2.3 ml, Anachem), pH 8.8 buffer (2.5 ml) and distilled water (5.0 ml) were mixed in a vacuum flask. The mixture was degassed by vacuum for 10 minutes. TEMED (6  $\mu\text{l}$ ) and 10% (w/v) ammonium persulphate (100  $\mu\text{l}$ ) were added. This was gently mixed and immediately poured between the glass plates of the electrophoresis apparatus (Mini-Protean II, Bio-Rad) assembled with 0.75-mm spacers. The gel was overlaid with propan-2-ol and left to polymerise for at least 1 hour. The stacking gel was prepared by mixing 30% acrylamide (0.83 ml), pH 6.8 buffer (0.63 ml), TEMED (5  $\mu\text{l}$ ), 10% (w/v) ammonium persulphate (50  $\mu\text{l}$ ) and water (3.4 ml). The stacking gel was mixed and immediately poured over the resolving gel.

### **Preparation and Electrophoresis of Samples**

Protein samples were prepared for electrophoresis by the addition of loading buffer (1% (w/v) SDS/ 30 mM Tris HCl (pH 6.8)/ 5% Glycerol/ 0.02% (w/v) bromophenol blue).  $\beta$ -mercaptoethanol (1% (v/v)) was added to disrupt disulphide linkages and the mixture was heated to 90°C for 5 minutes. The sample

was centrifuged briefly in a microcentrifuge to sediment any debris and collect the sample at the bottom. 15  $\mu$ l of the supernatant was loaded onto the gel and electrophoresed in 0.025M Tris/ 0.192 M glycine/ 0.1%(w/v) SDS, pH 8.4 at 10 V/cm until the dye entered the resolving gel. The current was then increased to 20 V/cm until the dye had migrated to roughly 0.5 cm of the end of the gel.

### **Staining of Gels**

Gels were transferred to a container with 5 volumes of Coomassie blue staining solution (0.25 g Coomassie Brilliant Blue R250 in 100 ml methanol:water:glacial acetic acid (45:45:10) filtered through Whatman No. 1 filter paper) and left gently agitating on a rotating platform for at least 4 hours. The gel was destained with several changes of methanol:water:acetic acid (45:45:10) until the bands were distinct and the background clear. The stained gel was photographed on an illuminated light box.

### **Western Blot**

To detect the presence of specific mismatch repair proteins, the SDS-PAGE gel was blotted onto a nitrocellulose membrane (Hybond ECL, Amersham). Antigen detection was carried out using the Enhanced Chemiluminescence (ECL) Plus Western Blot Kit (Amersham) according to the manufacturer's instructions. Briefly, the resolving gel was placed on a sheet of Hybond ECL cut to the same size as the gel. The gel/membrane was sandwiched between 2 sheets of Whatman 3MM chromatography paper of approximately same size as the gel/membrane. This was placed in a cassette between sponge pads and inserted into a blotting tank (Bio-Rad) containing enough transfer buffer (192 mM glycine/25mM Tris, pH 8.4/20% (v/v) methanol) to completely submerge the cassette. The protein was transferred overnight from the gel to the membrane by electrophoresis at 30V. The membrane was removed and placed in a plastic box (9.5 x 9.5 x 1.5 cm), blotted surface facing

upwards. Remaining protein binding sites were blocked with a 8% (w/v) solution of blocking reagent (Marvel milk powder) in TBS-T (10ml) with agitation on a rotating platform for 1 hour at room temperature. The blocking reagent mixture was poured off and the membrane washed for 20 minutes with 4 changes of TBS-T (10 ml). The primary antibodies of appropriate dilution in 4% (w/v) blocking agent in TBS were added to the membrane and agitated for 1 hour as before. The primary antibody solution was poured off and the membrane was washed with TBS-T as before. The horseradish peroxidase linked secondary antibody was used at an appropriate dilution, as determined by titration blotting experiments and was prepared in 4% (w/v) blocking agent in TBS-T (10 ml). The membrane was incubated with the secondary antibody for 1 hour followed by washing in TBS-T as before. Enhanced chemiluminescent (ECL) detection was carried out according to the manufacturer's instructions (Amersham).

### **Staining of Nitrocellulose Blots**

Amido black staining solution (0.1% (w/v) Amido Black/25 % (v/v) isopropanol/10% (v/v) acetic acid) was routinely used for the staining of nitrocellulose blots after blotting and ECL detection (Gershoni and Palade, 1982). The solution can detect microgram quantities of proteins transferred onto nitrocellulose membranes. The membrane was immersed in sufficient Amido Black staining solution to cover and stained for 1 minute. The membrane was destained by placing in an aqueous solution of Amido Black destain [25% (v/v) isopropanol/10% acetic acid] for 30 minutes. If a lighter background was necessary, the membrane was destained overnight. The destained membrane was then stored in deionized water or allowed to air dry.

### **Mobility Shift DNA-Binding Assay using Gel Electrophoresis**

The DNA binding assay using non-denaturing PAGE is a quick and sensitive method for detecting sequence-specific DNA-binding proteins. Proteins that bind specifically to a DNA fragment retard the mobility of the fragment during electrophoresis. If the DNA has been 5' [<sup>32</sup>P] end labelled the discrete bands corresponding to the individual protein DNA complexes can be detected by autoradiography. This assay was used for the detection of protein binding to a heteroduplex DNA containing a single GT mismatch.

### **Oligonucleotides used for Mobility Shift Assay**

Oligonucleotides were prepared by solid phase synthesis on an Applied Systems 380B DNA synthesiser. 34 base pair duplexes of general sequence AGC TTG GCT GCA GGN GGA CGG ATC CCC GGG AAT T (where N is C or T) were used. The complementary strand contained a G opposite the N position to form a perfectly matched G·C pair or a G·T mismatch.

### **Radioactive end-labelling of oligonucleotide**

The oligonucleotide was labelled in a 10 µl reaction containing 1.92 µM 34-mer oligonucleotide, buffer (50 mM Tris-HCl, pH 7.6, 10 mM MgCl<sub>2</sub> 5mM DTT, 0.1 mM spermidine, 0.1 mM EDTA), 3 µl [<sup>32</sup>P]ATP (10 µCi/µl) and 1 µl T4 polynucleotide kinase (10U/µl). The reaction was mixed, briefly centrifuged and incubated at 37°C for 3 h followed by 70°C for 10 minutes to stop the reaction. 2x annealing buffer (100 mM Hepes (pH 7.6)/40 mM MgCl<sub>2</sub>/200 mM KCl) was added to dilute the oligo to 100 nM.

### **Annealing of DNA Duplexes**

Annealing of the complementary strand to the end labelled strand was carried out in annealing buffer (50 mM Hepes (pH 7.6)/20 mM MgCl<sub>2</sub>/100 mM KCl.). The

mixture was heated to 70°C for 5 min to separate DNA single strands and allowed to cool slowly to room temperature to facilitate annealing. Annealing of non-labelled duplexes was carried out in the same way.

### **Buffer Exchange of Protein Extracts**

The buffer used for protein extraction is not compatible with the electro-mobility shift assay because it contains a high ammonium sulphate concentration, which can interfere with protein binding. Therefore the protein extracts were exchanged into gel shift buffer (25 mM Hepes (pH 7.6)/400 mM KCl/1 mM EDTA/1 mM benzamidine/20% (v/v) glycerol/5 mM DTT). This buffer was made without the DTT, which was added fresh each time. Bio-spin 30 columns (Bio-rad) were used for buffer exchanging, following the manufacturer's instructions.

### **Binding Reactions**

Reactions were carried out at 20°C in mismatch binding buffer (40 mM Hepes (pH 7.6)/10 mM MgCl<sub>2</sub>/8% Ficoll/4 mM DTT). Cell protein extracts (11 µg) were pre-incubated on ice for 15 min with 50 µg/ml poly(dI.dC).poly(dI.dC) in mismatch binding buffer (20 µl). An appropriate volume of 1 M KCl was added to each pre-incubation reaction to bring the salt concentration to 50 mM. To 25 f mol (0.5 µl of 50 nM) of <sup>32</sup>P-labelled heteroduplex was added 1 pmol (5 µl of 200 nM) of cold competitor oligonucleotide as appropriate. Oligonucleotide solution (11 µl) was added to 8.9 µl of the pre-incubation mixture, mixed, briefly centrifuged and left at room temperature for 20 minutes.

### **Preparation of Native Polyacrylamide Gel**

For the analysis of the binding reactions, 6% native polyacrylamide gel electrophoresis was used. Routinely, 500 ml of polyacrylamide gel mixture was prepared by mixing 10 ml of 40% (19:1) acrylamide solution, 12.5 ml of 10X TBE

buffer and 487.5 ml of water. Immediately prior to pouring 250  $\mu$ l of 10% ammonium persulphate and 15  $\mu$ l of TEMED was added to 80 ml of the polyacrylamide gel solution. A slab gel system was used for electrophoresis (Hoeffer) with gel size of 17 x 17 cm. One silicon treated glass plate was used with one non-silicon treated. The electrophoresis buffer used was 0.5X TBE (45mM Tris base/ 1 mM EDTA/ 45 mM boric acid) containing 0.05 %(v/v) Triton X100. The gel was pre-run for 1 hour at 20 V/cm, after which the buffers in the upper and lower chambers were mixed. 9.6  $\mu$ l of each sample was loaded onto the gel and 9.6  $\mu$ l of mismatch binding buffer containing 1% bromophenol blue was loaded into the end lane in order to follow the progress of the electrophoresis. The gel was run at 7 V/cm until the dye front had migrated to within 4 cm of the bottom of the gel. After electrophoresis the glass plate/gel sandwich was removed from the apparatus and the plates carefully separated. The gel remained attached to the non-siliconised glass plate. The gel was then pressed firmly against a piece of 3-MM Whatman paper, cut slightly larger than the gel. The 3-MM paper was carefully peeled away from the glass plate with the gel attached to the 3-MM paper. The gel was then covered in Saran wrap and dried in a gel dryer. When the gel was completely dry, it was placed in a film cassette and exposed to autoradiography film at  $-70^{\circ}\text{C}$  overnight.

### ***Measurement of thioguanine methylation by methylating agents***

#### **Reaction of deoxyguanosine and deoxy-6-thioguanosine with N-methyl-N-nitrosourea (MNU) and methyl methanesulphonate (MMS).**

Solutions of deoxyguanosine (dG) and deoxythioguanosine (dG<sup>s</sup>) were prepared by dissolving 5 mg of each in 500  $\mu$ l cacodylate (hydroxydimethylarsine oxide) buffer, (0.3 M, pH 7.6). For reaction with MMS, 100  $\mu$ l of the solution of each nucleoside i.e. approximately 3.4  $\mu$ moles, were added to separate eppendorf tubes (1.5 ml) and 16  $\mu$ l of an etherial solution of MMS (0.22 mmol/ $\mu$ l ether) was added. For reaction

with MNU, again 100  $\mu$ l aliquots were taken from each nucleoside solution and 7  $\mu$ l of MNU (0.09 mmol/ $\mu$ l ether) was added. Reactions were mixed and left to react overnight.

#### **Analysis by thin layer chromatography**

Thin layer chromatography analysis was performed using silica gel 60 F<sub>254</sub> coated aluminium sheets (Merck) cut to convenient size. Starting material (0.5  $\mu$ l) and reaction product were applied to the origin, a pencil line 1 cm from the bottom of the TLC plate. The TLC plates were resolved with CH<sub>3</sub>OH/CHCl<sub>3</sub> (1:4 by vol).

#### **Analysis by HPLC**

Nucleoside analysis was performed using reverse phase HPLC on a Varian 9010 system using a Nova-Pak C18, 3.9 x 150 mm column (Waters) with a Shimadzu SPD-10A UV spectrophotometric detector. The analogue output from the detector was passed through an A/D board (Strawberry Tree Computers, Inc., California, USA) to an Apple Macintosh. The data was graphed and the area under each peak calculated using Chrom<sup>TM</sup> software (Strawberry Tree). The gradient was formed from buffer A (50 mM KH<sub>2</sub>PO<sub>4</sub>) and buffer B (50 mM KH<sub>2</sub>PO<sub>4</sub> in CH<sub>3</sub>CN:H<sub>2</sub>O (2:1 by vol.)). The column was eluted at a flow rate of 1 ml/min with 90% buffer A/10% buffer B for 10 min, then with a linear gradient from 90% buffer A/10% buffer B to 40% buffer A/60% % buffer B over 10 min, and then to 20% buffer A/80%buffer B over 5 min and held at this buffer ratio for 3 minutes, then back to 90% buffer A/10% buffer B over 2 min.

#### **Measurement of the stability deoxy(6-methyl)thioguanine in acid**

The product of the reaction between dG<sup>S</sup> and MMS was collected by HPLC. This was confirmed to be deoxy(6-methyl)thioguanosine by its characteristic UV profile

between 220 and 350 nm. Aliquots (20  $\mu$ l) were taken, 2 $\mu$ l of HCl (1M) added, and heated at either 70°C or 90°C for 10, 20, 30 or 60 minutes. After hydrolysis, the reaction was again analysed by reverse phase HPLC to measure the amount of methylthioguanine formed under each reaction condition.

### **Reaction of DNA containing thioguanine with N-methyl-N-nitrosourea and methyl methanesulphonate**

#### **Synthesis of oligonucleotides**

For the study of the reaction of methylating agents with thioguanine in DNA, 11mer oligonucleotide duplexes were used. One duplex contained perfectly matched natural bases while the other contained a single thioguanine residue paired with a cytosine. The sequence of the top strand was CAGATXTACGC, where X was either G or G<sup>S</sup> and the sequence of the bottom strand was GCGTACATCTG. The non-thioguanine containing oligonucleotides were purchased from Sigma Genosys whereas the thioguanine containing oligonucleotide was prepared by solid phase synthesis on an Applied Systems 380B DNA synthesiser by Duncan Odom of California Institute of Technology, Pasadena, USA.

#### **Purification of oligonucleotides by FPLC**

Following synthesis, each oligonucleotide was purified by fast protein liquid chromatography (FPLC) on a Dionex BIOLC system with a Dionex variable wavelength detector using a Pharmacia monoQ HR5-5 column. The gradient was formed from buffer A (10 mM NaOH/0.2 M NaCl) and buffer B (1.2 M NaCl, 20 mM NaOH). All buffers were made using water purified by a milliQ water system (Millipore) and filtered using 0.4 $\mu$  nylon membrane filters (Millipore). The column was eluted at a flow rate of 1 ml/min with 95% buffer A/5% buffer B for 5 min, then with a linear gradient from 95% buffer a/5% buffer B to 75% buffer A/25 % buffer B over 2 min, and then to 60% buffer A/40%buffer B over 20 min, then to

100% buffer B over 2 min and back to 95% buffer A/5% buffer B over 2 minutes. The desired peak was collected and immediately neutralised with 1M acetic acid and desalted using a Nap25 (Sephadex G-25) column according to the manufacturer's instructions (Pharmacia Biotech).

### **Reaction of oligonucleotide duplexes with N-methyl-N-nitrosourea and methyl methanesulphonate**

Each single strand (0.25 OD i.e., approximately  $15 \times 10^9$  moles of oligonucleotide) of the oligonucleotide duplex was annealed to its complementary strand in cacodylate annealing buffer (25 mM cacodylate, pH 7.6, 10 mM  $MgCl_2$ , 50 mM KCl) by heating at 70°C for 5 min and allowing to slowly cool to room temperature. [ $^{14}C$ ]MMS (55 mCi/mmmole) and [ $^{14}C$ ]MNU (63 mCi/mmmole) were stored at -20°C in ether. [ $^{14}C$ ]methyl methanesulphonate was prepared by reaction between [ $^{14}C$ ]methyl iodide (Amersham) and silver methanesulphonate (Eastman Kodak) (Swann and Magee, 1968). N-nitroso-N-[ $^{14}C$ ]methylnitrosourea was prepared from [ $^{14}C$ ]methylamine (Amersham) by the method of Cox and Warne 500,000 dpm, was added to the annealed duplex solution. The reaction was left at room temperature overnight. The reaction was then transferred into a 50 ml pear-shaped flask which was attached to a vacuum freeze-drying apparatus and dried for at least 2 hours. The drying procedure was performed in order to remove the [ $^{14}C$ ]methanol produced by hydrolysis of the methylating agents. After drying, the reaction was resuspended in 100  $\mu$ l  $H_2O$  and 1M HCl (11  $\mu$ l) so that the final concentration was 0.1 M HCl. For depurination of the DNA, the solution was heated at 70°C for 40 minutes followed by neutralisation with 1M NaOH. The methylated purines produced by acid hydrolysis were separated by reverse phase HPLC on a Varian 9010 system using a 25cm x 0.4 cm stainless steel column packed with Hamilton HXC 8.0 (10-15  $\mu$ m part 77833) cation exchange resin with a Shimadzu SPD-10A UV spectrophotometric detector. The signal from the UV

detector was collected using Chrom<sup>TM</sup> for Apple Macintosh (Strawberry Tree Computers, Inc., California, USA). The sample was eluted with ammonium formate (0.4 M, pH 4.5) with the column in a 60°C water bath. Fractions (1ml) were collected for scintillation counting.

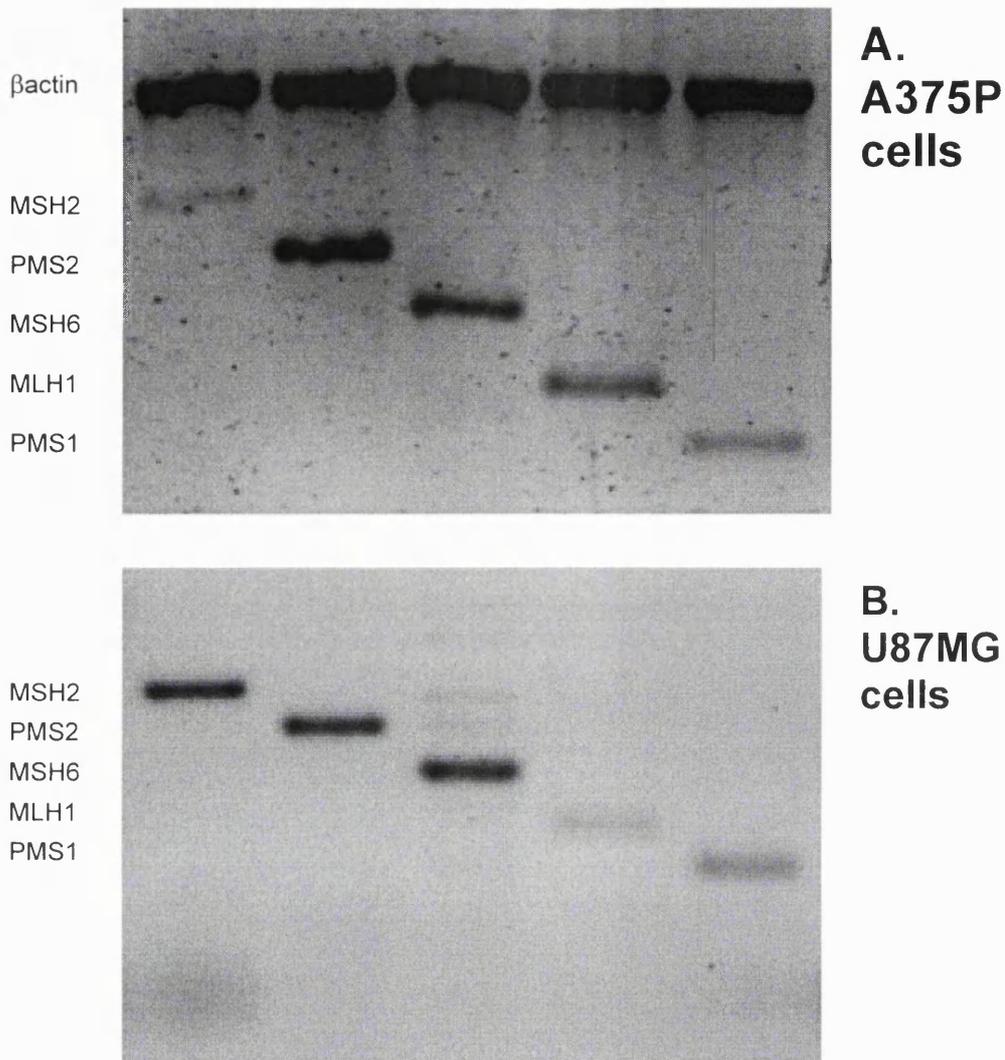
## Results

### ***Synergy between thioguanine and temozolomide in vivo***

#### **a. Confirmation that cell lines are post-replicative mismatch repair proficient**

Before testing the effect of temozolomide and thioguanine on the U87MG and A375P xenografts the cell lines were tested to confirm that they were post-replicative mismatch repair competent. Firstly, the expression of the mRNA for the mismatch repair genes, *hMSH2*, *hPMS2*, *hMLH1*, *hMSH6* and *hPSM1* was determined by semi-quantitative RT-PCR (Fig. 2.1). The expression of the  $\beta$ -actin gene was determined at the same time in order to normalise the expression of each mismatch repair gene relative to  $\beta$ -actin expression. Both cell lines express *hMSH2*, *hPMS2*, *hMSH6*, *hMLH1* and *hPSM1*. The expression of *hMSH2* mRNA is lower in U87MG cells than A375P cells and *hMLH1* mRNA expression is lower in A375P than U87MG. Expression of all the other mRNAs appears equal.

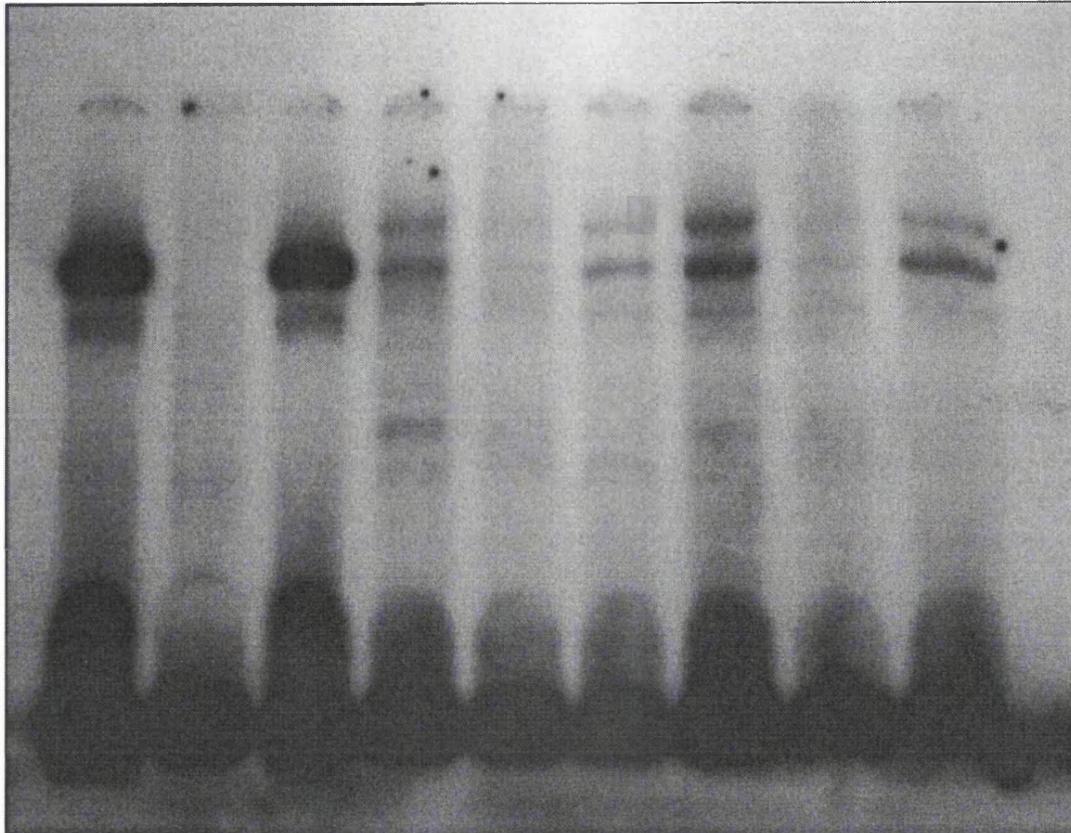
Secondly, the mismatch binding activity in U87MG and A375P cells was determined using the electro-mobility shift (gel shift) assay (fig 2.2). Whole cell protein extracts of each cell line was prepared. The extracts were incubated with a  $^{32}\text{P}$  end-labelled oligonucleotide duplex of 34 base pairs containing a single GT mismatch. DNA-protein binding was observed when the GT heteroduplex was incubated with HeLa, A375P and U87MG protein extract. The formation of the [ $^{32}\text{P}$ ]-labelled protein-DNA complex could be prevented by addition of 100 fold excess non-labelled, GT duplex oligonucleotide, but excess, non-labelled, perfectly matched oligonucleotide did not compete for GT binding, indicating that the binding was specific for the mismatch. Thirdly, western blotting was used to detect the amount of *hMSH2* and *hMLH1* protein in U87MG and A375P cells (fig. 2.3).



**Figure 2.1. Expression of mRNA for mismatch repair genes in A375P cells**

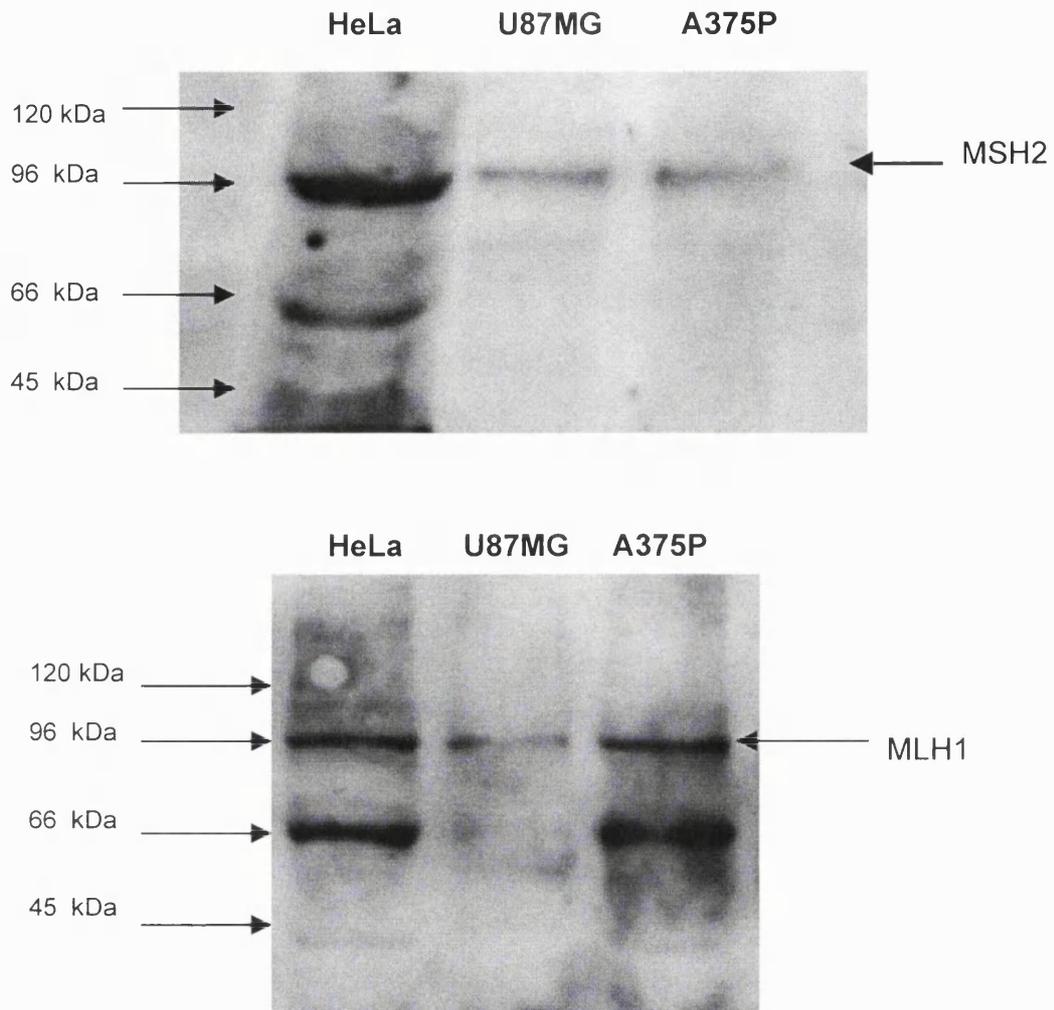
RT-PCR was used to measure the expression of the mRNAs for  $\beta$ -actin, hMSH2, hPMS2, hMLH1 and hMSH6 in A375P cells (A) and hMSH2, hPMS2, hMLH1 and hMSH6 in U87MG cells (B). The mRNAs for each of these mismatch repair genes was found to be expressed in both cell lines.

| Competitor | HeLa |     |     | U87MG |     |     | A375P |     |     |
|------------|------|-----|-----|-------|-----|-----|-------|-----|-----|
|            | None | +GT | +GC | None  | +GT | +GC | None  | +GT | +GC |



**Figure 2.2. Mismatch binding by protein extracts of HeLa and U87MG cells**

Protein extracts from HeLa, U87MG and A375P cells were tested for their binding to a synthetic radiolabelled duplex oligonucleotide containing a single GT mismatch. In all cells lines, no binding was detected when excess non-labelled GT containing oligonucleotide was added and competition with perfectly matched non-labelled duplex DNA did not result in any loss of binding. This result shows that U87MG and A375P cells contain a protein that is able to bind to GT mismatches which is likely to be MutS $\alpha$ .



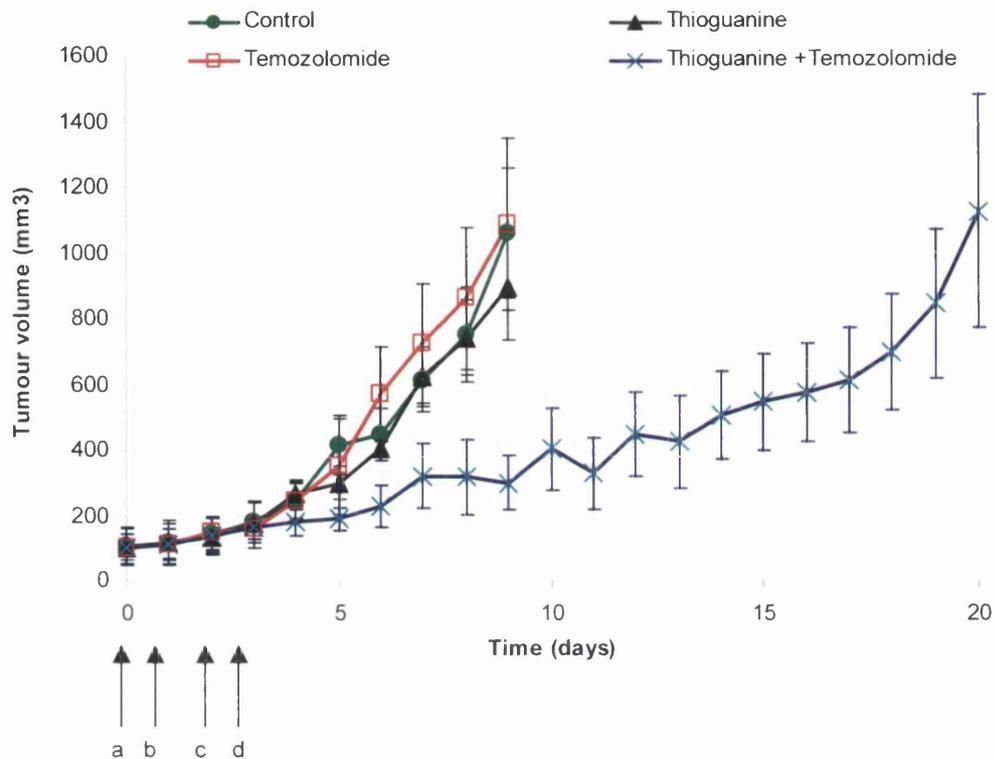
**Figure 2.3. Expression of hMSH2 and hMLH1 proteins in U87MG and A375P cells.**

Western blot was used to detect hMSH2 and hMLH1 protein in U87MG and A375P cells compared with HeLa cells. hMSH2 is a 100 kDa protein and hMLH1 is an 85 kDa protein.

**b. Establishment of xenografts in nude mice and test of effect of thioguanine and temozolomide**

The athymic nude-mouse xenograft model is widely used for the study of anti-cancer therapy. It has been an important tool in the pre-clinical study of temozolomide (Wedge and Newlands, 1996; Wedge *et al.*, 1997). In this study, the growth of both gliomablastoma and melanoma xenografts was measured after combined treatment with temozolomide and thioguanine. In both cases, the xenografts were established by the injection of  $1 \times 10^7$  cells into the right hand flank of the animal. When the tumour in this first mouse had grown, it was removed and cut into cubes 2 mm on each side and these cubes introduced into new mice. The xenografts were serially passaged 6 times before being introduced into the mice used for these experiments. The first xenografts took 28 days to grow to 100 cu mm but after the 5<sup>th</sup> passage, the glioma xenografts grew to 100 cu mm after 8 days and the melanoma xenografts grew to 100 cu mm after 7 days. After the 6<sup>th</sup> passage, each xenograft was transplanted onto 50 mice. The tumours were measured daily and dosing began when the mean tumour volume was 100 cu mm. On the first day of dosing xenograft sizes ranged from those that were too small to accurately measure to 400 cu mm, however, only those that were between 70 cu mm and 200 cu mm were included in the experiment. The animals were randomly assigned to the 4 dosing groups, control, thioguanine only, temozolomide only, thioguanine and temozolomide. For the glioma experiment, there were 7 mice per group and in the melanoma experiment there were 10 mice per group. Thioguanine was given by intraperitoneal injection in 4 daily doses at 4mg/kg-body weight. A single intraperitoneal dose of 5 mg/kg temozolomide was given 8 hours after the final thioguanine dose. The daily dose of thioguanine administered was chosen on the basis of the data of Wang *et al* (Wang *et al.*, 1991) and the dose of temozolomide was chosen from Wedge and Newlands' data (Wedge and Newlands, 1996). Daily measurement of the tumours continued until the volume reached the

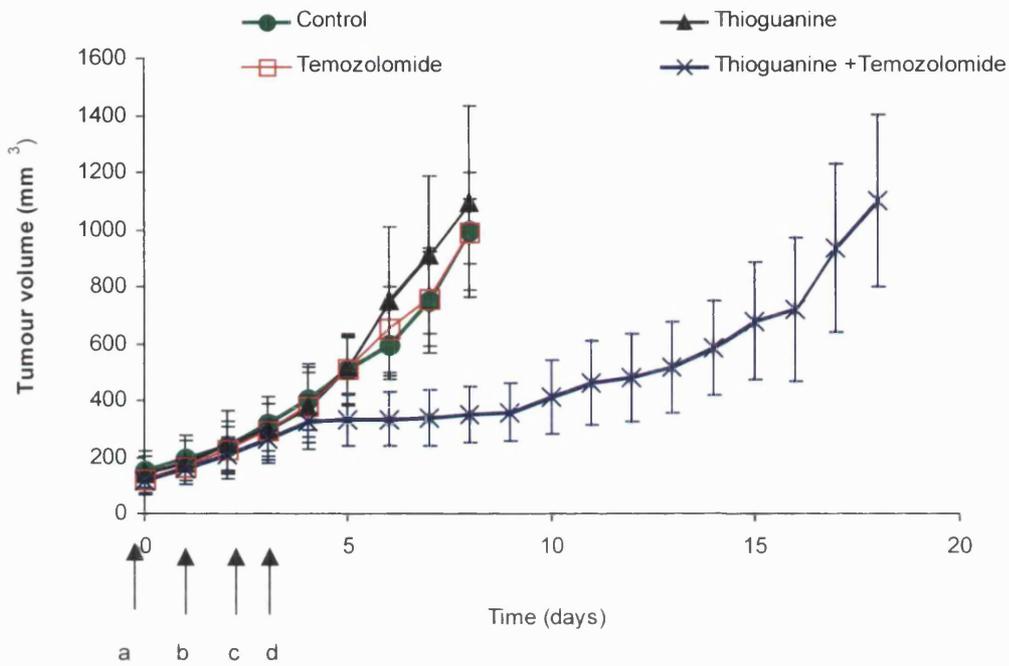
allowed limit of 1500 cu mm. Fig 2.4 and 2.5 show that the combination of thioguanine and temozolomide strongly inhibits the growth of glioblastoma and melanoma. When given alone neither thioguanine alone nor temozolomide had any effect on glioblastoma growth. However, treatment with both thioguanine and temozolomide strongly inhibited the growth of the tumours, such that they did not grow to 1000 cm<sup>3</sup> until 20 days after dosing began (i.e. 27 days after implantation). When given alone, neither thioguanine alone, nor temozolomide alone had any effect on melanoma growth, since the xenografts also grew to 1000 mm<sup>3</sup> after 9 days. However, once again treatment with both thioguanine and temozolomide strongly inhibited the growth of the tumours such that they did not grow to 1000 cm<sup>3</sup> until 21 days after dosing (i.e. 17 days after implantation).



- a thioguanine dosing begins
- b 2<sup>nd</sup> dose of thioguanine
- c 3<sup>rd</sup> dose of thioguanine
- d 4<sup>th</sup> dose of thioguanine followed by dose of temozolomide 8 h later

**Figure 2.4. Growth of glioblastoma xenografts after treatment with thioguanine and temozolomide**

The graph shows the mean volume  $\pm$  the SD. On the 8<sup>th</sup> day after transplantation the mice were randomised into 4 groups each of 7 mice and immediately given thioguanine (4 mg/kg i.p.). They were given 3 more doses on day 9, 10 and 11. A single dose of temozolomide (5mg/kg i.p.) was given 8 hours after the final dose of thioguanine.



- a thioguanine dosing begins
- b 2<sup>nd</sup> dose of thioguanine
- c 3<sup>rd</sup> dose of thioguanine
- d 4<sup>th</sup> dose of thioguanine followed by dose of temozolomide 8 h later

**Figure 2.5. Growth of melanoma xenografts after treatment with thioguanine and temozolomide**

The graph shows the mean volume  $\pm$  the SD. On the 9<sup>th</sup> day after transplantation the mice were randomised into 4 groups each of 7 mice and immediately given thioguanine (4 mg/kg i.p.). They were given 3 more doses on day 10, 11 and 12. A single dose of temozolomide (5 mg/kg i.p) was given 8 hours after the final dose of thioguanine.

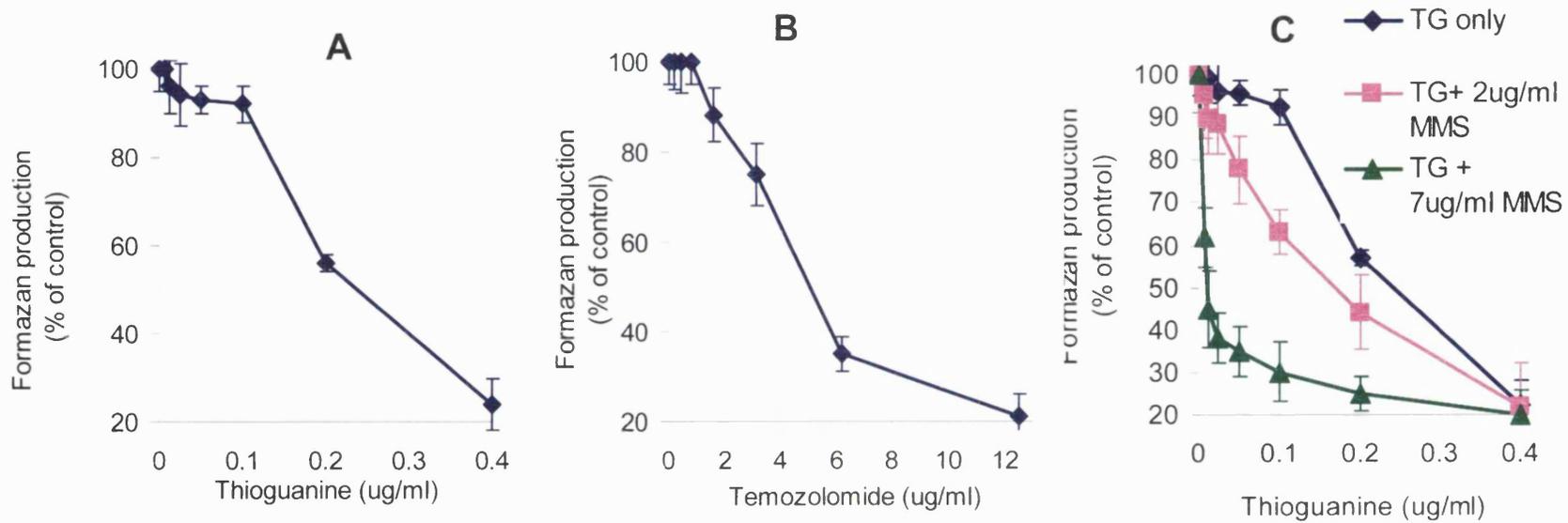
### **Synergy between thioguanine and methylating agents in vitro**

The *in vivo* experiment showed a strong synergy between thioguanine and temozolomide. To investigate further this cytotoxic synergy a series of tissue culture experiments was done. The best way of measuring cell death in response to drug treatment in culture is by clonogenic assay. This involves plating cells at low density so that colonies grow from a single cell. After the cells have adhered to the tissue culture dish they are treated followed by staining and counting of colonies after colonies of a suitable size have formed. Many cells have poor plating efficiency and are therefore unsuitable for clonogenic assays.

Attempts were made to assess cytotoxicity using the clonogenic assay with U87MG and A375P, however these cells have a low plating efficiency and were not strongly adherent to the dish. It was therefore very difficult to count the number of colonies formed because each time the medium was changed, loosely adhered cells would detach and form new smaller colonies around the original one. Coating of the tissue culture plates with fibronectin or vitronectin, proteins that are found in the extracellular matrix deposited by cultured adherent cells did not improve the adhesion of U87MG and A375P cells. An alternative method of assessing cytotoxicity, the 3-(4,5-dimethylthiazol-2-yl)-2,5, diphenyltetrazolium bromide (MTT) assay was therefore used for these cells. This assay does not directly measure cell death but measures the number of viable cells at some time after the drug is given.

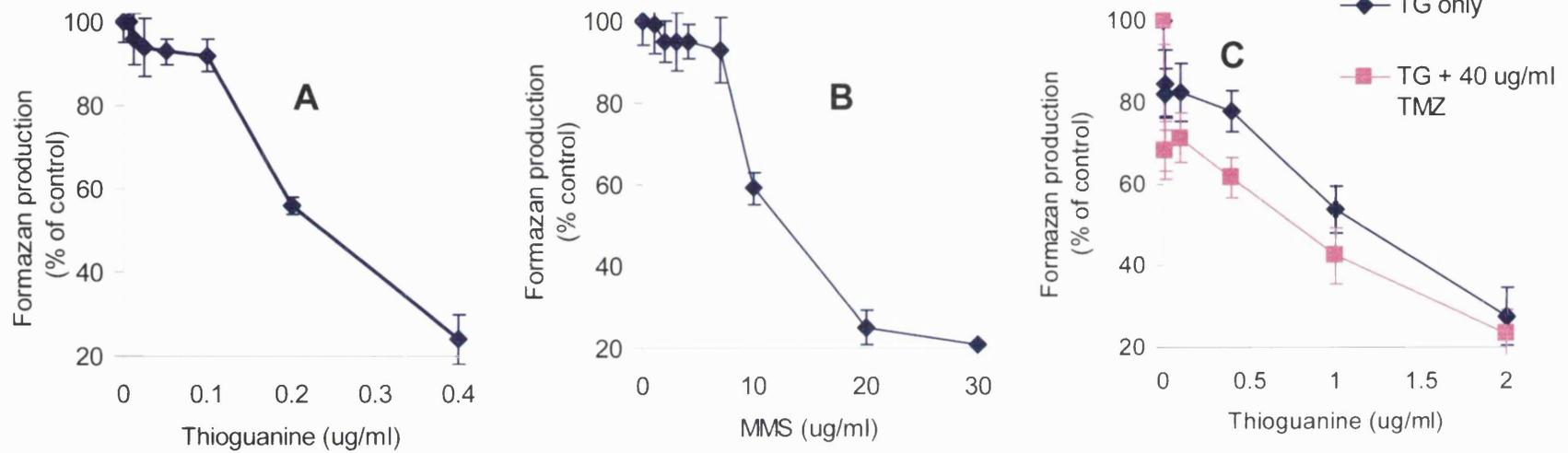
A great deal of time was spent optimising this assay, but it was not possible to obtain satisfactory results for thioguanine and methylating agent cytotoxicity. The assay involved treatment of cells with thioguanine and/or methylating agent followed by withdrawal of the drug to allow the cells to grow such that upon

exposure to MTT, live cells will convert the MTT to a purple product whose absorbance can be read in a spectrophotometer. A major limitation of the assay was the fact that the control readings were usually very high, and up to 10 fold dilutions were necessary to produce absorbances between 0.1 and 1.0. However this assay allowed a comparison to be made between the effect of temozolomide and the effect of another methylating agent methyl methanesulphonate. The results of these experiments are given in figure 2.6 (effect of thioguanine and temozolomide on glioblastoma cells), figure 2.7 (effect of thioguanine and methyl methansulphonate on glioblastoma cells), figure 2.8 (effect of thioguanine and temozolomide on melanoma cells) and figure 2.9 (effect of thioguanine and methyl methanesulphonate on glioblastoma cells).



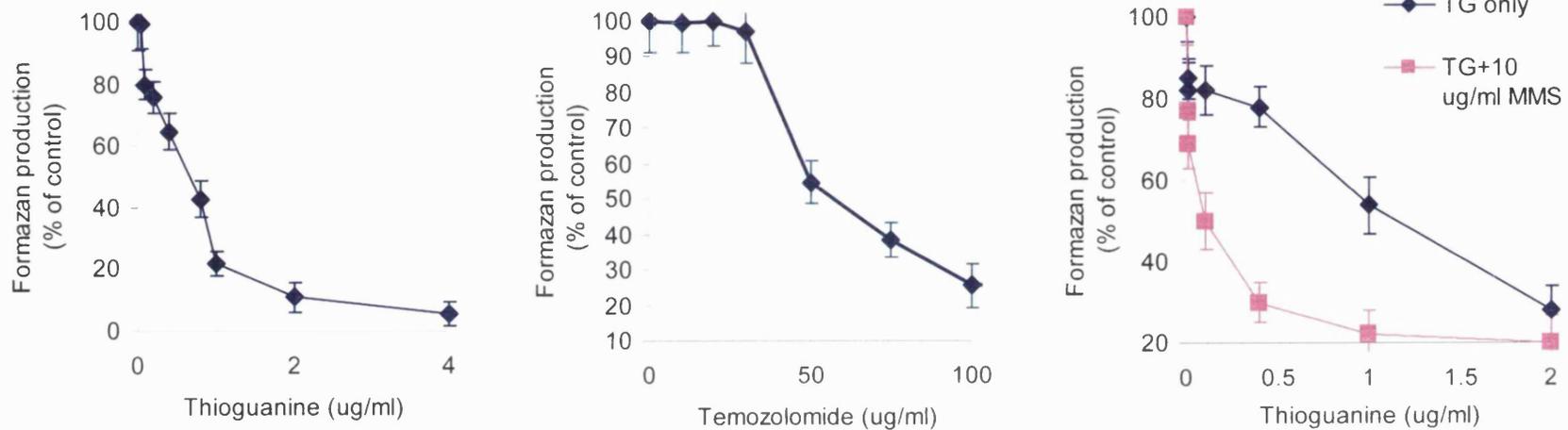
**Figure 2.6 Growth of U87MG glioblastoma cells after treatment with thioguanine and temozolomide**

U87MG cells were treated with a range of doses of thioguanine (A) and temozolomide (B) to obtain the dose response curve for each drug with this cell line. The doses of temozolomide (0.39 and 0.78  $\mu\text{g/ml}$ ) used in combination with thioguanine (C) had no appreciable effect on formazan production when given alone



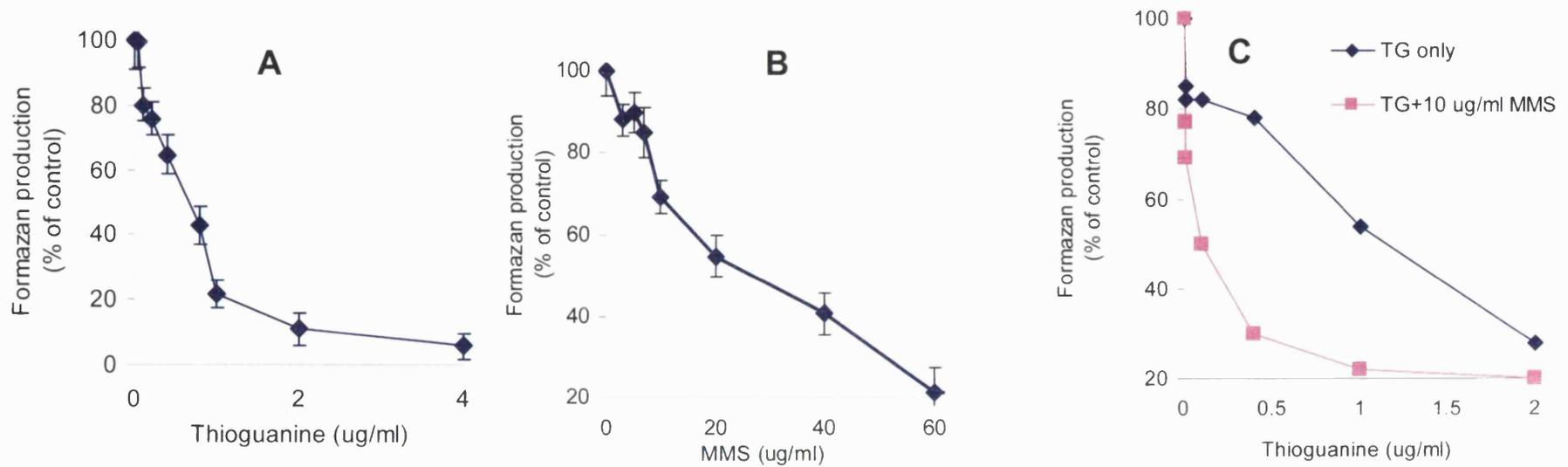
**Figure 2.7 Growth of U87MG glioblastoma cells after treatment with thioguanine and methyl methanesulphonate**

U87MG cells were treated with a range of doses of thioguanine (A) and methyl methanesulphonate (B) to obtain the dose response curve for each drug with this cell line. The doses of MMS (2 and 7  $\mu\text{g/ml}$ ) used in combination with thioguanine (C) had no appreciable effect on formazan production when given alone



**Figure 2.8 Growth of A375P after treatment with thioguanine and temozolomide**

A375P cells were treated with a range of doses of thioguanine (A) and temozolomide (B) to obtain the dose response curve for each drug with this cell line. The dose of temozolomide (40  $\mu\text{g/ml}$ ) used in combination with thioguanine (C), had no appreciable effect on formazan production when given alone



**Figure 2.9 Growth of A375P after treatment with thioguanine and methyl methanesulphonate**

A375P cells were treated with a range of doses of thioguanine (A) and MMS (B) to obtain the dose response curve for each drug with this cell line. The dose of MMS (10  $\mu\text{g/ml}$ ) used in combination with thioguanine (C), had no appreciable effect on formazan production when given alone

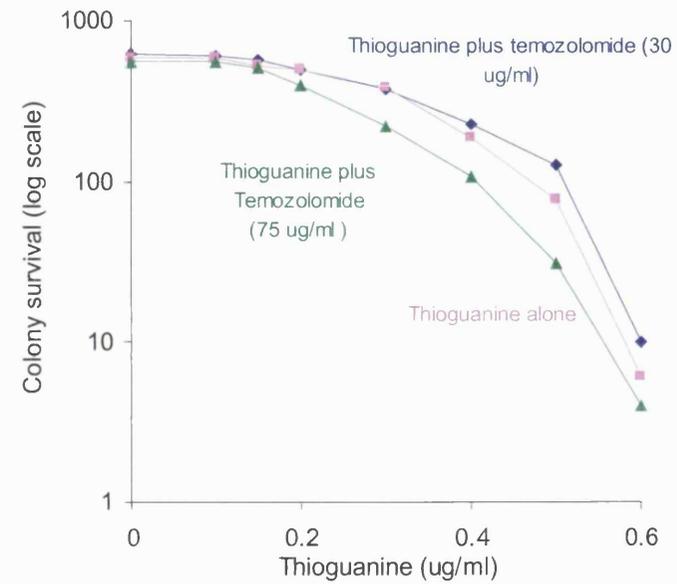
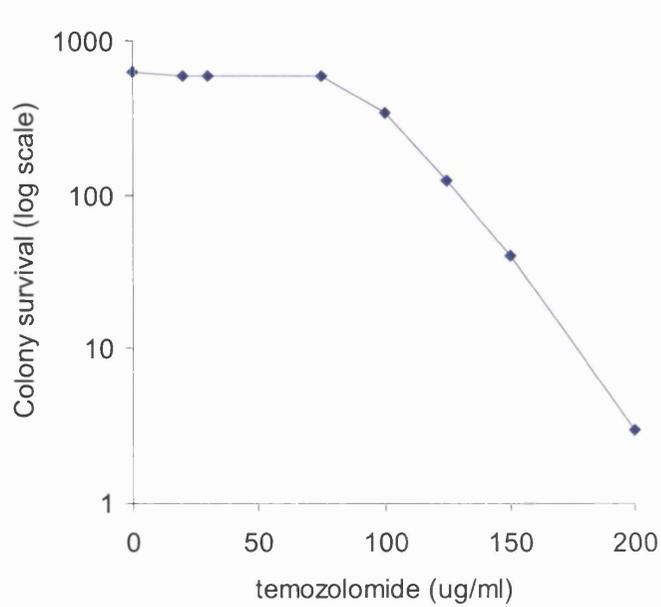
**Table 2.2 Toxicity data for treatment of A375P and U87MG cells with thioguanine, temozolomide and methyl methanesulphonate**

|                                 | IC <sub>50</sub> (ug/ml) |       |
|---------------------------------|--------------------------|-------|
|                                 | U87MG                    | A375P |
| <b>Thioguanine</b>              | 0.24                     | 1.2   |
| <b>Temozolomide</b>             | 3.0                      | 50    |
| <b>Methyl methanesulphonate</b> | 16.9                     | 30    |

The results from the *in vitro* experiments with U87MG and A375P cells suggest that temozolomide has only a marginal effect on thioguanine toxicity but that MMS has a great effect. Since this result was a consistent one between both cell lines, it appeared to be an accurate result. However, an additional way of confirming that this is a true result would be to obtain the same result with a different assay system. For this reason, HeLa cells were used. HeLa cells have a high plating efficiency and are therefore suitable for assessing colony forming ability after treatment with the drugs.

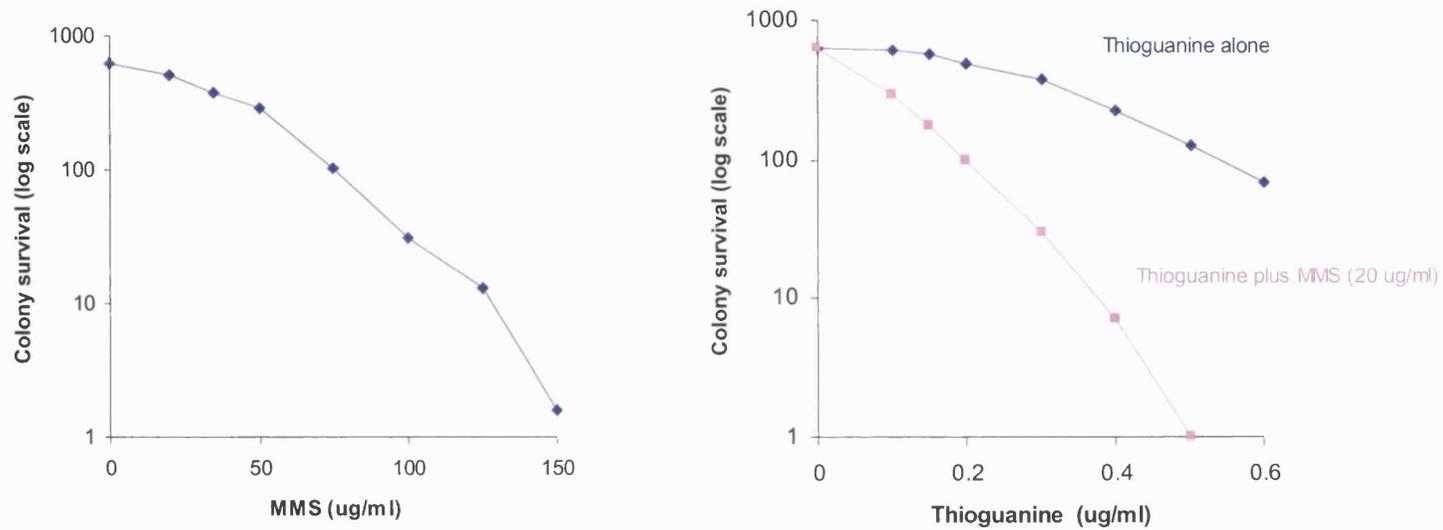
Figure 2.10 shows the result of treatment of HeLa cells with thioguanine and temozolomide. The doses of temozolomide of 30 and 75  $\mu\text{g}/\text{ml}$  that were used in combination with thioguanine were chosen because they had no effect when given alone. 30  $\mu\text{g}/\text{ml}$  temozolomide had no effect on thioguanine toxicity whereas 75  $\mu\text{g}/\text{ml}$  temozolomide had a very marginal effect. However as with the experiments with A375P and U87MG cells, methyl methanesulphonate had a great effect on toxicity (figure 2.11). 20  $\mu\text{g}/\text{ml}$  methyl methanesulphonate had no effect when given alone, however when given after thioguanine, it increased the toxicity of thioguanine such that the dose of thioguanine that resulted in the toxicity of 50% of the cells decreased from 0.42 to 0.14  $\mu\text{g}.\text{ml}$ .

These results show that the same pattern of synergy was observed using a clonogenic method of determining drug cytotoxicity as was obtained with U87MG and A375P cells using the MTT assay, i.e., minimal synergy between thioguanine and temozolomide and noted synergy between thioguanine and methyl methanesulphonate.



**Figure 2.10 Growth response curve for HeLa cells treated with thioguanine and temozolomide**

The left hand graph shows the dose response curve for HeLa cells treated with temozolomide alone and the right hand graph shows the combined effect of thioguanine and temozolomide. Temozolomide doses of 30 and 75  $\mu\text{g/ml}$  were used in combination with thioguanine because these doses had no appreciable effect on colony formation when given alone.



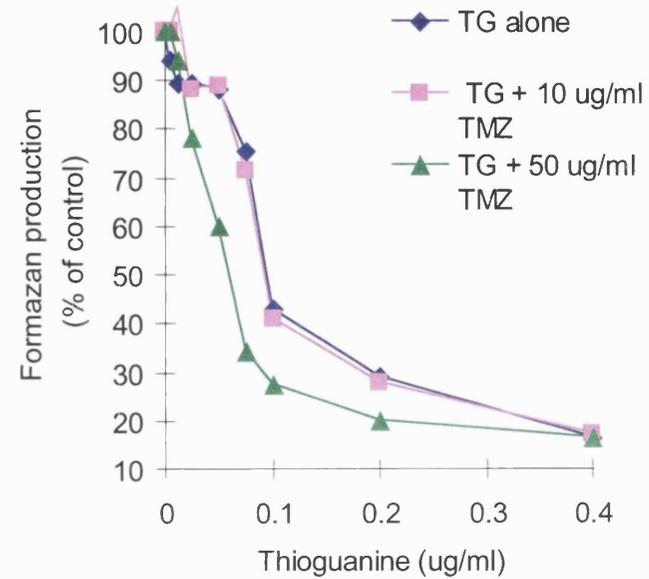
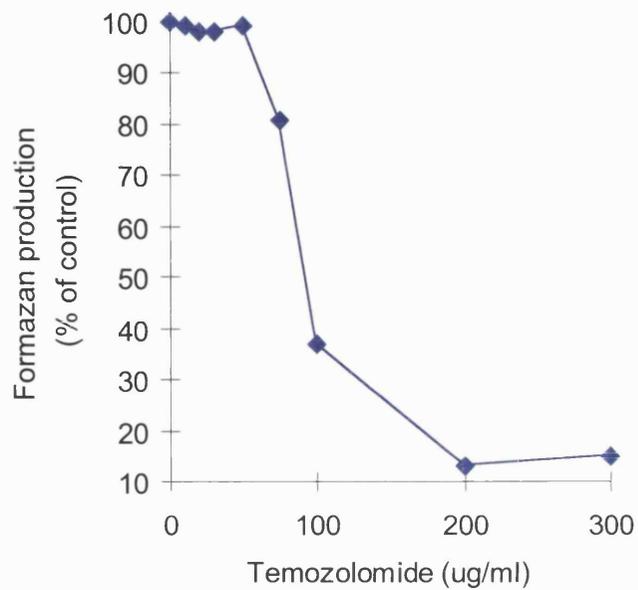
**Figure 2.11 Growth response curve for HeLa cells treated with thioguanine and methyl methanesulphonate**

The left hand graph shows the dose response curve for HeLa cells treated with MMS alone and the right hand graph shows the combined effect of thioguanine and MMS. As can be seen in the graph on the left the MMS dose (20  $\mu\text{g/ml}$ ) used in conjunction with thioguanine had no appreciable effect on survival when given alone.

### **Comparison of clonogenic and MTT assays using HT29 cells**

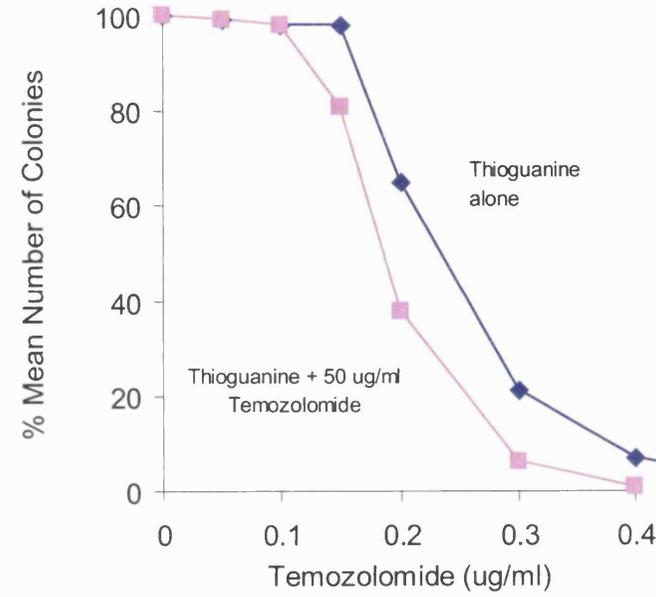
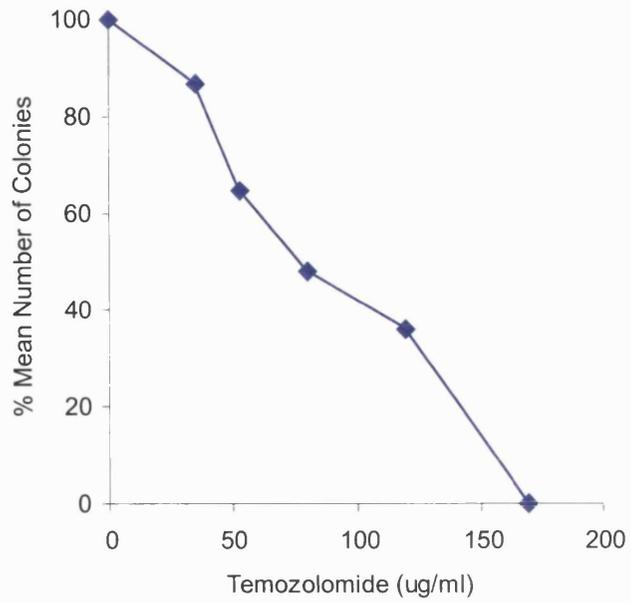
A consistent pattern of toxicity was obtained when comparing the MTT assay results for U87MG and A375P cells with the clonogenic assay using HeLa cells. As further confirmation of this result, the HT29 cell line, which has a high plating efficiency, was used for the direct comparison of the results obtained with the MTT assay and clonogenic assay after treatment with thioguanine and temozolomide.

The absolute concentrations resulting in 50% survival for the both assays were different. For example, the concentration of thioguanine alone that gave 50% survival was 0.28  $\mu\text{g}/\text{ml}$  for the clonogenic assay (figure 2.13) compared with 0.1  $\mu\text{g}/\text{ml}$  for the MTT assay (figure 2.12). However comparison of the percentage reduction in thioguanine concentration that gave 50% survival in combination with 50  $\mu\text{g}/\text{ml}$  temozolomide shows that the 2 different methods of determining cytotoxicity gave similar results. With both the clonogenic and MTT assays, there was a 30% reduction in the dose of thioguanine required to kill 50% of the cells when given with 50  $\mu\text{g}/\text{ml}$  temozolomide.



**Figure 2.12 MTT assay to measure the effect of HT29 cells treated with thioguanine and temozolomide**

Note that 10 and 50  $\mu\text{g/ml}$  temozolomide had no appreciable effect on formazan production



**Figure 2.13 Clonogenic assay to measure the effect of HT29 cells treated with thioguanine and temozolomide**

Note that 50  $\mu\text{g/ml}$  temozolomide had no appreciable effect on colony formation

**Reaction of methylating agents with thioguanine containing nucleotides**

The *in vivo* and *in vitro* experiments to determine whether synergy exists between thioguanine and temozolomide gave inconsistent results. The *in vivo* experiments using the nude mouse xenograft model indicated that thioguanine and temozolomide are synergistic in their cytotoxicity in both glioblastoma and melanoma, but with the same cell lines in tissue culture, temozolomide did not result in significant synergy with thioguanine. However in sharp contrast to this result it was found that another methylating agent, methyl methanesulphonate gave synergistic cytotoxicity with thioguanine. Although temozolomide and methyl methanesulphonate are both methylating agents there is a profound difference in their chemistry. As it seemed possible that the difference seen in the experiments with cells could be explained by this difference in chemistry the following experiments were done

**Reaction of deoxythioguanosine with methyl methanesulphonate and N-methyl-N-nitrosourea**

Deoxythioguanosine and deoxyguanosine in aqueous buffered solution was mixed with an equimolar amount of methyl methanesulphonate in a small volume of ether and left to react overnight. Thin layer chromatography analysis was performed to give a qualitative estimate of product formation. Reaction of deoxyguanine with methyl methanesulphonate gave no visible product, however there was roughly 50% conversion to product from the reaction of deoxythioguanosine with methyl methanesulphonate. To obtain an accurate quantitation of product formation and to confirm that the product was deoxymethylthioguanosine, reverse phase HPLC analysis was performed. The elution was monitored at 309 and 340 nm and shown in figure 2.14. The spectral characteristics of deoxythioguanosine and deoxy(6-methylthio)guanosine is shown in figure 2.13 and table 2.3.

Figure 2.13 Spectral characteristics of deoxythioguanosine and deoxy(6-methylthio)guanosine

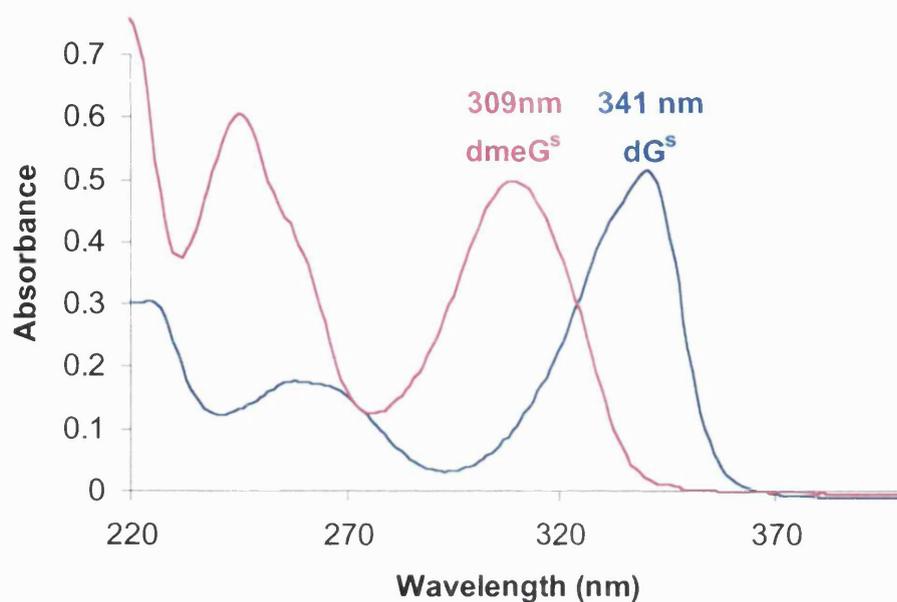


Table 2.3 Molar extinction coefficients and wavelength of maximum absorbance guanine and thioguanine nucleosides

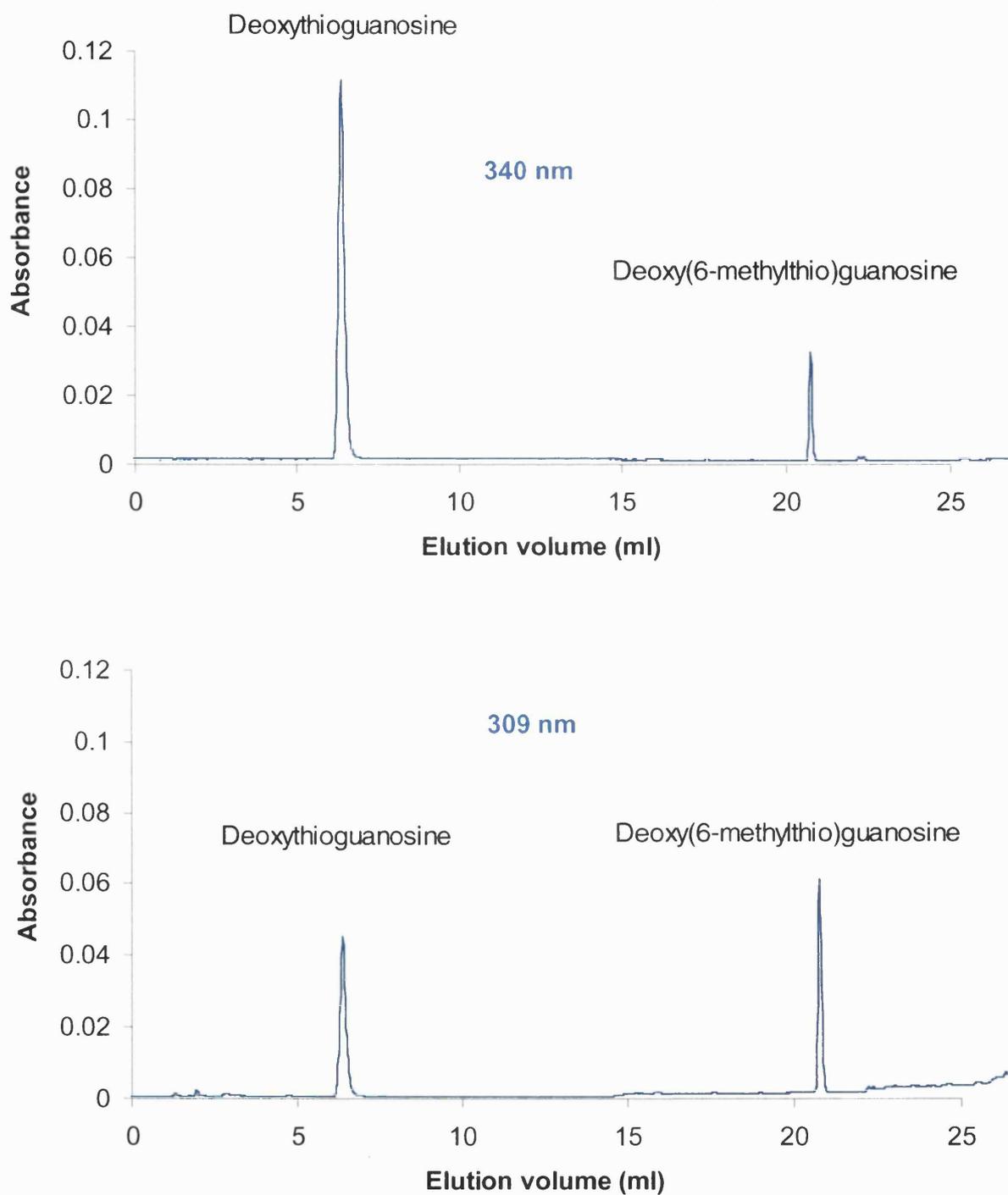
| Deoxy(6-methylthio)guanosine    |                    |                    |
|---------------------------------|--------------------|--------------------|
| $\lambda_{\max}$ 259 nm, 309 nm | Ratio 309/341= 8   | $E_{309} = 7,400$  |
| $\lambda_{\min}$ 230 nm, 278 nm |                    |                    |
| Deoxythioguanosine              |                    |                    |
| $\lambda_{\max}$ 259nm, 341 nm  | Ratio 309/341= 0.2 | $E_{341} = 20,000$ |
| $\lambda_{\min}$ 240 nm, 295 nm |                    |                    |

Using the molar extinction coefficients for deoxythioguanosine and deoxymethylthioguanosine, it is possible to calculate the amount of product formed. By taking into account the different molar extinction coefficients at this wavelength, there was 50% conversion of deoxythioguanosine to deoxymethylthioguanosine after reaction with an equimolar concentration of methyl methanesulphonate, at room temperature overnight. The ratio of the integrated peak areas at 309 nm confirms this result.

The HPLC was repeated with injection of another 20  $\mu$ l sample from the same reaction mixture. Each peak was collected as it eluted from the column. The UV absorbance spectrum of each peak between 220 and 400 nm was measured in a spectrophotometer. The characteristic UV profile for each peak confirmed that the peak at 6 minutes was deoxythioguanosine and the peak at 21 minutes was deoxymethylthioguanosine.

As described in the introduction (figure 1.3) temozolomide produces the same methylating species- the methyldiazonium cation- as N-methyl-N-nitrosourea. Since N-[ $^{14}$ C]methyl-N-nitrosourea is much easier to make than temozolomide and N-methyl-N-nitrosourea is easier to handle than temozolomide, the following experiments were done with N-methyl-N-nitrosourea. Deoxythioguanosine was also reacted with the  $S_N1$  methylating agent, N-methyl-N-nitrosourea. Deoxythioguanosine in cacodylate buffer was reacted overnight with an equimolar concentration of N-methyl-N-nitrosourea. There was no product detected by thin layer chromatography analysis. Reverse phase HPLC analysis of this reaction was performed in the same way as the dG<sup>S</sup> + methyl methanesulphonate reaction. The elution was again monitored at 340 and 309 nm, and shown in figure 2.15. Using the same conditions HPLC conditions at 340 nm as for the reaction with methyl

methanesulphonate, there was no deoxymethylthioguanosine product visible. However at 309 nm there was a very small product peak.



**Figure 2.14 Reverse phase HPLC analysis of the reaction of deoxythioguanosine with methyl methanesulphonate**

The absorbance of the eluate was measured at two wavelengths, 309 nm and 340 nm. The top graph shows the elution profile when the absorbance was measured at 340 nm and the bottom the absorbance profile at 309 nm.

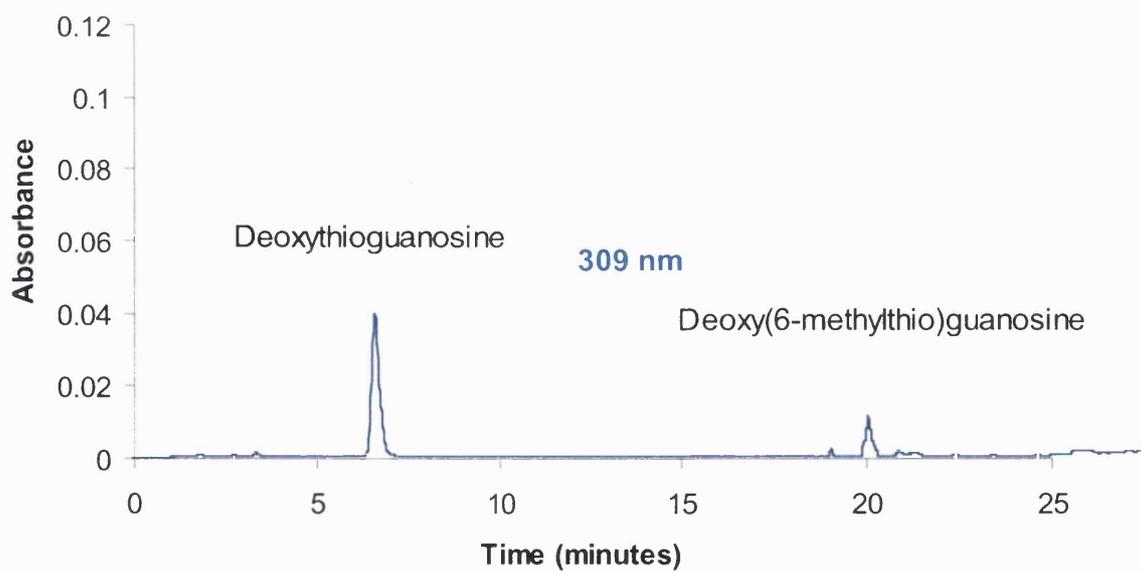
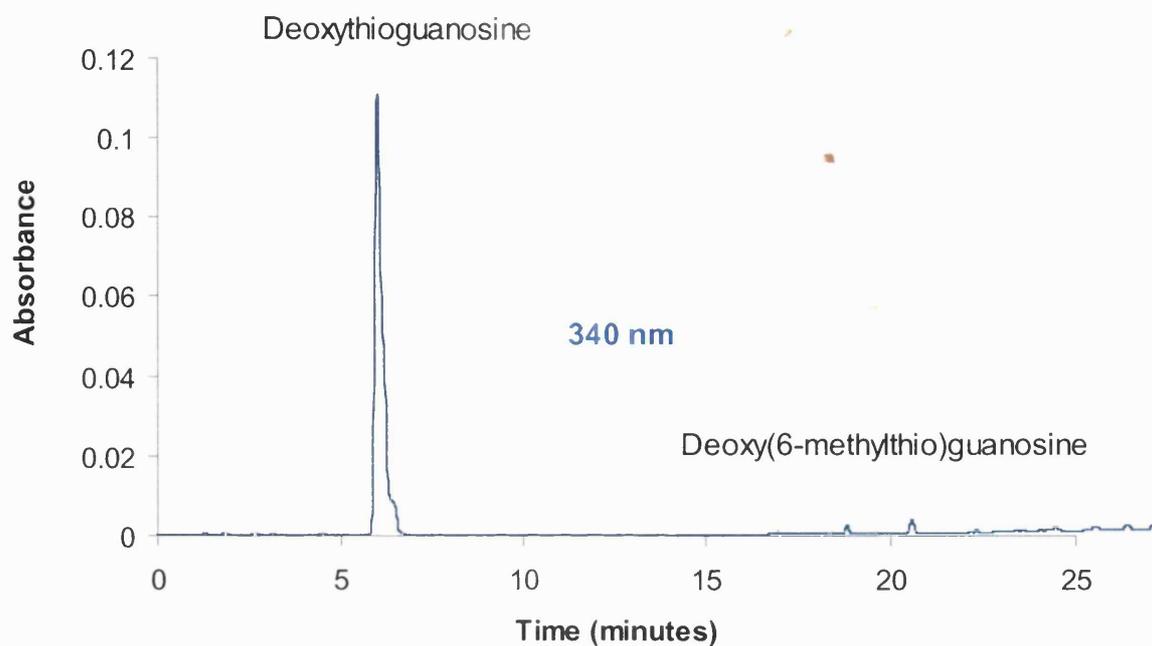
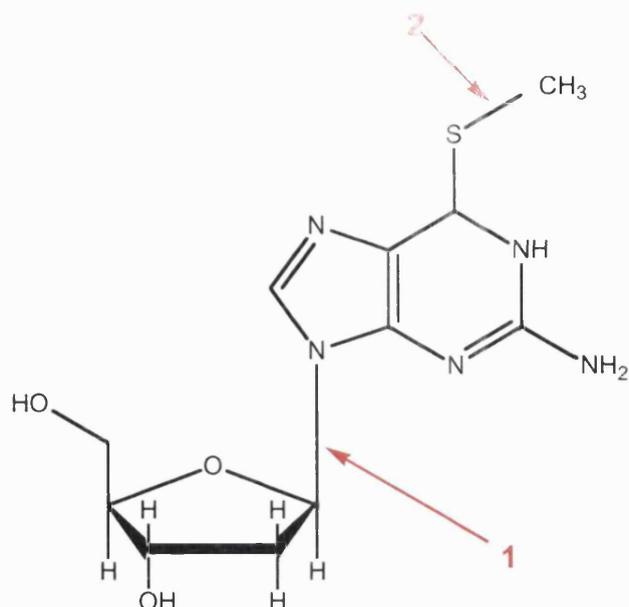


Figure 2.15 Reverse Phase HPLC analysis of the reaction of deoxythioguanosine with N-methyl-N-nitrosourea

## **Determination of optimal conditions for DNA hydrolysis for measurement of DNA methylation**

### **Measurement of methylthioguanine stability**

The results in the previous section immediately suggested that methyl methanesulphonate was much more effective in methylating the sulphur of deoxythioguanosine than N-methyl-N-nitrosourea. To see whether methyl methanesulphonate was also much more effective than N-methyl-N-nitrosourea in methylating the sulphur of thioguanine residues in DNA, the following experiments were done. The most convenient and reliable way of measuring methylation at the DNA level is to separate the bases by cation exchange chromatography after acid hydrolysis of the methylated DNA. However the use of acid presents problems. The production of O<sup>6</sup>-methylguanine was missed by early workers on nitrosamines because the instability of the O-CH<sub>3</sub> link to the acid hydrolysis used in analysis of the methylation destroyed the O<sup>6</sup>-methylguanine (Loveless, 1969). Thus before measurement of the products of methylation of DNA it was necessary to ensure that the S<sup>6</sup>-methylthioguanine would survive the hydrolysis conditions.



**Figure 2.16 Structure of deoxymethylthioguanosine. The bond labelled 1 is the glycosidic bond. The bond labelled 2 is the bond between the sulphur and the methyl adduct. It is crucial that bond 2 is not broken during acid hydrolysis.**

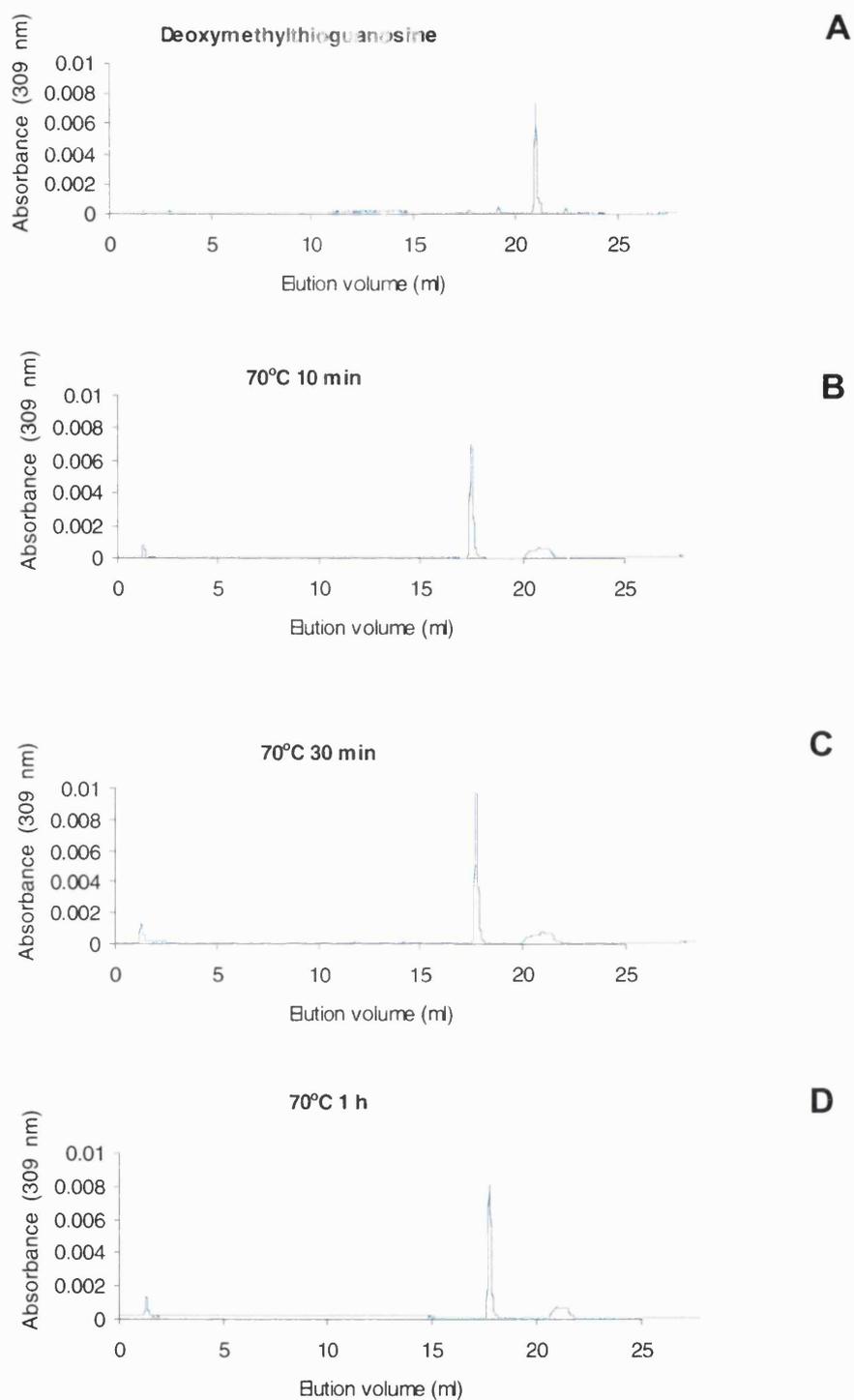
The  $\text{CH}_3\text{-S}$  bond is expected to be relatively unstable in deoxy(6-methylthio)guanosine but relatively stable in the base, methylthioguanine. Therefore it is very important that acid hydrolysis conditions are used that result in cleavage of the glycosidic bond first. It was shown by Lawley and Thatcher (Lawley and Thatcher, 1970) that  $\text{O}^6$ -methylguanine can be released quantitatively from DNA without appreciable loss of the methyl group when the DNA is treated with 0.1 M HCl at  $70^\circ\text{C}$ . Therefore these conditions were used in a trial with deoxy(6-methylthio)guanosine.

Firstly, untreated deoxy(6-methylthio)guanosine was analysed by reverse phase HPLC, as shown in figure 2.17(A). A peak was obtained at 21 minutes, which was expected for deoxy(6-methylthio)guanosine under these conditions. Deoxy(6-methylthio)guanosine was then treated with 0.1 M HCl at  $70^\circ\text{C}$  for 10, 30 and 60

neutralised by the addition of  $\text{KH}_2\text{PO}_4$  buffer 1M NaOH. The optimal conditions for neutralisation were determined prior to reaction.

There was complete breakdown of deoxy(6-methylthio)guanosine to methylthioguanine at 10 minutes as shown in figure 2.17 (B). The methylthioguanine product had an elution time of 17 minutes. This peak was confirmed to be methylthioguanine by the HPLC of standard methylthioguanine under the same conditions. If there had been loss of the methyl group under these conditions, deoxythioguanosine would have been produced. This has an elution time of 7 minutes but there was no peak detected at 7 minutes. However, it is also possible that the bond between the sulphur and the purine ring could be cleaved to produce guanine. Any loss of guanine would not have been detected directly in this experiment since the HPLC was monitored at 309 nm. If there has been loss of methylthioguanine as guanine, it is minimal. After further acid hydrolysis for 30 minutes and 1 hour there was no evidence of loss of methylthioguanine (see figures 2.17(C) and 2.27(D)). Therefore it seems that the glycosidic bond is easily cleaved under these conditions and the  $\text{CH}_3\text{-S}$  bond remains intact.

The stability of deoxy(6-methylthio)guanosine was also studied at  $90^\circ\text{C}$  with 0.1 M HCl. The results of this set of experiments are not shown, however the same pattern was observed. The cleavage of the glycosidic bond was favoured over the cleavage of the  $\text{CH}_3\text{-S}$  bond.



**Figure 2.17 Stability of deoxy(6-methylthio)guanosine to acid**

Reverse phase HPLC of deoxy(6-methylthio)guanosine (A) before acid treatment and the resulting methylthioguanine after treatment with 0.1M HCl at 70°C for 10 minutes (B), 30 minutes (C) and 1 hour (D).

### Determination of the optimal conditions for acid hydrolysis of DNA

The experiments in the previous section showed that treatment of deoxy(6-methylthio)guanosine nucleoside with 0.1 M HCl at 70°C for up to 1 hour did not result in the loss of the methyl adduct from the sulphur of thioguanine. However, since this analysis was performed at the nucleoside level, it may not represent the optimal conditions needed for cleavage of the glycosidic bond of purines at the DNA level. For this reason, a set of experiments was performed to determine the minimum conditions needed for complete depurination. A 34mer oligonucleotide was treated with 1M HCl for 1 hour at 90°C, resulting in the ratio of G:A that would be expected after complete depurination of the DNA. The integrated peak ratios were calculated for each reaction and table 2.4 summarises the G:A ratios obtained under the conditions tested. There was incomplete purination after 10 and 30 minutes, however after 40 minutes hydrolysis was complete. It was therefore decided that for the study of methylation of DNA that the methylating agent treated DNA should be hydrolysed in 0.1 M HCl for 40 minutes at 70°C before cation exchange HPLC.

| Hydrolysis conditions   | Guanine (AU) | Adenine (AU) | G:A absorbance ratio |
|-------------------------|--------------|--------------|----------------------|
| 1M HCl, 90°C, 1 hour    | 0.041        | 0.0108       | 3.79                 |
| 0.1 M HCl, 10 min, 70°C | 0.0845       | 0.075        | 1.13                 |
| 0.1 M HCl, 30 min, 70°C | 0.1365       | 0.0775       | 1.76                 |
| 0.1 M HCl, 40 min, 70°C | 0.040        | 0.0110       | 3.64                 |

**Table 2.4. Depurination of DNA by acid hydrolysis.**

This table summarises the results of reverse phase HPLC analysis after treatment with 0.1M HCl for 10, 30 and 40 minutes. The correct G:A absorbance ratio was obtained after treatment for 40 minutes.

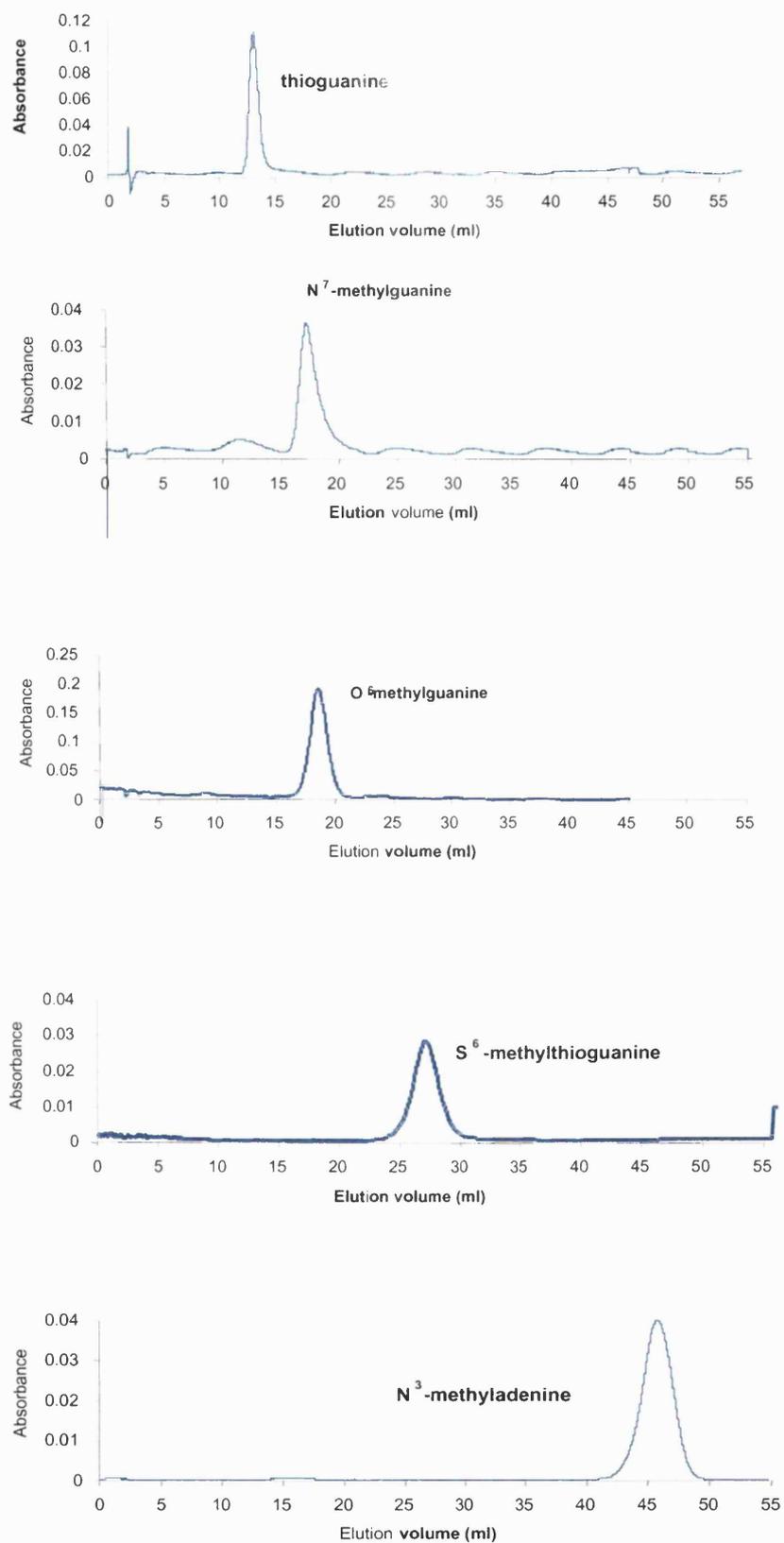


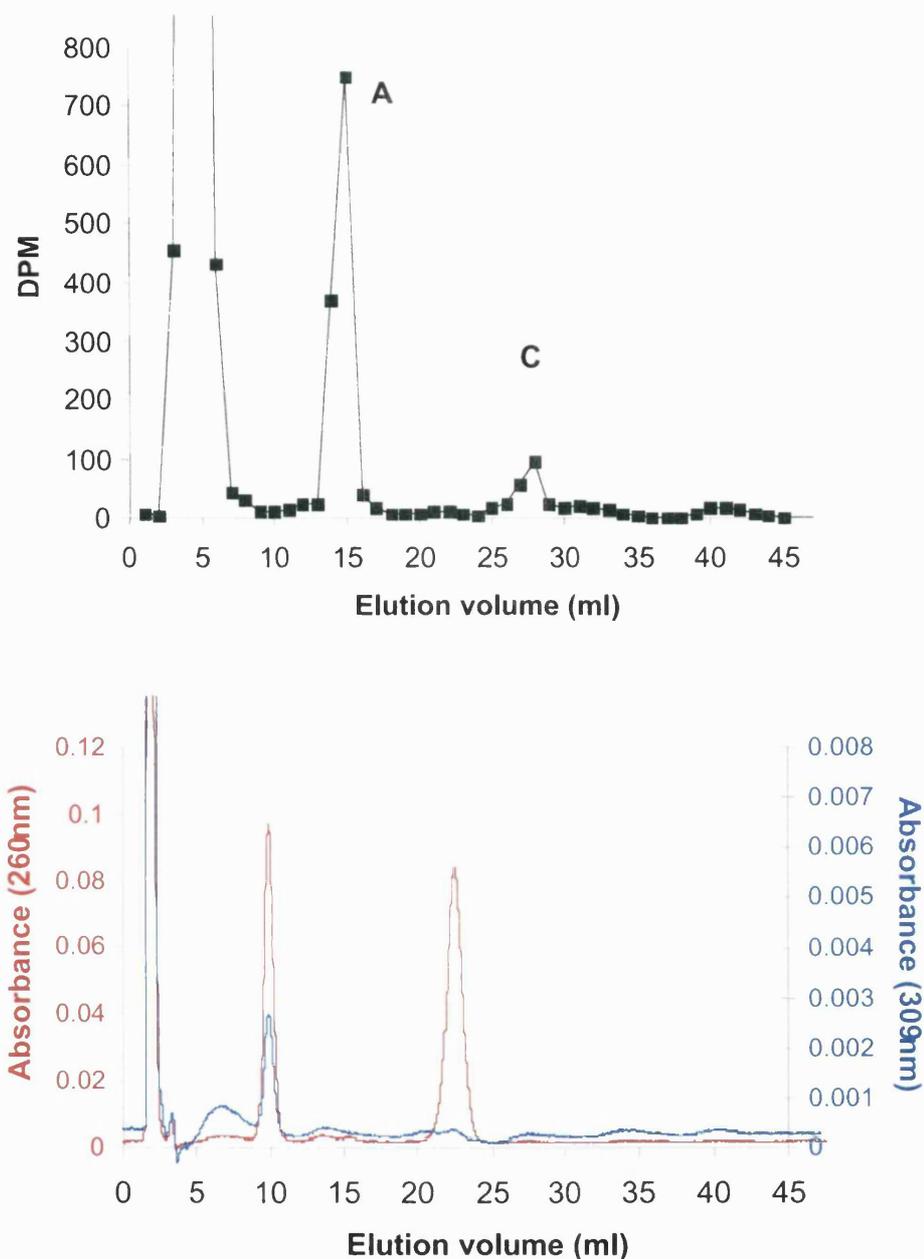
Figure 2.18 Cation exchange HPLC of base standards

The methylation of DNA containing thioguanine by methyl methanesulphonate and N-methyl-N-nitrosourea was studied. DNA duplexes of 11 nucleotides were used. One duplex contained perfectly matched bases whereas the other contained a single thioguanine, which was paired with cytosine. The sequences of the oligonucleotides used are shown in table 2.5. The elution times of the bases of interest was determined using standards (figure 2.18).

**Table 2.5. Sequences of oligonucleotides used for the study of DNA methylation by methyl methanesulphonate and N-methyl-N-nitrosourea**

| Oligo name | Sequence                  |
|------------|---------------------------|
| ThioG-top  | CAGATG <sup>s</sup> TACGC |
| G-top      | CAGATGTACGC               |
| C-bottom   | GCGTACATCTG               |

Figure 2.19 shows the results of the reaction of the parent duplex (non-thioguanine containing) with [<sup>14</sup>C]MMS. The peaks on the HPLC profile at 260 nm are guanine at 11 minutes and adenine at 24 minutes. Guanine, unlike adenine has some UV absorbance at 309 nm. The collected fractions were counted, revealing 3 main peaks. The first peak, eluting in the first 3 to 6 minutes was large, containing a total of 8250 DPM. The second peak, N<sup>7</sup>-methylguanine and O<sup>6</sup>-methylguanine eluted after 15 minutes with 1119 dpm and the third, N<sup>3</sup>-methyladenine was at 28 minutes with 154 dpm. Taking into account that there are 1.2 Gs for every A in the DNA duplex, MMS has a 6 times preference for N<sup>7</sup>-meG over N<sup>3</sup>-meA. Methyl methanesulphonate has a 7 times preference for N<sup>7</sup>-meG over N<sup>3</sup>-meA (Beranek, 1990), therefore the data obtained in this study is reasonably close to published data.



**Figure 2.19 Reaction of [ $^{14}\text{C}$ ]methyl methanesulphonate with an 11mer duplex oligonucleotide containing only naturally occurring bases A, C, G and T.**

Peak A:  $\text{N}^7$ -methylguanine and  $\text{O}^6$ -methylguanine

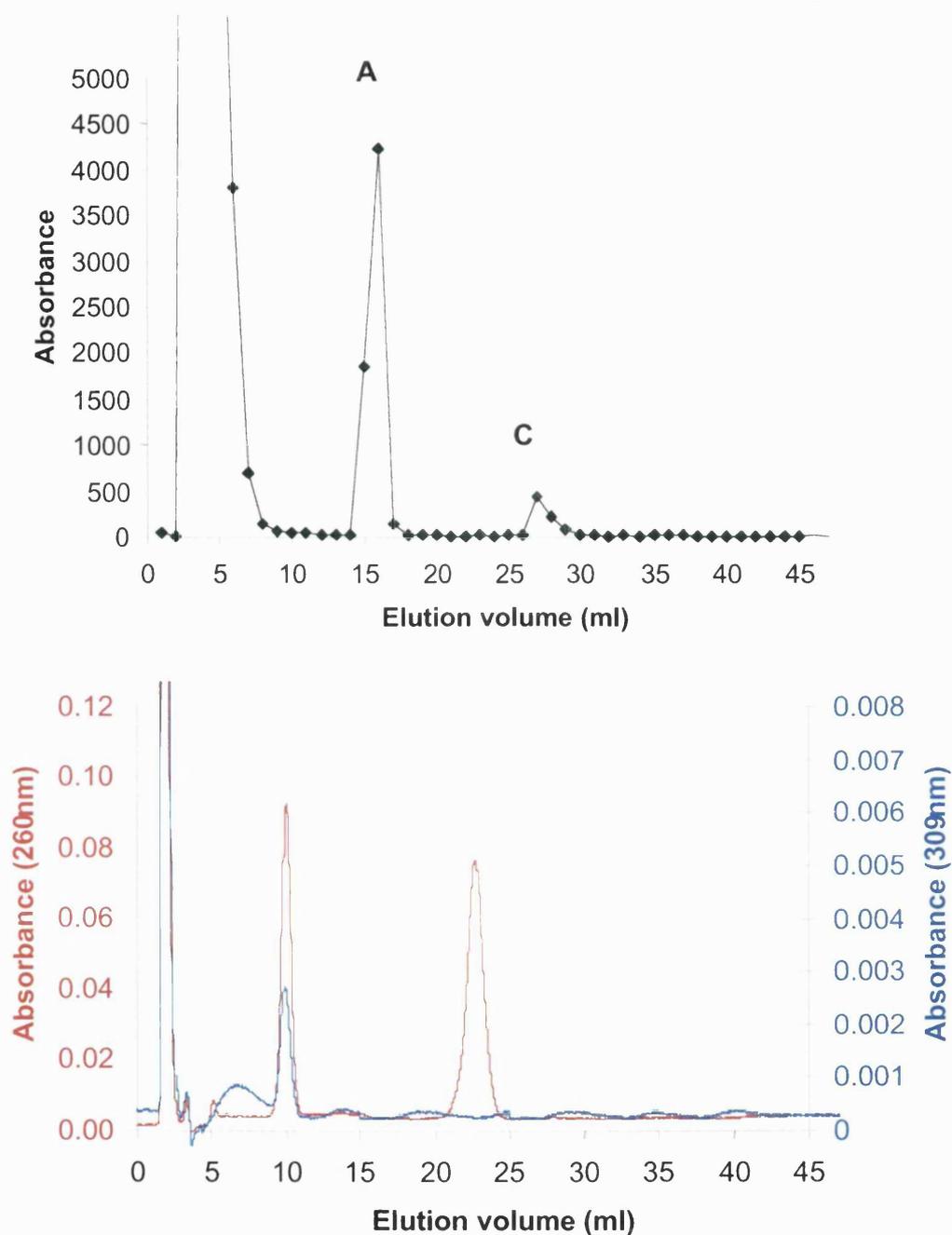
Peak C:  $\text{N}^3$ -methyladenine

A perfectly matched 11mer duplex oligonucleotide was reacted with [ $^{14}\text{C}$ ]-labelled MMS. After treatment with acid, cation exchange HPLC (bottom panel) with collection of eluted material and scintillation counting (top panel) was used to analyse the reaction products. Note that the full scale for the radioactivity is 8000 dpm, while the full scale for the next graph, figure 2.20 is 5000 dpm.

The same procedure was followed for the analysis of the reaction of the parent DNA duplex with [<sup>14</sup>C]MNU. The results of this are shown in figure 2.20. Guanine again eluted after 11 minutes and adenine after 24 minutes. As with the reaction of [<sup>14</sup>C]MMS with DNA, there were no other peaks visible on the UV-HPLC profile.

The collected fractions gave a similar pattern of peaks to that obtained with [<sup>14</sup>C]MMS. Three clear peaks were obtained, the first of which was between 3 and 6 minutes, the second was at 15 minutes and the third at 28 minutes. The N<sup>7</sup>-meG peak contained 6212 DPM while the N<sup>3</sup>-A peak contained 730 DPM. Since there were 1.2 times guanine for every adenine, the ratio of N<sup>7</sup>-meG/N<sup>3</sup>-meA is 7.09. Therefore, MNU has a 7 times preference for N<sup>7</sup>-meG over N<sup>3</sup>-meA. This is close to the published data of 8 for N<sup>7</sup>-meG/N<sup>3</sup>-meA (Beranek, 1990).

It is unclear why there is a high number of radioactive counts between 2 and 6 minutes. Since the results for the methylation of DNA by MMS and MNU obtained here are the same as expected results, it is unlikely that the counts represent [<sup>14</sup>C]CH<sub>3</sub> that has been cleaved during the acid hydrolysis treatment. Additionally, the conditions used (0.1 M HCl, 70°C, 40 minutes) corresponds to the minimum conditions required for complete depurination of the DNA.



**Figure 2.20 Reaction of N-methyl-N-[ $^{14}\text{C}$ ]methylurea with an 11 mer duplex oligonucleotide containing only naturally occurring bases**

Peak A: N<sup>7</sup>-methylguanine and O<sup>6</sup>-methylguanine

Peak C: N<sup>3</sup>-methyladenine

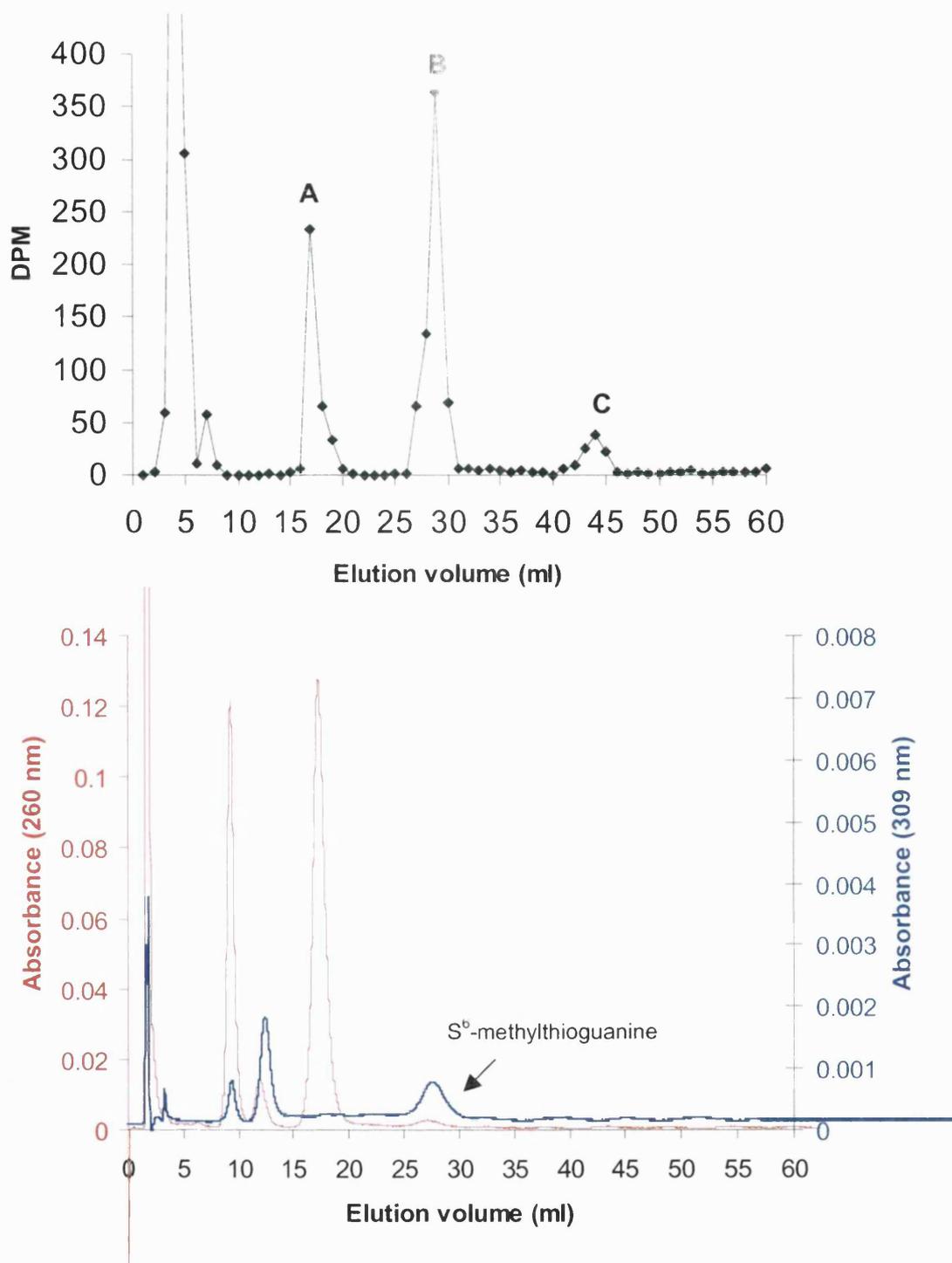
A perfectly matched 11mer duplex oligonucleotide was reacted with [ $^{14}\text{C}$ ]-labelled MNU. After treatment with acid, cation exchange HPLC (bottom panel) with collection of eluted material and scintillation counting (top panel) was used to analyse the reaction.

DNA duplex containing a single thioguanine residue paired with cytosine was reacted with [ $^{14}\text{C}$ ]MMS and [ $^{14}\text{C}$ ]MNU. The reaction was left overnight at room temperature before drying under vacuum, resuspension and acid hydrolysis. Fractions of 1ml were collected from the HPLC for scintillation counting.

The reaction of the thioguanine containing duplex DNA with [ $^{14}\text{C}$ ]MMS is shown in figure 2.21. There are three purine peaks detected at 260 nm. The first peak is guanine at 9 minutes and the second very small peak is thioguanine at 12 minutes and the third is adenine at 16 minutes. The peak at 14 minutes is confirmed to be thioguanine by its higher absorbance at 309 nm. There is a third peak visible at 309 nm, but not 260 nm that elutes after 25 ml, properties characteristic of S<sup>6</sup>-methylthioguanine.

The 1ml fractions collected were counted revealing four peaks. The first peak eluted between 2 and 7 mls and contained a total of 8250 dpm. The second peak eluted after 15 and 20 ml, corresponding to N<sup>7</sup>-methylguanine, the third peak eluting after 25 to 30 ml is S<sup>6</sup>-methylthioguanine and the fourth peak, N<sup>3</sup>-methyladenine eluted after 40 to 45 ml. The radioactive counts for these peaks are summarised in table 2.6.

Similarly, the reaction of the thioguanine containing duplex with [ $^{14}\text{C}$ ]MNU is shown in figure 2.22. The pattern of peaks obtained by UV detection at 260 and 309 nm was the same, with guanine at 9 minutes, thioguanine at 12 minutes and adenine at 16 minutes. There was however, no S<sup>6</sup>-methylthioguanine peak detected by UV at 260 or 309 nm. The radioactive counts corresponding to N<sup>7</sup>-methylthioguanine, S<sup>6</sup>-methylthioguanine and N<sup>3</sup>-methylguanine collected after this reaction are also summarised in table 2.6.



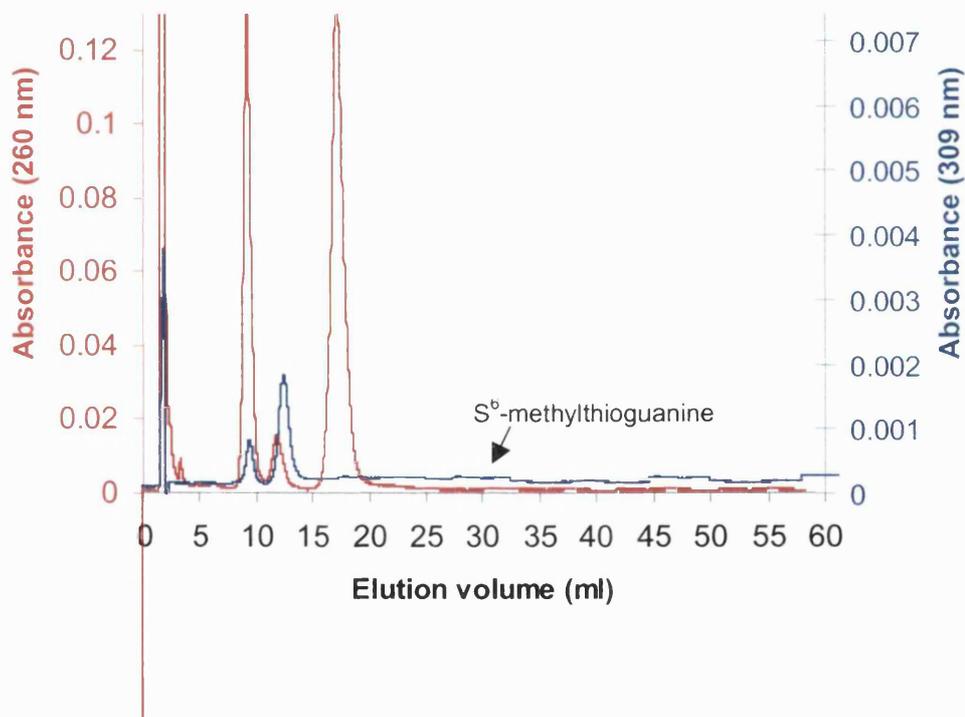
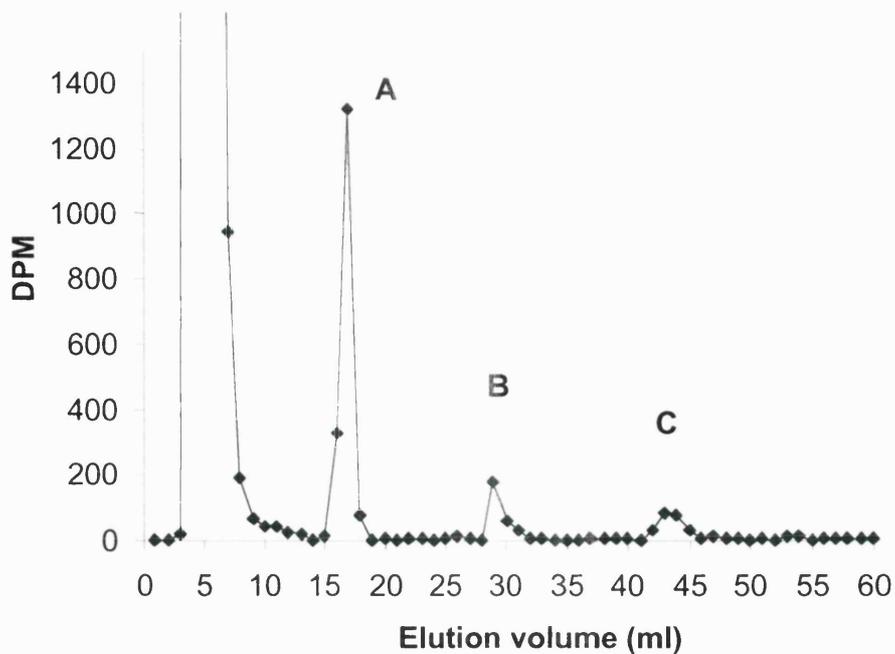
**Figure 2.21. Reaction of methyl methanesulphonate with a thioguanine containing oligonucleotide duplex**

Peak A: N<sup>7</sup>-methylguanine and O<sup>6</sup>-methylguanine

Peak B: S<sup>6</sup>-methylthioguanine

Peak C: 3-methyladenine

A thioguanine 11mer duplex oligonucleotide was reacted with [<sup>14</sup>C]-labelled MMS. After treatment with acid, cation exchange HPLC (bottom panel) with collection of eluted material and scintillation counting (top panel) was used to analyse the reaction. Sufficient S<sup>6</sup> methylthioguanine was produced for it to be detected by its absorbance at 309 nm (bottom panel).



**Figure 2.22. Reaction of *N*-methyl-*N*-nitrosourea with a thioguanine containing oligonucleotide duplex**

Peak A:  $N^7$ -methylguanine and  $O^6$ -methylguanine

Peak B:  $S^6$ -methylthioguanine

Peak C: 3-methyladenine

A thioguanine 11mer duplex oligonucleotide was reacted with [ $^{14}C$ ]-labelled MNU. After treatment with acid, cation exchange HPLC (bottom panel) with collection of eluted material and scintillation counting (top panel) was used to analyse the reaction. Note that there is very little radioactivity in the fractions (B) where  $S^6$ -methylthioguanine would be expected and no evidence of  $S^6$ -methylthioguanine in the  $A_{309}$  profile (bottom panel).

**Table 2.6. Reaction of thioguanine containing oligodeoxynucleotide duplexes with [<sup>14</sup>C]N-methyl-N-nitrosourea and [<sup>14</sup>C]methyl methanesulphonate**

|  | <b>MNU<br/>DPM</b> | <b>MMS<br/>DPM</b> |
|--|--------------------|--------------------|
| <b>N<sup>7</sup>-methylguanine</b>     | 1800               | 350                |
| <b>N<sup>3</sup>-methyladenine</b>     | 200                | 80                 |
| <b>S<sup>6</sup>-methylthioguanine</b> | 220                | 600                |
| <b>Ratio 3meA:7meG</b>                 | 0.11:1             | 0.22:1             |
| <b>Ratio S<sup>6</sup>meG:7meG</b>     | 0.12:1             | 1.71:1             |

The above table shows that methyl methanesulphonate has a greater preference for the production of S<sup>6</sup>-methylthioguanine than does N-methyl-N-nitrosourea. The ratio of S<sup>6</sup>-methylthioguanine to 7-methylguanine is 14 times greater after reaction with methyl methanesulphonate than N-methyl-N-nitrosourea.

## Discussion

Interest in possible synergy between thioguanine and methylating agents originally arose from work in our laboratory on the methylating N-nitroso compounds that are cytotoxic through O<sup>6</sup>-methylguanine adducts in DNA and subsequent recognition by proteins of the post replicative mismatch repair pathway. When it was discovered that reason why the cytotoxicity of thioguanine is mediated via the same repair system as N-methyl-N-nitrosourea is because the thioguanine residues in DNA are methylated by S-adenosylmethionine (Swann *et al.*, 1996) it was realised that the combination of thioguanine and methylating agents could have potential in anti-cancer chemotherapy.

To investigate whether such synergy exists, U87MG and A375P were the cell lines of choice because they have been used by others to study the *in vitro* and *in vivo* cytotoxicity of a clinically used methylating agent, temozolomide and other methylating agents (Baer *et al.*, 1993; Wedge and Newlands, 1996; Wedge *et al.*, 1996a; Wedge *et al.*, 1996b). It could be assumed that both U87MG and A375P cells have a functional mismatch repair pathway because of their observed sensitivity toward methylating agents (Wedge *et al.*, 1996a). But more direct evidence that these cell lines are mismatch repair proficient was obtained from the western blot, RT-PCR and gel shift experiments. The western blot experiment showed that both cell lines contained a roughly equal amount of MSH2, despite the RT-PCR result suggesting that there is lower expression of hMSH2 mRNA in U87MG cells compared with A375P. The mRNAs for hPMS2, hMSH6, hMLH1 and hPMS1 were expressed at approximately equal levels in both cell lines. Functional MutS $\alpha$  was demonstrated by the presence of GT-specific binding activity in a gel shift assay with the GT binding activity being slightly higher in

U87MG cells than in A375P. Unlike HeLa cell protein extract, which contains one GT binding activity, both U87MG and A375P cells contain 2 specific GT binding proteins. This binding pattern has previously been observed in our laboratory in a third cell line, HT29 and has been reported using recombinant MutS $\alpha$  and MutS $\beta$  (Marra and Schar, 1999). However, addition of excess non-labelled heteroduplex containing a single looped nucleotide did not result in the removal of one of the GT specific U87MG and A375P bands, suggesting that one of the bands is not due to MutS $\beta$  binding. Despite this uncertainty, these experiments suggested that both U87MG and A375P cell lines are mismatch repair proficient. Interestingly, it has been reported that resistance to methylating agents and 6-thioguanine need not be due to complete loss of a mismatch repair function but can be quantitatively related to GT mismatch binding and MSH2 expression (Dorsch *et al.*, 1997).

Glioma xenografts of U87MG cells and melanoma xenografts of A375P cells served as models for testing the *in vivo* response to treatment with thioguanine and temozolomide. Thioguanine alone at 4 mg/kg had no effect on the growth of glioma and melanoma xenografts. This was expected since thioguanine is known to be ineffective against solid tumours and has been confirmed in a previous study using glioma xenografts in which therapeutic interaction between thiopurines and various alkylating drugs was investigated (Wang *et al.*, 1991). The chloroethylnitrosoureas were used in combination with thioguanine in this study, which are bifunctional since they have a halogen and a nitroso group. 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) reacts with DNA at the N-7 of guanine to form chloroethyl, hydroxyethyl and aminoethyl compounds and reaction to form mono-hydroxyethyl adducts occurs at the O<sup>6</sup> of guanine and the N-3 of cytosine. Additionally, there are two ethano compounds formed that have a 3,N<sup>4</sup> ethano ring on cytosine or a 1,N<sup>6</sup> ethano ring on adenine (Singer and Grunberger, 1983).

Cross-links can be formed between reactive groups, such as through the N-7 of guanine to form diguanyl ethane. The chemotherapeutic effect of 1,3-bis(2-chloroethyl)-1-nitrosourea is attributed to their crosslinking activity. Wang *et al* (Wang *et al.*, 1991) found synergy between thioguanine and the chloroethylnitrosoureas, BCNU and 1-(2-chloroethyl)-3-(2,5-dioxo-3-piperidyl)-1-nitrosourea (PCNU). However in contrast to the data presented in this thesis, they found that combination of thiopurines and the methylating agent procarbazine was not significantly more active than procarbazine alone. The reason for their failure is not clear.

Treatment of the nude mice with temozolomide (5 mg/kg) had no effect on the growth of the glioma as expected from the data of Wedge and Newlands (Wedge and Newlands, 1996). In this study, they were interested in the enhancement of temozolomide activity in glioma xenografts upon pretreatment with O<sup>6</sup>-benzylguanine, an irreversible inhibitor of O<sup>6</sup>-methylguanine-DNA alkyltransferase (MGMT). O<sup>6</sup>-benzylguanine bound MGMT is inactive and is subsequently degraded therefore cellular MGMT activity can only be restored by *de novo* protein synthesis. Since MGMT affords protection against methylating agent cytotoxicity, inhibition of this activity would be expected to increase the toxicity of methylating agents. Consistent with this expectation, Wedge and Newlands found that when 40 mg/kg O<sup>6</sup>-benzylguanine was administered before a single dose of 5 mg/kg temozolomide, a 10 day delay in glioma xenograft growth resulted. The data presented in this thesis shows that pretreatment with 3 consecutive daily doses of 4 mg/kg thioguanine had a similar effect to this treatment with O<sup>6</sup>-benzylguanine producing a 10 day growth delay for glioma xenografts.

In similar studies with melanoma (A375P) xenografts the same group showed that a single dose of temozolomide of 5 mg/kg would have no effect on the xenograft (Wedge *et al.*, 1997), which is consistent with the results reported now. In their study Wedge *et al* examined the dependency of the dosing schedule on toxicity. A single dose of O<sup>6</sup>-benzylguanine at 35 mg/kg followed by 100 mg/kg temozolomide was found to result in a 7 day growth delay. When the temozolomide was divided into 5 daily doses of 20 mg/kg, the growth delay was 18 days. The melanoma xenografts presented in this thesis were given 3 daily doses of 4 mg/kg thioguanine and a final dose of 5 mg/kg temozolomide resulting in a growth delay of 9 days compared with control. Therefore, in combination with thioguanine, a dose of temozolomide that is 20 times lower has the same effect as that achieved with a single dose O<sup>6</sup>-benzylguanine for melanoma xenografts.

The enhancement of temozolomide toxicity by pretreatment with thioguanine is proposed to be a better clinical alternative to O<sup>6</sup>-benzylguanine pretreatment. Administration of O<sup>6</sup>-benzylguanine will deplete MGMT in normal tissues as well as the tumour. Failure of MGMT to repair O<sup>6</sup>-methylguanine results in O<sup>6</sup>-methylguanine:thymine mispairs, which will lead to an increased rate of apoptosis and/or mutation rate in normal tissues. An important beneficial feature of the thioguanine-temozolomide combination is that MGMT is unable to remove the methyl group from S<sup>6</sup>-methylthioguanine (Yarosh and Day, 1986). Therefore the MGMT activity of the tumour is irrelevant, as demonstrated by the fact that the same dose of temozolomide gave good synergy *in vivo* in the xenograft with high MGMT (A375P) activity as well as the low (U87MG). However, when given alone, quite different doses of temozolomide are required for xenograft growth delay. To achieve a 10-day growth delay with U87MG xenograft, a dose of 10 mg/kg temozolomide is needed whereas with A375P, 200 mg/kg is required

(Wedge and Newlands, 1996; Wedge *et al.*, 1997). A further benefit of thioguanine and temozolomide therapy is that the basis of thioguanine toxicity depends on its incorporation into DNA so is more likely to be incorporated into the rapidly dividing tumour than normal tissue.

Experiments using the nude mouse xenograft model are expensive and take a long time so it was not possible to perform more than one study with each of the glioblastoma and melanoma tumour types. Although good synergy was obtained with the chosen doses for both xenografts, ideally a more thorough study of dosing would have been done. With more time and resources, the minimum synergistic doses of each drug could have been determined, since it is possible that the concentrations of thioguanine and temozolomide used were higher than necessary. It would also have been desirable to study the effectiveness of thioguanine and other methylating agents such as the S<sub>N</sub>2 methylator, methyl methanesulphonate. For these reasons, the study of thioguanine and methylating agent synergy was continued *in vitro*.

There is a wide range of MGMT activities among different cell types. U87MG cells have a low MGMT activity of 2.5 fmol/mg protein (Wedge *et al.*, 1996a) while the activity in A375P cells is relatively high at 95 fmol/mg protein (Wedge *et al.*, 1997). This means that A375P cells would be more resistant to temozolomide cytotoxicity than U87MG because the methyl adduct of O<sup>6</sup>-methylguanine is more easily repaired in A375P cells. A comparative *in vitro* study demonstrated a linear relationship between MGMT activity and the IC<sub>50</sub> value obtained from a single 1 h treatment with temozolomide (Wedge *et al.*, 1996a). Such a linear relationship has also been found between MGMT activity and sensitivity towards N-methyl-N'-nitro-N-nitrosoguanidine and 5-(3-methyltriazene-1-yl)imidazole-4-carboxamide

(MTIC), the hydrolysis product of temozolomide (figure 1.3) (Scudiero *et al.*, 1984; Tisdale, 1987). As expected, U87MG cells with low MGMT activity are a lot more sensitive towards methylating agents than A375P with high MGMT activity. For example, the  $IC_{50}$  for A375P cells after temozolomide treatment is roughly 50 times greater than that for U87MG, which exactly correlates with the MGMT activity in these cell lines. To ensure the lack of synergy was a true result and not because of the MTT assay itself, the clonogenic assay was used with HT29 and HeLa cells, but a similar lack of synergy was observed. However, the  $S_N2$  methylating agent, methyl methanesulphonate caused a large increase in thioguanine toxicity with both cell lines *in vitro*.

The inactivation of MGMT activity, has been the most extensively studied means of increasing methylating agent toxicity *in vitro* (Aida *et al.*, 1987; Dolan *et al.*, 1985; Gerson *et al.*, 1988; Marathi *et al.*, 1994; Wedge *et al.*, 1996a; Wedge *et al.*, 1996b; Wedge *et al.*, 1997). These studies have found that potentiation of a single dose of methylating agent by MGMT depletion results in an enhancement of cytotoxicity of up to 5 fold. But, as one would expect, MGMT inhibition in cell lines that already have very low activity, such as U87MG, has no effect on methylating agent toxicity (Wedge *et al.*, 1996a). This is in contrast to the *in vivo* combination of  $O^6$ -benzylguanine and temozolomide with U87MG xenografts where there was synergy (Wedge and Newlands, 1996). The results of treating U87MG cells with thioguanine and temozolomide in this thesis follow a similar pattern of good synergy *in vivo* but not *in vitro*.

The reason for the good synergy with methyl methanesulphonate but not temozolomide *in vitro* may be due to the very different chemistry of these two methylating agents. Methyl methanesulphonate reacts with DNA with a  $S_N2$

mechanism, reacting mainly with the N-7 of guanine, the N-1 and N-3 of adenine and the N-3 of cytosine. N-methyl-N-nitrosourea, N-methyl-N'-nitro-N-nitrosoguanidine and temozolomide are examples of  $S_N1$  methylators that react with DNA via the formation of a reactive methyldiazonium ion. Such agents, unlike  $S_N2$  methylators, react with all four oxygens in DNA, the O<sup>6</sup> of guanine, the O<sup>2</sup> and O<sup>4</sup> of uracil or thymine and the O<sup>2</sup> of cytosine.

The analysis of DNA containing thioguanine is possible due to the different spectral characteristics of guanine, thioguanine and methylthioguanine. Guanine, like the other bases in DNA has a peak of UV absorbance at 260 nm whereas thioguanine has an absorbance maximum at 340 nm and methylthioguanine has an absorbance maximum at 309 nm. The methylation of thioguanine was firstly studied at the nucleoside level. The reaction of deoxythioguanosine with N-methyl-N-nitrosourea was compared with its reaction with methyl methanesulphonate. After reaction of deoxythioguanosine with methyl methanesulphonate there was a 50% conversion to deoxy(6-methylthio)guanosine. However, when deoxythioguanosine was reacted with N-methyl-N-nitrosourea there was very little conversion to deoxy(6-methylthio)guanosine. This result indicated that there is more favourable methylation of thioguanine with an  $S_N2$  methylating agent than with an  $S_N1$  methylator. However, more convincing evidence would come from the reaction of methylating agents with thioguanine containing DNA rather than just a thioguanine nucleoside. A duplex oligonucleotide of 11 bases containing a single thioguanine nucleotide was used to study the reaction of methylating agents with thioguanine containing DNA.

The most reliable and convenient way of measuring the products of reaction of DNA with methylating agents is by base analysis by cation exchange

chromatography. After the DNA has been reacted with the methylating agent, the DNA is depurinated by treatment with acid. However studies on DNA methylation missed the production of O<sup>6</sup>-methylguanine because the acid hydrolysis used in the analysis destroyed the O<sup>6</sup>-methylguanine (Loveless, 1969). For this reason a number of experiments were done to check the stability of 6-methylthioguanine under the conditions used for the acid hydrolysis of DNA. A series of experiments confirmed that deoxy(6-methylthio)guanosine is completely broken down to methylthioguanine in less than 10 minutes at 70°C indicating that the glycosidic bond is cleaved first. It is possible that methylguanine could have been further broken down to guanine. Comparison of the amount of methylthioguanine with the starting material, deoxy(6-methylthio)guanosine suggests that if there is loss of methylguanine as guanine then it is very minimal. Treatment of deoxymethylthioguanosine with 0.1 M HCl for 30 minutes and 1 hour did not cause any further breakdown of methylthioguanine, showing that after cleavage of the glycosidic bond, the molecule is relatively stable to acid treatment. The optimum conditions for depurination of DNA was further confirmed by the treatment of an oligonucleotide with acid under various conditions. It was important to use the correct conditions for acid hydrolysis of the DNA because if the conditions were not vigorous enough, there would be incomplete depurination of the DNA resulting in an inaccurate measurement of methylated product. The expected result was obtained after treatment with 0.1 M HCl for 40 minutes at 70°C. Therefore, these were the conditions used for hydrolysis of DNA that had been reacted with methylating agents.

Firstly, a duplex oligonucleotide of 11 nucleotides in length containing natural bases was used to study methylation by [<sup>14</sup>C]methyl methanesulphonate and [<sup>14</sup>C]N-methyl-N-nitrosourea. The result showed that after methylation with

[<sup>14</sup>C]methyl methanesulphonate there is a ratio of N<sup>7</sup>methylguanine:N<sup>3</sup>methyladenine of 6:1. This is close to the published data which states that the ratio of N<sup>7</sup>methylguanine:N<sup>3</sup>methyladenine is 7:1. Similarly after reaction of DNA with [<sup>14</sup>C]N-methyl-N-nitrosourea, the ratio of N<sup>7</sup>methylguanine:N<sup>3</sup>methyladenine was 7:1, close to the published ratio of 8:1. This result shows that is a valid method of measuring DNA methylation by S<sub>N</sub>1 and S<sub>N</sub>2 methylators. This result also confirms that the DNA was double stranded during the reaction. If the oligonucleotide had been single stranded then a different pattern of methylation would have been expected. For example, N<sup>3</sup>-methyladenine represents 10.4% of the total methylation resulting from the methylation of double stranded DNA with methyl methanesulphonate whereas when single stranded DNA is methylated with methyl methanesulphonate, 1.4% of the total methylation is on the N-3 of adenine (Singer and Grunberger, 1983).

The same procedure was therefore followed to study the methylation pattern of a duplex oligonucleotide containing a single thioguanine residue. The purpose of this experiment was to see whether there was favourable methylation of the S-6 of thioguanine by N-methyl-N-nitrosourea and methyl nitrosourea. When the thioguanine containing DNA duplex was methylated with radioactively labelled N-methyl-N-nitrosourea, there was no detectable formation of S<sup>6</sup>-methylthioguanine by UV, however there was a peak of radioactivity at the expected elution volume for S<sup>6</sup>-methylthioguanine. The relative peak ratios were calculated and it was found that there is a 0.6:1 preference for formation of S<sup>6</sup>-methylthioguanine compared with N<sup>7</sup>-methylguanine after reaction with N-methyl-N-nitrosourea. When there is an oxygen in place of sulphur in the ratio for the relative amounts of O<sup>6</sup>-methylguanine:N<sup>7</sup>-methylguanine is 0.03:1 (Singer and Grunberger, 1983).

Therefore there is a 20 times greater production of S<sup>6</sup>-methylthioguanine than O<sup>6</sup>-methylguanine after reaction with N-methyl-N-nitrosourea.

After reaction with radioactively labelled methyl methanesulphonate, there was an 8 times preference for the formation of S<sup>6</sup>-methylthioguanine compared with N<sup>7</sup>-methylguanine. The ratio of O<sup>6</sup>-methylguanine:N<sup>7</sup>-methylguanine in non-thioguanine containing DNA after reaction with methyl methanesulphonate is 0.09:1 (Singer and Grunberger, 1983). Therefore there is approximately 90 times more S<sup>6</sup>-methylthioguanine produced than O<sup>6</sup>-methylguanine after reaction with methyl methanesulphonate.

This result might explain why the *in vitro* results display good synergy between thioguanine and methyl methanesulphonate but not with temozolomide. The combination of thioguanine and temozolomide had an effect of the tumour xenograft growth *in vivo*. In light of the HPLC results, the obvious next experiment would be the treatment of nude mice bearing xenografts with thioguanine and methyl methanesulphonate.

The results reported here show that temozolomide plus thioguanine is far more effective than temozolomide alone on glioblastoma and melanoma xenografts and therefore suggests that this combination may be more effective than temozolomide alone on these tumours in man. This raises two questions; first is this discovery likely to be of clinical use and second as similar enhancement of temozolomide action has been produced by pretreatment with O<sup>6</sup>-benzylguanine, whether this combination of temozolomide and methylating agent is preferable to temozolomide plus O<sup>6</sup>-benzylguanine, which is currently undergoing clinical trials. Treatment of humans with O<sup>6</sup>-benzylguanine is likely to increase the risk of cancer

in other tissues since it depletes O<sup>6</sup>-methylguanine-DNA-methyltransferase. There is no need to inhibit methyltransferase activity when using thioguanine and temozolomide. Thioguanine has been used clinically for many years. Since both thioguanine and temozolomide could be used in combination at considerably lower doses than in isolation and there could be less problems with thioguanine than with O<sup>6</sup>-benzylguanine, thioguanine with methylating agent therapy is worthy of clinical trials.

The experiments described in this chapter were carried out to test the prediction that thioguanine and temozolomide would be synergistic in their cytotoxic action. As discussed in the introduction this prediction was based on the supposition that thioguanine and temozolomide both owe their cytotoxic action to the 6-methylated bases, S<sup>6</sup>-methylthioguanine and O<sup>6</sup>-methylguanine they produce in DNA and recognition by proteins of the post-replicative mismatch repair system. Because temozolomide would be expected to react much more readily with the sulphur of thioguanine residues in DNA than with the oxygen of guanine, the incorporation of only a small number of thioguanine residues into the DNA before the dose of temozolomide would result in a significant increase in the total amount of 6-methylated bases in the DNA and thus in the degree of cell death. The result of the experiment (fig 2.4 and 2.5) are as predicted and thus support the hypothesis on which the prediction was based. It was assumed that the xenografts used in this work were mismatch repair proficient on the basis of a number of experiments performed on the U87MG and A375P cell lines before injection into the mice. Both cell lines were found to express the mRNA for hMHS2, hPMS2, hMSH6, hMLH1 and hPMS1. An oversight here was that the expression of hMSH3, the partner of hMSH2 in hMutS $\beta$ , was not determined. The importance of MSH3 expression and cellular response to cytotoxic anticancer drugs has recently been

demonstrated in a study where the overexpression of MSH3 was found to be accompanied by significantly lower growth inhibition after treatment with temozolomide, thioguanine and N-methyl-N'-nitro-N-nitrosoguanidine (Pepponi *et al.*, 2001). This is because overexpression of MSH3 sequesters MSH2 and therefore lowers the amount of hMutS $\alpha$  protein (Drummond *et al.*, 1997). Both U87MG (glioblastoma) and A375P (melanoma) cell lines were found to have GT binding activity by gel shift and were found to express the hMSH2 and hMLH1 protein by western blot. While these experiments do not suggest that U87MG and A375P cells are mismatch repair deficient, they do not conclusively show that they are mismatch repair proficient either. The functional mismatch repair assay as used in chapter 3 (see page 166) or the demonstration of absence of microsatellite instability would provide more convincing evidence for the mismatch repair proficiency of these cell lines. However, there are no reports in the literature of the A375P and U87MG cell lines being mismatch repair deficient therefore all available evidence suggests that the cells in culture and the xenografts derived from them are mismatch repair proficient.

However, despite the demonstration of synergy *in vivo*, it is too early to say that the result proves the hypothesis particularly as the synergism was not seen when the two drugs were used on the glioblastoma and melanoma cells in tissue culture (fig 2.6 and 2.8). This failure with the cultured cells may not be crucial as a test of the prediction because a similar pattern of synergism against the xenograft with no synergism against the cells in culture was seen when temozolomide and O<sup>6</sup>-benzylguanine were tested together (Wegde *et al.*, 1996; Wedge *et al.*, 1996a). But the failure to demonstrate synergism in tissue culture does emphasize that there might be an explanation for the synergism on the xenografts other than the hypothesis outlined above. Evidence in support of a role for mismatch repair

system in temozolomide cytotoxicity *in vivo* comes from a study in which temozolomide was used as a single agent against 15 independently derived xenografts (Middlemas *et al.*, 2000). The sensitivity of each tumour was compared with the expression of MGMT, MSH2 and MLH1. Each tumour classified as highly sensitive or of intermediate sensitivity had low levels of MGMT and demonstrated detectable MSH2 and MLH1. Tumours classified as having low sensitivity fell into two groups: those with high MGMT and detectable expression of MSH2 but undetectable or marginal MLH1 and those with a whole range of MGMT but with undetectable or marginal MLH1. The authors conclude that high levels of MGMT predict intrinsic resistance to temozolomide but for tumours with low or undetectable MGMT, mismatch repair status is an important determinant of response. The U87MG (glioblastoma) tumours used in this thesis have low (< 15 fmol/mg protein) MGMT activity while A375P (melanoma) have high (95 fmol/mg protein) MGMT activity. Therefore if we assume that both U87MG and A375P tumours express MSH2 and MLH1 on the basis of the western blot experiments (fig 2.3), the synergy seen *in vivo* between thioguanine and temozolomide would appear to be independent of MGMT activity.

Since the synergism was seen only when the tumour was growing as a xenograft in nude mice. It is possible that this growth of the xenograft in mice plays a fundamental role in the synergism. When we obtained this result, we discussed the possibility that the effect of the second drug was primarily to damage blood supply to the tumour by for example damaging the vasculature. An explanation of this sort might also be considered for the experiments with temozolomide and O<sup>6</sup>-benzylguanine reported by Wedge *et al* (1996 and 1996a). One cannot explain why depletion of the MGMT in the U87MG tumour with low MGMT activity could have such a large effect unless the effect of the O<sup>6</sup>-benzylguanine is on the

vasculature of the tumour rather than on the tumour itself. Unfortunately, investigation of such a possibility could only be undertaken by a laboratory with experience in histology and was outside the capability of our laboratory. An alternative suggestion has been that thioguanine might act like O<sup>6</sup>-benzylguanine and deplete O<sup>6</sup>-alkylguanine-DNA-alkyltransferase levels. This would be consistent with two aspects of the results shown in fig 2.4. First the magnitude of the increase in the anti-tumour effect which thioguanine produces is similar to the increase in the magnitude of the increase produced by O<sup>6</sup>-benzylguanine (Wedge *et al.*, 1996b). Second thioguanine and the O<sup>6</sup>-alkylguanine-DNA-alkyltransferase inhibition by O<sup>6</sup>-benzylguanine were effective against the xenograft but ineffective against the human cells in culture (Wedge *et al.*, 1996; Wedge *et al.*, 1996a). O<sup>6</sup>-benzylguanine inactivates the alkyltransferase by reacting with the cysteine in the active site of the protein which normally acts as the receptor for the alkyl groups of O<sup>6</sup>-alkylguanine and O<sup>4</sup>-alkythymine residues in DNA (reviewed by Pegg *et al.*, 2000). In the case of thioguanine the proposed depletion of the O<sup>6</sup>-alkylguanine-DNA-alkyltransferase would be produced by S<sup>6</sup>-methylthioguanine arising either from the chemical methylation of thioguanine residues in DNA by S-adenosylmethionine (Swann *et al.*, 1996) or by the enzymic methylation of the free base by thiopurine methyltransferase (E.C. 2.1.1.67). Mice like humans, have a thiopurine methyltransferase but because methylation is an important detoxification pathway for thioguanine, azathioprine and mercaptopurine most of the whole animal studies have been done in man. Measurements of the amount of methyltransferase (Dooley and Maddocks, 1985) and the very large difference in the effectiveness of the thioguanine group of drugs between people with high and low levels of thiopurine methyltransferase suggests that formation of S<sup>6</sup>-methylthiopurine is an important pathway for thioguanine. If the O<sup>6</sup>-alkylguanine-DNA-alkyltransferase is depleted by dosing the animals with thioguanine then the free base S<sup>6</sup>-

methylthioguanine rather than the S<sup>6</sup>-methylthioguanine residues in DNA is the most probable culprit. This is because only a very small proportion of the administered thioguanine is incorporated into DNA and only a very small proportion of that is methylated (Swann *et al.*, 1996). As mentioned above, Wedge *et al* showed synergism between temozolomine and O<sup>6</sup>-benzylguanine against the same glioblastoma xenograft used in the experiments described in this thesis but failed to show synergism against the glioblastoma cells in tissue culture (Wedge *et al* 1996; Wedge *et al.*, 1996a). The only explanation the authors offer was 'some efficacy is derived from a pharmacokinetic interaction of temozolomide with O<sup>6</sup>-benzylguanine *in vivo*'. It is not clear what they meant by this statement but one could suggest another explanation. One reason that O<sup>6</sup>-benzylguanine has proved more effective in depleting O<sup>6</sup>-alkylguanine-DNA-alkyltransferase *in vivo* than some 6-substituted guanines which react with the pure alkyltransferase more readily than it does is that a substantial part of the effectiveness of O<sup>6</sup>-benzylguanine is due to its metabolite 8-oxo-O<sup>6</sup>-benzylguanine (Roy *et al.*, 1995). O<sup>6</sup>-benzylguanine is extremely insoluble so the production of an active metabolite makes a significant contribution to its effectiveness. The oxidation of the 8-position is carried out by cytochrome P450 (Roy *et al.*, 1995). It is unlikely that the glioblastoma cells are capable of this metabolism so there would be a real difference between the fate of O<sup>6</sup>-benzylguanine in the xenograft and tissue culture experiments, which might explain the different results which Wedge observed. It could be envisaged that there might be a similar explanation for the difference in the effect of thioguanine and temozolomide against xenografts and against cells in culture. 8-oxoguanine is a prominent metabolite of thioguanine and although no report could be found in the literature it seems likely that S<sup>6</sup>-methylthioguanine is converted to 8-oxo-S<sup>6</sup>-methylthioguanine. Taken alone the discussion above might suggest that a plausible explanation for the synergism between thioguanine and

temozolomide is that the S<sup>6</sup>-methylthioguanine produced from the thioguanine depleted the O<sup>6</sup>-alkylguanine-DNA-alkyltransferase. However it is unlikely to be the correct explanation because S<sup>6</sup>-methylthioguanine reacts extremely slowly with O<sup>6</sup>-alkylguanine-DNA-alkyltransferase. The human alkyltransferase reacts with S<sup>6</sup>-methylthioguanine residues in DNA 100,000 thousand times more slowly than it does with O<sup>6</sup>-methylguanine residues (Swann *et al.*, 1996). Similarly studies of O<sup>6</sup> and S<sup>6</sup>-substituted purines showed that S<sup>6</sup>-benzylguanine did not inactivate the human alkyltransferase. Consistent with this Yarosh and Day (1986) found that S<sup>6</sup>-methylthioguanine had no effect on alkyltransferase levels in cells with tissue culture.

An experiment to test the combination of thioguanine and methyl methanesulphonate *in vivo* is currently being carried out in this laboratory. It is hoped that this experiment will address some of the unanswered questions relating to synergy between thioguanine and methylating agents. Methyl methanesulphonate produces very little O<sup>6</sup>-methylguanine so it would be expected that S<sup>6</sup>-methylthioguanine be the main DNA adduct associated with cytotoxicity. MGMT does not repair S<sup>6</sup>-methylthioguanine residues very efficiently so if the proposed mechanism of thioguanine toxicity were correct then MGMT should not be depleted in the xenografts. It is intended that MGMT levels will be measured in this experiment. Maintenance of MGMT activity will demonstrate that there is no inhibition of its activity by any S<sup>6</sup>-methylthioguanine free base.

## **Chapter 3**

# **Thioguanine Resistance and Defective Mismatch Repair**

## Chapter 3

### Thioguanine resistance and defective mismatch repair

#### Introduction

This chapter is concerned with the production of cells defective in post-replicative mismatch repair. A functional post-replicative mismatch repair pathway is vital for the maintenance of genomic integrity and defects in this pathway are well documented in both hereditary and sporadic human cancers. In particular, hereditary non-polyposis coli colorectal cancer (HNPCC) has been shown to be caused by an inherited mutation in one allele of one of the genes involved in post replicative mismatch repair. Mutations in hMSH2 are the most common causes of HNPCC, closely followed by mutations in hMLH1 (Peltomaki, 1997). HNPCC accounts for 1-5 % of all colorectal cancers. The familial clustering of colorectal tumours is associated with an increased incidence of endometrial and to a lesser extent, stomach, urinary tract and ovarian carcinoma among family members (Papadopoulos *et al.*, 1995). Tumours in HNPCC patients develop when the remaining functional mismatch repair allele is lost by mutation. However, sporadic tumours with loss of post-replicative mismatch repair in colon (Herman *et al.*, 1998), gastric (Leung *et al.*, 1999), and endometrial cancers (Gurin *et al.*, 1999) are most commonly deficient in the protein hMLH1 in the absence of mutation in the hMLH1 gene. Rather, there is downregulation of hMLH1 as a result of promoter hypermethylation (Herman *et al.*, 1998; Kane *et al.*, 1997; Wheeler *et al.*, 1999). Karran (Karran and Bignami, 1994) has proposed that the down regulation of mismatch repair in sporadic cancers in the colon is a result of prolonged exposure to N-nitroso compounds in the form of bile acids.

Attempts to localize the gene predisposing to HNPCC by genetic linkage analysis with microsatellite sequences revealed an unusual phenotype of HNPCC tumours.

Instead of the one or two distinct parental alleles, tumour DNA microsatellites were found to be extremely polymorphic. Lengthened and shortened versions of a number of mono and dinucleotide repeat loci on a number of difference loci were seen, referred to as microsatellite instability (MIN) (Umar *et al.*, 1994). At least 90% of HNPCC colorectal tumours exhibit MIN. The level of MIN in sporadic colon adenocarcinomas is much lower, at 15%. MIN in sporadic cancers is also observed in non-colonic tumours in non-HNPCC patients such as endometrial or stomach, as well as tumours that are not associated with HNPCC, such as non-small cell carcinoma of the lung. Generally, microsatellite changes associated with HNPCC are early changes so can be considered to be a direct consequence of defective mismatch repair whereas MIN in sporadic cancers occurs later, possibly reflecting a non-specific genome destabilization. This observation first suggested that whereas mismatch repair gene mutations occur early in the progression of HNPCC, some other changes must be occurring early in sporadic colon cancer (Eshelman and Markowitz, 1995). Colorectal cancer is a stepwise process that from normal colonic mucosa leads to carcinoma through premalignant lesions known as adenomas. Recently evidence has accumulated suggesting that the early changes referred to as aberrant crypt foci can be considered premalignant and these foci exhibit microsatellite instability (Pedroni *et al.*, 2001). Aberrant crypt foci are seen on the colonic mucosal surface of humans and animals treated with colon carcinogens (Tudek *et al.*, 1989).

For some cytotoxic drugs such as thioguanine, temozolomide and cisplatin, the mismatch repair system provides a link between recognition of DNA damage and downstream effectors of an apoptotic response such as p53 (Anthony *et al.*, 1996; Duckett *et al.*, 1999). Cisplatin Aebi *et al.* 1996; (Brown *et al.*, 1997) doxorubicin (Toft *et al.*, 1999) and N-methyl-N-nitrosourea (Karran and Bignami, 1992) have

been shown to convert mismatch proficient cells to mismatch repair deficient cells *in vitro*. If thioguanine were also capable of converting mismatch repair proficient cells to mismatch repair deficient cells it would provide evidence in support of Karran's hypothesis and might explain one of the problems associated with the use of the thioguanine prodrug azathioprine. Azathioprine is an immunosuppressive drug taken by renal transplant recipients to prevent transplant rejection. It is metabolised to an active intracellular metabolite, 6-thioguanosine (Lennard *et al.*, 1984). Increased levels of 6-thioguanine has been found in the circulation of patients taking azathioprine (Lennard *et al.*, 1984). There is an increased incidence of skin cancer in renal transplant recipients (Bouwes-Bavinck *et al.*, 1996) and several mechanisms have been proposed to account for this increase increased risk, including loss of immune surveillance (Liddington *et al.*, 1989), solar radiation (Bouwes-Bavinck *et al.*, 1996), and viral infection (Kiviat, 1999).

Renal transplant recipients under chronic immunosuppression have a 27-40% cumulative risk of developing either a basal cell or squamous cell carcinoma within the first 20 years following transplantation (London *et al.*, 1995; McGregor and Proby, 1995). Basal cell and squamous cell carcinomas account for approximately 90% of all skin malignancies (Preston and Stern, 1992). . Basal cell carcinomas arise from cells of the lowermost layers of the epidermis. Squamous cell carcinomas may occur anywhere on the skin, although mainly arise in sun-damaged skin. The limits of the neoplasm are not well defined and often extend beyond the visible margin of the lesions. The edges of the nodules or plaques are indurated and red-yellowish in colour. Histologically, the neoplasm consists of nests and islands of atypical keratinocytes. Squamous cell carcinoma may occasionally infiltrate among nervess, blood vessels and lymphatics. Recurrence is more frequent in squamous cell carcinomas with deep infiltration and poor differentiation (Preston and Stern,

1992). The risk of metastases depends on the clinical setting in which the lesion arises. The lowest risk is for squamous cell carcinoma originating on sun damaged skin. In contrast, squamous cell carcinomas arising in skin not exposed to the sun seem to bear a higher risk of metastases. Metastases usually develop in the lymph nodes and subsequently extend to other organs (Gloster and Brodland, 1996).

Epidemiological data has long implicated sun exposure as a risk factor for sporadic non-melanoma skin cancer (Giles *et al.*, 1988). The direct absorption of UVC (200 - 290 nm) or UVB (290 - 320 nm) by DNA leads primarily to photoproducts joining adjacent pyrimidines. The resulting mutations are mainly CC to TT double base substitutions and C to T substitutions at dipyrimidine sites and are unique to UV light. Double base mutations are rarely produced by other mutagens; hydrogen peroxide and cisplatin cause occasional double mutations but not CC to TT. Therefore because CC to TT base changes are only known to be caused by UV, their presence identifies UV as the mutagen. Brash *et al* in 1991 found that the majority of squamous cell carcinomas of the skin contain mutations in the p53 tumour suppressor gene and that these mutations were the type caused by UV radiation (Brash *et al.*, 1991). Since squamous cell carcinomas in renal transplant recipients are most commonly on the sun exposed surfaces of the skin, UV light has been proposed to be a contributing factor for the induction of skin cancer in immunosuppressed as well as immune competent individuals (Leffell and Brash, 1996).

Since there is a higher incidence of squamous cell carcinoma in renal transplant recipients compared with the general population UV-light cannot be the sole causative agent. The general assumption is that immune suppression is the major contributing factor in squamous cell carcinoma of renal transplant recipients. It was

proposed that cancerous cells would normally be eliminated by a healthy immune system and failure of this system for any reason would allow progression to cancer to occur (Kinlen, 1992). There are additional consequences of impaired immune surveillance, namely that immunosuppression may allow the proliferation of mismatch repair defective cells (Harwood *et al.*, 2001). Immunological surveillance is particularly severe on mismatch repair defective tumours (Branch *et al.*, 1995). Mismatch repair defective cells have a high rate of frame shift mutations. When proteins derived from the frameshifted genes are processed by the cell during normal protein turnover, they will generate a significant number of foreign peptides, thus effective immune surveillance may act to reduce the incidence of repair defective tumours (Harwood *et al.*, 2001).

Renal transplant patients were the most widely studied candidates for immune suppression in the 1980s, however more recently, the AIDS epidemic has provided another opportunity for the study of the effects of immune impairment of cancer risk. Kaposi's sarcoma is the most common tumour associated with human immunodeficiency virus type 1 infection occurring in approximately 30% of HIV-1 infected homosexual men (Reitz *et al.*, 1999). The incidence of Kaposi's sarcoma is 100,000 fold greater in this risk group relative to the general population (Biggar *et al.*, 1996). The peculiar epidemiology of Kaposi's sarcoma in different HIV-transmission groups indicates that an environmental or transmissible agent other than HIV must be involved in Kaposi's sarcoma pathogenesis (Reitz *et al.*, 1999). Whereas 20% of homo and bisexual AIDS patients develop Kaposi's sarcoma, only 1% of age and sex matched AIDS patients with haemophilia suffered from Kaposi's sarcoma. Therefore the high incidence of Kaposi's sarcoma in AIDS patients cannot be explained solely by impaired immune surveillance allowing overproliferation of the HIV virus.

The first report of cancer in renal transplant patients first appeared in the literature in the 1960s (Kinlen, 1992). The first cohort studies performed found a three fold increased incidence of a few cancers including non-Hodgkin's lymphoma, skin cancer and mesenchymal tumours. It was notable that none of the fatal cancers that are common in western societies was represented in this group. The development of non-Hodgkin's lymphoma in the renal transplant patients was characterised by a very short induction period after transplantation, often within six months of transplantation (Hoover and Fraumeni, 1973). Altered immunity of transplant patients to certain viruses was found, suggesting that the lymphomas might also be of viral origin (Kinlen, 1992). The Epstein-Barr virus (EBV) was detected in the malignant cells of transplant patients and immunosuppressive therapy could allow the reactivation of EBV (Crawford *et al.*, 1981). Cancerous cells of transplant patients have also been found to contain human papillomavirus DNA (Barr *et al.*, 1989).

The most common cancer in transplant patients is skin cancer (Bouwes-Bavinck *et al.*, 1996). In the general population the great majority of skin cancers are basal-cell type, squamous-cell carcinoma is the most common in renal transplant recipients (Bouwes-Bavinck *et al.*, 1996). These skin cancers occur mostly on the light exposed surfaces of the skin as in the general population and in addition, and have been found to contain human papillomavirus DNA (Barr *et al.*, 1989).

The work in this chapter aims to investigate whether thioguanine can convert normal mismatch repair competent to mismatch repair deficient cells therefore providing evidence that the azathioprine associated skin tumours have arisen, at

least in part because of defects in the mismatch repair pathway. This chapter describes the production of mismatch repair defective HT29 cells in tissue culture after prolonged exposure to thioguanine. Resistance to thioguanine can arise either because of defective mismatch repair or because of loss of hypoxanthine guanine phosphoribosyl transferase. To ensure that only mismatch repair defective cells were selected, O<sup>6</sup>-methylguanine-DNA methyltransferase activity was depleted with O<sup>6</sup>-benzylguanine and the cells treated with a toxic concentration of N-methyl-N-nitrosourea. RNA and protein was extracted from surviving clones and reverse transcription to cDNA followed by polymerase chain reaction (RT-PCR) was used to measure expression of the mRNA for the mismatch repair genes and western blotting was used to determine the presence of the mismatch repair proteins. The results of these experiments showed that the hMLH1 protein was absent in several clones with accompanying loss of mRNA expression. The absence of hMLH1 expression in the N-methyl-N-nitrosourea resistant clones was found to be caused by methylation silencing of the hMLH1 promoter rather than by mutation of the gene.

## Materials and Methods

### ***Isolation of N-methyl-N-nitrosourea thioguanine resistant clones from HT29 cultures grown in medium containing thioguanine***

HT29 cells were maintained as subconfluent monolayers in Dulbecco's modified Eagle medium (Gibco, Paisley, UK) containing thioguanine (0.075 µg/ml) for 4 months. The medium was supplemented with 10% (v/v) heat inactivated fetal calf serum (Gibco, Paisley, UK), L-glutamine (2mM), penicillin (100 U/ml) and streptomycin (100 µg/ml). After the period of thioguanine treatment, the cells were detached, counted and 800 cells per 40 mm tissue culture dish were plated. HT29 cells that had never been treated with thioguanine were also plated at the same density. There were 2 treatment groups for both thioguanine and non-thioguanine treated cells.

For the first group, the cells were allowed to attach for 24h after plating before the medium was removed and replaced with medium containing O<sup>6</sup>-benzylguanine (25 µM). After 3 hours, the medium was replaced with N-methyl-N-nitrosourea (2 mM) in PBS and left for 30 minutes and replaced with medium containing O<sup>6</sup>-benzylguanine (25 µM). After 10 days, surviving colonies were either fixed in absolute methanol, stained with 1% (w/v) crystal violet in methanol and counted or were transferred into separate flasks and grown until there were enough cells for 4 pellets of 1 x 10<sup>7</sup> cells.

The second group was exposed to O<sup>6</sup>-benzylguanine only. The cells were allowed to attach for 24h, the medium removed and replaced with medium containing O<sup>6</sup>-benzylguanine (25 µM). After 3h the medium was replaced with PBS and left for

30 min and again replaced with medium containing O<sup>6</sup>-benzylguanine. The cells were allowed to grow for a further 10 days until colonies of approximately 50 cells formed and were fixed in absolute methanol, stained with 1% (w/v) crystal violet in methanol and counted.

### ***Western Blot analysis of mismatch repair protein expression in thioguanine resistant HT29 clones***

#### **Preparation of Protein Extracts**

Protein was extracted as described by Nedderman and Jiricny (Nedderman and Jiricny, 1993). All procedures were carried out in a 4°C cold room. Frozen pellets of  $1 \times 10^7$  cells were quick thawed in a 37°C water bath and allowed to swell in three cell volumes of hypotonic buffer (25mM Hepes/NaOH, pH 7.8, 1mM EDTA, 2mM dithiothreitol, 1mM phenylmethylsulfonyl fluoride, 0.5 mM spermidine and 0.1 mM spermine) at 4°C for 20 min. The cells were homogenised in a glass Dounce homogeniser with 20 strokes of a tight fitting pestle, glycerol added to give a final concentration of 20% (v/v), followed by a solution of saturated neutralized (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (11ml/100 ml of extract). The suspension was stored on ice for 30 minutes to allow complete mixture and the extract cleared by centrifugation in a Beckman ultracentrifuge using a Ti-70 rotor at 370,000g (60,000 rpm) for 90 min at 4°C. The resulting supernatant was stored in 20 µl aliquots at -70°C.

#### **SDS-PAGE of Protein**

This was based on the original method of Laemmli (Laemmli, 1970). To make a 7% resolving gel, 30% (w/v) acrylamide/0.8% (w/v) methylene-bisacrylamide (2.3 ml, Anachem), pH 8.8 buffer (2.5 ml) and distilled water (5.0 ml) were mixed in a vacuum flask. The mixture was degassed by vacuum for 10 minutes. TEMED (6 µl) and 10% (w/v) ammonium persulphate (100 µl) were added. This was gently mixed and immediately poured between the glass plates of the electrophoresis

apparatus (Mini-Protean II, Bio-Rad) assembled with 0.75-mm spacers. The gel was overlaid with propan-2-ol and left to polymerise for at least 1 hour. The stacking gel was prepared by mixing 30% acrylamide (0.83 ml), pH 6.8 buffer (0.63 ml), TEMED (5  $\mu$ l), 10% (w/v) ammonium persulphate (50  $\mu$ l) and water (3.4 ml). The stacking gel was mixed and immediately poured over the resolving gel.

### **Preparation and Electrophoresis of Samples**

Protein samples were prepared for electrophoresis by the addition of loading buffer (1% (w/v) SDS/ 30 mM Tris HCl (pH 6.8)/ 5% glycerol/ 0.02% (w/v) bromophenol blue).  $\beta$ -mercaptoethanol (1% (v/v)) was added and the mixture was heated to 90°C for 5 minutes. The sample was centrifuged briefly in a microcentrifuge to sediment any debris and collect the sample at the bottom. The supernatant (15  $\mu$ l) was loaded onto the gel and electrophoresed in 0.025M Tris/ 0.192 M glycine/ 0.1%(w/v) SDS, pH 8.4 at 10 V/cm until the dye entered the resolving gel. The current was then increased to 20 V/cm until the dye had migrated to roughly 0.5 cm of the end of the gel.

### **Staining of Gels**

Gels were transferred to a container with 5 volumes of Coomassie blue staining solution (0.25 g Coomassie Brilliant Blue R250 in 100 ml methanol:water:glacial acetic acid (45:45:10) filtered through Whatman No. 1 filter paper) and left gently agitating on a rotating platform for at least 4 hours. The gel was destained with several changes of methanol:water:acetic acid (45:45:10) until the bands were distinct and the background clear. The stained gel was photographed on an illuminated light box.

## **Western Blot**

To detect the presence of specific mismatch repair proteins, the SDS-PAGE gel was blotted onto a nitrocellulose membrane (Hybond ECL, Amersham). Antigen detection was carried out using the Enhanced Chemiluminescence (ECL) Plus Western Blot Kit (Amersham) according to the manufacturer's instructions. Briefly, the resolving gel was placed on a sheet of Hybond ECL cut to the same size as the gel. The gel/membrane was sandwiched between 2 sheets of Whatman 3MM chromatography paper of approximately same size as the gel/membrane. This was placed in a cassette between sponge pads and inserted into a blotting tank (Bio-Rad) containing enough transfer buffer (192 mM glycine/25mM Tris, pH 8.4/20% (v/v) methanol) to completely submerge the cassette. The protein was transferred overnight from the gel to the membrane by electrophoresis at 30V. The membrane was removed and placed in a plastic box (9.5 x 9.5 x 1.5 cm), blotted surface facing upwards. Remaining protein binding sites were blocked with a 8% (w/v) solution of blocking reagent (Marvel milk powder) in TBS-T (10ml) with agitation on a rotating platform for 1 hour at room temperature. The blocking reagent mixture was poured off and the membrane washed for 20 minutes with 4 changes of TBS-T (10 ml). The primary antibodies of appropriate dilution in 4% (w/v) blocking agent in TBS were added to the membrane and agitated for 1 hour as before. The primary antibody solution was poured off and the membrane was washed with TBS-T as before. The horseradish peroxidase linked secondary antibody was used at an appropriate dilution, as determined by titration blotting experiments and was prepared in 4% (w/v) blocking agent in TBS-T (10 ml). The membrane was incubated with the secondary antibody for 1 hour followed by washing in TBS-T as before. Enhanced chemiluminescent (ECL) detection was carried out according to the manufacturer's instructions (Amersham).

### **Staining of Nitrocellulose Blots**

Amido black staining solution (0.1% (w/v) Amido Black in isopropanol:acetic acid:H<sub>2</sub>O 25:10:65 by vol) was routinely used for the staining of nitrocellulose blots after blotting and ECL detection (Gershoni and Palade, 1982). The solution can detect microgram quantities of proteins transferred onto nitrocellulose membranes. The membrane was immersed in Amido Black staining solution and stained for 1 minute. Excess Amido Black was removed by immersing the membrane in isopropanol:acetic acid:H<sub>2</sub>O 25:10:65 by volume for 30 minutes. If a lighter background was necessary, the membrane was left in this solution overnight. The destained membrane was then stored in deionized water or allowed to air dry.

**Reverse transcription-polymerase chain reaction to measure the expression of mRNA of mismatch repair genes in thioguanine resistant HT29 clones**

All procedures used to detect mismatch repair gene mRNA were the same as those described in chapter 2.

**In vitro Mismatch repair assay to measure repair in thioguanine resistant HT29 clones**

The *in vitro* mismatch repair assay for the measurement of mismatch repair activity in the thioguanine resistant HT29 clones was performed with the help of Dr Peter Karran and Mr Peter MacPherson at the Imperial Cancer Research Fund, Clare Hall laboratories, South Mimms, UK.

**Preparation of protein extract of HT29 clones for the *in vitro* mismatch repair assay**

Cells in the exponential phase of growth (1 litre at  $6.8 \times 10^5$  cells/ml) were harvested and washed with PBS. For the extraction of protein, all buffers used were at 4°C, all procedures performed on ice and all rotors and centrifuges were pre-cooled to 0-4°C. The cell pellet was resuspended by gentle pipetting in 250 ml hypo/sucrose buffer (20 mM HEPES-KOH pH 7.5, 5 mM KCl, 1.5 mM MgCl<sub>2</sub> 0.5 mM dithiothreitol (DTT), 0.25 M sucrose) followed by centrifugation at 4,000 rpm for 10 min in Sorvall 250 ml bottles. The supernatant was carefully removed and the pellet was resuspended by gently pipetting in 240 ml Hypo buffer (20 mM HEPES-KOH pH7.5, 5 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.5 mM DTT) and again centrifuged at 4,000 rpm in Sorvall 250 ml bottles for 10 min. The supernatant was removed, the cells transferred as thick slurry to a cold Dounce glass 15 ml homogeniser and allowed to swell on ice for 30 min. Aprotinin (50 µl, 10mg/ml)

was added and the cells were disrupted with 20-30 strokes. The suspension was transferred to 15 ml glass Sorvall tubes and allowed to stand on ice for 15-20 min followed by centrifugation at 10,000 rpm in a Sorvall SA600 rotor for 20 min and 50,000 rpm in a Beckmann TLA120 rotor for 1h. The supernatant was snap frozen in liquid N<sub>2</sub>, aliquoted and stored at -70°C.

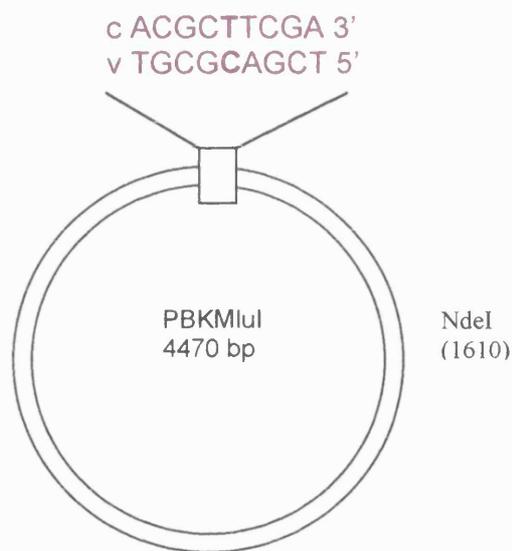
### **Preparation of plasmid substrates for the *in vitro* mismatch repair assay**

This experiment was carried out with Mr Peter MacPherson at the Imperial Cancer Research Laboratories, Clare Hall. The substrate for the assay is derived from a pBK-CMV phagemid (Stratagene) that contains a 211 base pair Pvu I/Pst I fragment of HK7 M13 (Varlet *et al.*, 1990). This 211 base pair fragment contains a sequence that can be used create specific mismatches within restriction endonuclease sites. For the experiment here, this sequence contained a TC mismatch as shown in figure 3.1 (Hampson *et al.*, 1997). Repair of the TC mismatch creates an MluI restriction site. After incubation of substrate with protein extract, digestion with MluI will be diagnostic for mismatch repair activity.

To prepare the TC containing substrate, the replicative form of the plasmid containing an AT pair at position 1030 was purified and linearized by digestion with NdeI at position 1610. The single stranded viral form containing a C at position 1030 was also purified by standard techniques (Sambrook *et al.*, 1989). The linear double stranded DNA was then mixed with a 2-fold excess of the single stranded DNA and the mixture was adjusted to 50% formamide (Hampson *et al.*, 1997).

The purified 4470 base pair molecules contain a CT mispair that inactivates a *Mlu* I restriction site. The mismatched T is 580 base pairs away from a single nick in the 5' direction. Most substrate preparations contained a small amount of reannealed

linearized plasmids. The restriction endonuclease digestion products of these contaminating molecules are well resolved from the diagnostic repair products and do not interfere with the assay.



**Figure 3.1** The substrate for the *in vitro* mismatch repair assay

Substrates containing a CT mismatch were derived from pBK-CMV molecules containing a subcloned 211 bp region of HK7. Viral DNA containing a C at position 1030 was annealed with an excess of linearised double stranded plasmid containing an AT pair at position 1030 to create a CT mismatch. The CT mismatch is located 580 base pairs 5' from a single stranded nick. Correction of the mismatch creates a MIul restriction site that is diagnostic for mismatch repair.

### Mismatch repair assay

Each reaction (25  $\mu$ l) contained 30 mM HEPES-KOH pH 8.0, 0.7 mM MgCl<sub>2</sub>, 0.5 mM DTT, 0.1 mM each dNTP (Pharmacia Biotech), 4 mM ATP, 40 mM phosphocreatine, 1 mg creatine phosphokinase (rabbit type I), 40 ng (141 fmol) of substrate and cell extract (150  $\mu$ g of protein). Each reaction was performed in triplicate. The reactions were incubated at 37°C for 20 min followed by termination by addition of EDTA (1.5  $\mu$ l of 0.5 M to give a final concentration of 10 mM). Each reaction was mixed well and stored on ice before addition of Proteinase K (3.75  $\mu$ l of 1mg/ml). SDS (3.75  $\mu$ l of 10% (w/v)) and glycogen (0.5  $\mu$ l) were added to each reaction, mixed well and incubated for 1 h at 37°C. DNA was extracted by standard phenol-chloroform extraction and digested with 10 U Mlu I (New England Biolabs) for 1h at 37°C. Each digestion contained DNA solution (15.2  $\mu$ l), NEB buffer 2 (1.8  $\mu$ l) and Mlu I (1  $\mu$ l of 10, 000 U/ml). After digestion, the samples separated on a 0.8 % agarose gel containing ethidium bromide at 10V/cm for approximately 1h.

An *Mlu* I site is located at position 463, and digestion of the uncorrected substrate generates linear molecules of 4470 base pairs. Digestion of molecules that have undergone nick-directed repair of TC to GC to create the second *Mlu* I site produces two fragments of 3,900 base pairs and 567 base pairs. Digestion of the small amount of contaminating matched linear molecules generated during the annealing reaction produces traces of fragments of 3.3 and 1.17 kilobase pairs that are resolved from the products of mismatch repair. Therefore, the presence of a 3.9 kilobase pair fragment that is resolved from 4.47 kilobase pair molecules is diagnostic for mismatch correction

### **Promoter methylation assays in MHL1 deficient HT29 clones**

#### **Genomic DNA extraction**

Genomic DNA was extracted by phenol chloroform extraction as follows. After suspension in homogenisation buffer cells were broken in a Duall homogeniser with 10 passes of the pestle. An equal volume of phenol:chloroform:isoamylalcohol was added to the homogenised cells ( $1 \times 10^6$ ) in a 1.5 ml eppendorf tube and mixed until an emulsion formed. The organic and aqueous phases were separated by centrifugation at 1600 g for 3 min at room temperature and the aqueous, DNA containing upper phase was transferred to a fresh 1.5 ml eppendorf tube. The extraction was repeated until there was no protein visible at the interface between the organic and aqueous phases. An equal volume of chloroform was added to the pooled aqueous phases and extraction repeated. The DNA was recovered by precipitation with ethanol and pelleted by centrifugation at 1600g. The pellet was washed several times with 70% ethanol and resuspended in TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA).

#### **Digestion of genomic DNA for the analysis of hMHL1 promoter methylation**

The method used for analysis of methylation of the promoter of hMLH1 was based on that of Kane *et al* (1997). Three reactions were set up for each genomic DNA sample, digestion with Hpa II, MspI or uncut control. HpaII and MspI have the same recognition sequence, CCGG, however MspI will cut DNA even if the internal cytosine is methylated, whereas Hpa II will not cut the methylated sequence. The digestion reactions (20  $\mu$ l) contained genomic DNA (250  $\mu$ g), 75 U Hpa II (1.5  $\mu$ l of 50,000 U/ml, New England Biolabs) or 150 U Msp I (1.5  $\mu$ l of 100,000 U/ml, New England Biolabs) with the appropriate buffer from New

England Biolabs. For the undigested control reaction, water was added in the place of enzyme. All reactions were incubated at 37°C for 4 hours, followed by 65°C for 30 min to inactivate the restriction enzyme.

### **PCR of DNA from each digestion to analyse cleavage of the MLH1 promoter and analysis of PCR products**

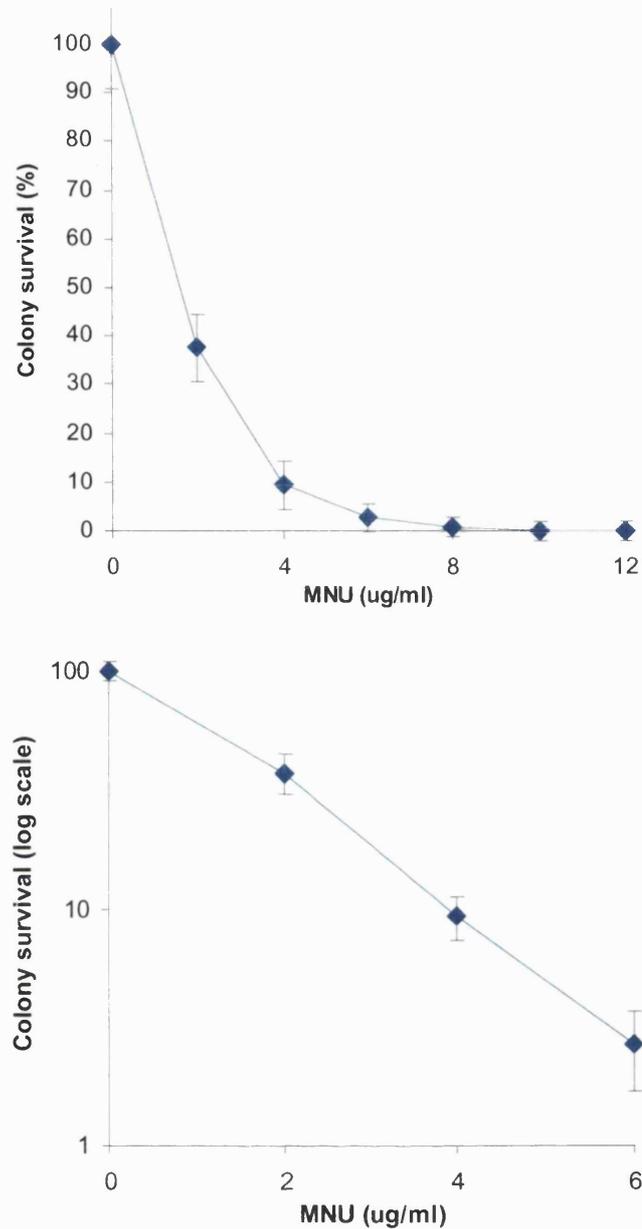
Aliquots of each digestion reaction (10 µl) were used for PCR analysis of the MLH1 promoter. The primers used were hMLH1-pro-s3 (5'-CCA CAT ACC GCT CGT AGT ATT CGT GC-3') and hMLH1-pro-a3 (5'-CCT CAG TGC CTC GTG CTC ACG TTC-3') which gave a product of 603 base pairs in length if an intact MLH1 promoter was present after digestion. The 5' end primer hMLH1-pro-s3 binds to the nucleotide 670 base pairs before the A of the ATG start sequence and the 5' end of primer hMLH1-pro-S3 binds to the nucleotide 67 base pairs before the A of the ATG start sequence. There were 4 Hpa II sequences within the amplified region at positions -567, -527, -347 and -341. Each 50 µl PCR reaction contained 25 ng DNA, 25 µl HotStarTaq Master Mix (Qiagen) (5 U/µl HotStarTaq DNA polymerase, 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 50 µM each dNTP) and 25 pmol of each primer. The samples were heated to 95°C for 15 min to activate the Hot Start Taq polymerase followed by 33 cycles of 94°C for 30s, 55°C for 30s, 72°C for 30s and a final elongation step of 72°C for 7 min. The samples were analysed by electrophoresis on a 1.2% agarose gel.

## Results

### ***Selection of N-methyl-N-nitrosourea resistant clones from the HT29 cells treated with thioguanine***

The colorectal carcinoma cell line HT29 was grown in medium containing 0.075 µg/ml thioguanine for 4 months. Mismatch repair defective cells among the thioguanine treated population were selected by treatment with 25 µM O<sup>6</sup>-benzylguanine and 2 mM N-methyl-N-nitrosourea (MNU). It had previously been shown by Aquilina *et al* (Aquilina *et al.*, 1998) that a 2 h exposure to 25 µM O<sup>6</sup>-benzylguanine was sufficient to inactivate more than 90% of the O<sup>6</sup>-methylguanine-DNA methyltransferase (MGMT) activity in HT29 cells. Since MGMT reduces the cytotoxicity of MNU by removing the methyl group from the resulting O<sup>6</sup>-methylguanine residues in DNA, depletion of MGMT activity would increase the toxicity of MNU. Exposure to 25 µM O<sup>6</sup>-benzylguanine for 24 h after MNU treatment resulted in a 6-fold potentiation of MNU toxicity. This enabled the use of much lower doses of MNU to select for mismatch repair defective cells, thus minimising the handling of this highly carcinogenic chemical.

Colonies were stained and counted after treatment with 25 µM O<sup>6</sup>-benzylguanine alone. When O<sup>6</sup>-benzylguanine was followed by treatment with MNU, a mean survival of 0.12 % resulted with the thioguanine treated cells but there were no surviving clones from the non-thioguanine treated cells. A total of 14 surviving colonies from 2 separate selection experiments were grown up for analysis of mismatch repair status. Clones 1 to 10 were obtained from the first selection experiment and clones 11 to 14 were obtained from the second.

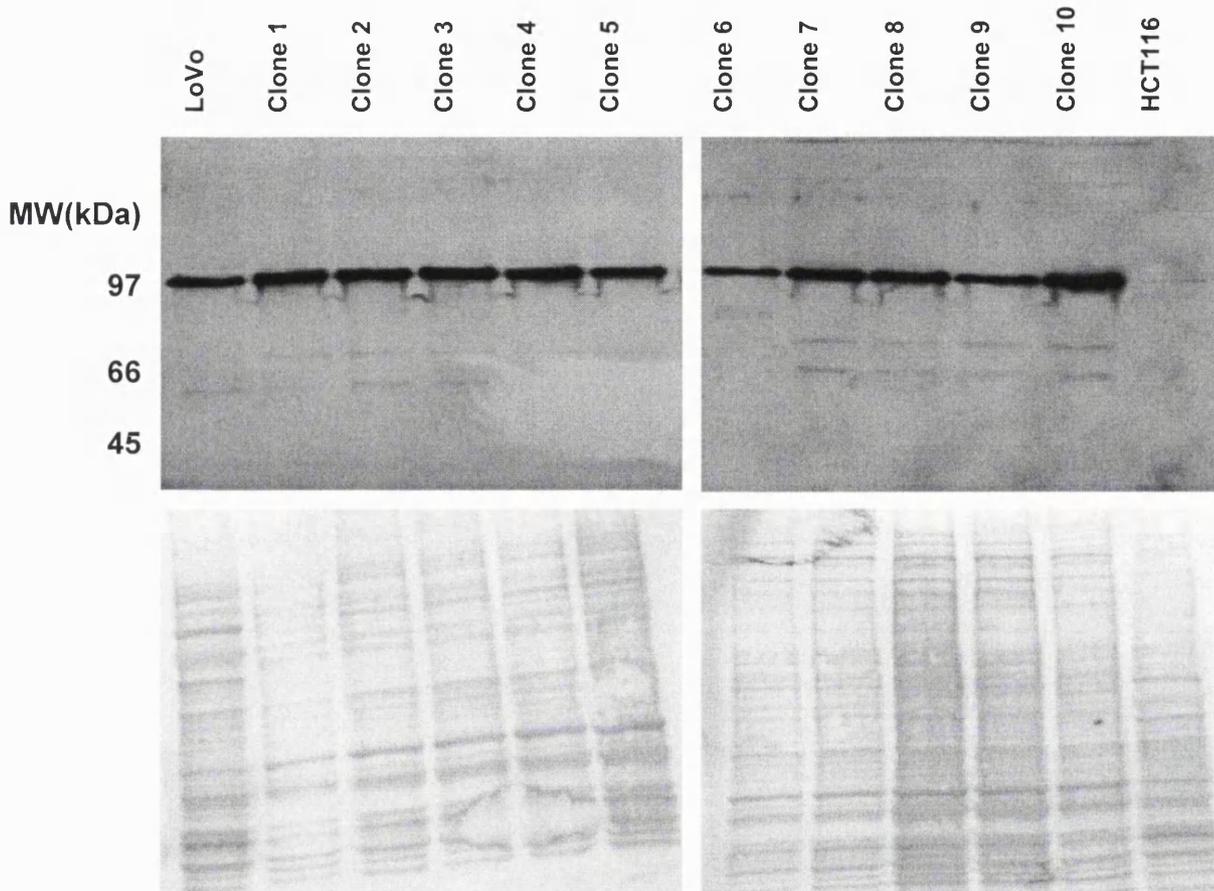


**Figure 3.2 Growth of HT29 cells after treatment with O<sup>6</sup>-benzylguanine and N-methyl-N-nitrosourea (MNU)**

Normal HT29 cells were treated with 25  $\mu$ M O<sup>6</sup>-benzylguanine 3 hours before treatment with MNU at 2, 4, 6, 8, 10 and 12  $\mu$ g/ml. The fresh medium containing 25  $\mu$ M O<sup>6</sup>-benzylguanine was added 24 hours after addition of MNU. This result shows that all the cells are killed by 10  $\mu$ g/ml MNU, which is 21 times lower than the dose used to select for MNU resistant cells after prolonged treatment with thioguanine

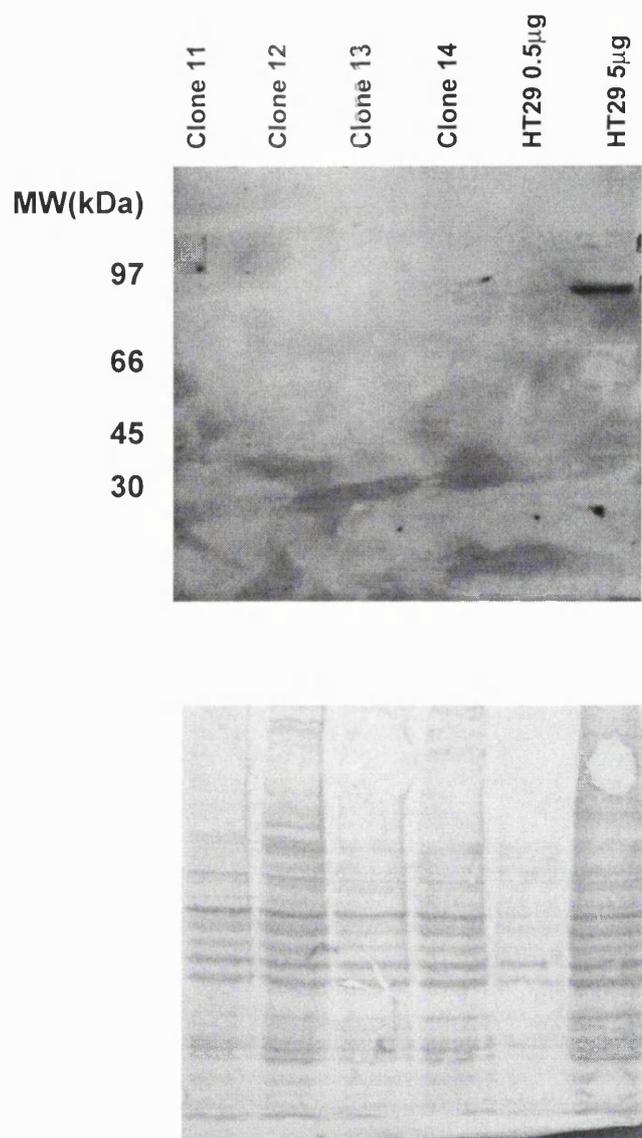
***Western blot analysis to detect mismatch repair proteins in the N-methyl-N-nitrosourea resistant clones***

The absence of either hMSH2 or hMLH1 has been found to be the most common cause of mismatch repair deficiency in both hereditary and sporadic colon cancers, therefore the presence of these proteins was determined in the N-methyl-N-nitrosourea resistant HT29 clones. Four of the clones were found to be negative for hMLH1 (fig 3.3) and one clone was found to be negative for hMSH2 (fig 3.6). The hMLH1 negative clones were also found to be negative for hPMS2, the partner of hMLH1 in the MutL $\alpha$  heterodimer



**Figure 3.3 Western blot showing reaction of total protein from N-methyl-N-nitrosourea resistant HT29 clones with hMLH1 antibody**

10 of the 14 N-methyl-N-nitrosourea resistant clones were positive for hMLH1. Protein extracts from these 10 clones (designated 1 to 10) were simultaneously electrophoresed on SDS-polyacrylamide gels, transferred to nitrocellulose membranes and blotted with anti-hMLH1 antibody. The two upper pictures show the hMLH1 specific band revealed by ECL and the same membranes stained with amido black to check loading are shown in the lower pictures. The LoVo cell line is positive for hMLH1 (but negative for hMSH2) and the HCT116 cell line is negative for hMLH1.



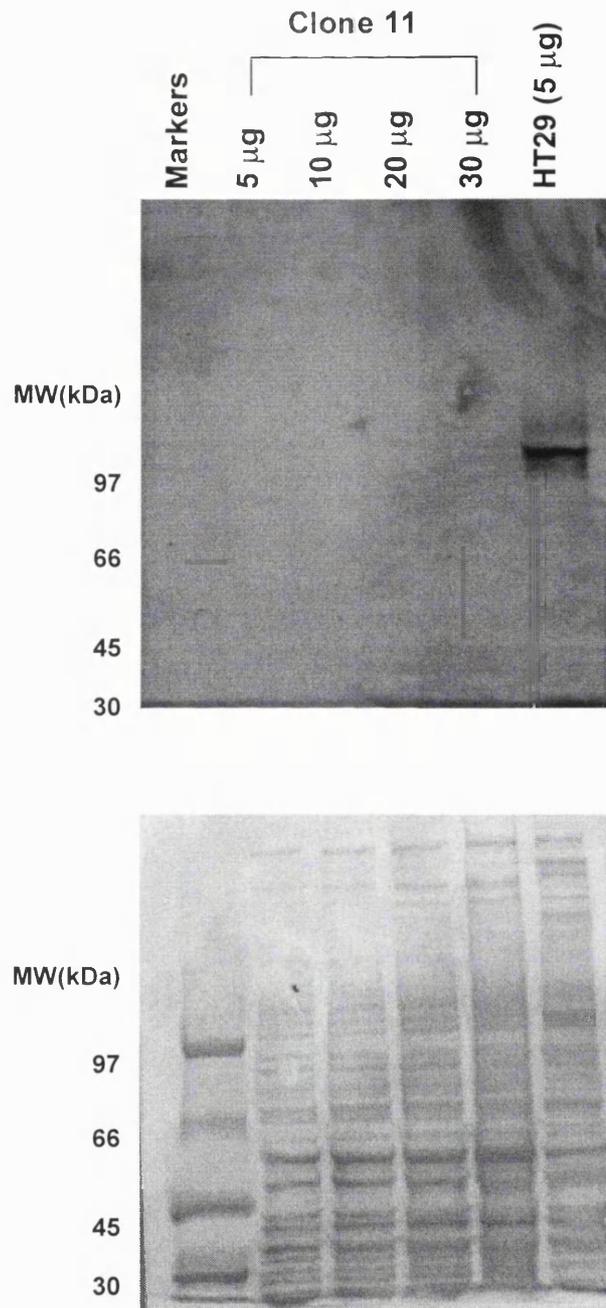
**Figure 3.4 Western blot showing reaction of total protein from N-methyl-N-nitrosourea resistant HT29 clones and normal HT29 cells with hMLH1 antibody**

4 of the 14 N-methyl-N-nitrosourea resistant clones were found to be negative for hMLH1 (designated clones 11 to 14). This was confirmed by repeating the western blot procedure for these clones 3 times. Protein extracts from the 4 hMLH1 negative clones, together with protein from untreated HT29 cells were electrophoresed on an SDS-polyacrylamide gel, transferred to nitrocellulose membrane and blotted with anti-hMLH1 antibody. An hMLH1 specific band was visible when 5µg of normal HT29 total protein extract was loaded but not with 0.5 µg (upper picture). The same membrane was stained with amido black to check loading and transfer (lower picture).

One of the hMLH1 negative clones, clone 11 was used to investigate further the apparent absence of the protein. 5 $\mu$ g of clone 11 protein did not produce a hMLH1 band but 5  $\mu$ g of normal HT29 protein did. Increasing amounts of clone 11 protein were run on a gel, blotted and probed with anti-hMLH1 to see if it was possible to detect a hMLH1 band when larger amounts of protein were loaded onto the gel. However, as figure 3.5 shows, there was no band for hMLH1 detected even with 30  $\mu$ g of clone 11 protein. It is therefore possible to conclude that one of the hMLH1 negative clones, clone 11 has at least 6 times less hMLH1 than the parental HT29 cells line.

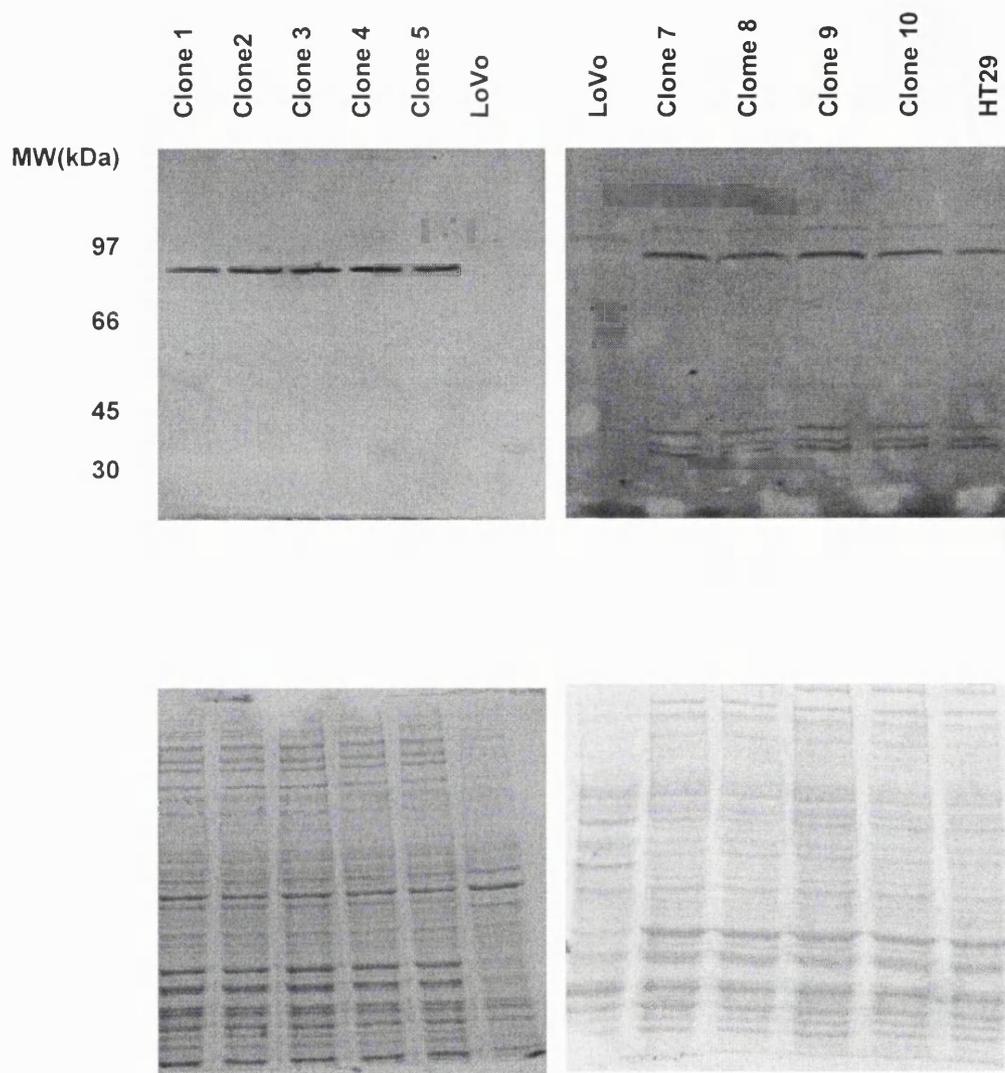
The presence of hMSH2 in the 14 N-methyl-N-nitrosourea resistant clones, using total protein from LoVo cells and normal HT29 as controls, was also determined by western blot. Figure 3.6 shows that clones 1 to 10 are positive for hMSH2 and figure 3.7 shows that clones 11, 12 and 13 are also positive for this protein, however clone 14 appears to lack hMSH2.

The protein hMLH1 is paired with hPMS2 to form the heterodimer hMutS $\alpha$ , in the postreplicative mismatch repair pathway. Cells that are negative for hMLH1 have also been found to lack hPMS2, which is presumed to be because this protein will be short lived in the absence of its partner (Brown *et al.*, 1997). Figure 3.8 shows the result of the western blot for hPMS2 with the hMLH1 negative clones 11, 12, 13 and 14. All 4 were also found to be negative for hPMS2.



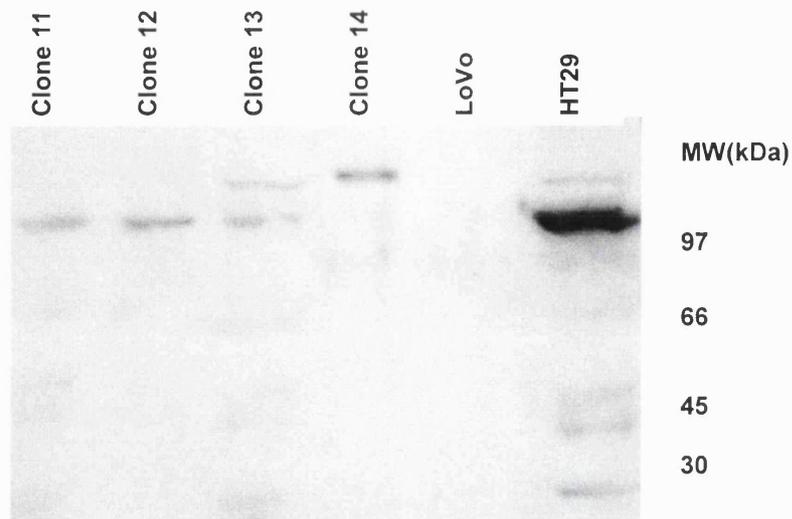
**Figure 3.5 Western blot showing reaction of increasing amounts of total protein from a N-methyl-N-nitrosourea resistant HT29 clone with hMLH1 antibody**

5,10,20 and 30 µg of protein of the hMLH1 negative clone, clone 11 were immunoblotted with anti-hMLH1 and compared with 5 µg of normal HT29 total protein extract (top panel). The nitrocellulose membrane was stained with amido black to check the loading and transfer. There is no hMLH1 visible even when 30 µg of clone 11 was used, therefore there is at least 6 times less hMLH1 in clone 11 than the untreated HT29 cells.



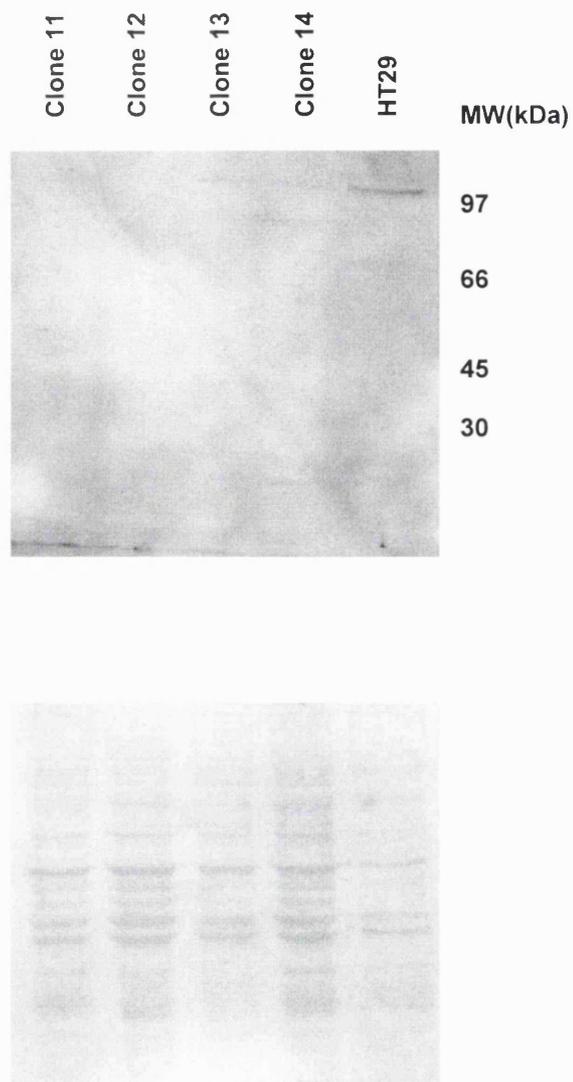
**Figure 3.6 Western blot showing reaction of total protein from N-methyl-N-nitrosourea resistant HT29 clones with hMSH2 antibody**

Protein extracts from N-methyl-N-nitrosourea resistant HT29 clones 1 to 10 together with the hMSH2 negative cell line LoVo and normal HT29 were electrophoresed on a SDS-polyacrylamide gel, transferred to nitrocellulose membrane and blotted with anti-hMSH2 antibody (top panel). The same membrane was stained with amido black to check loading and transfer (bottom panel).



**Figure 3.7 Western blot showing reaction of total protein from N-methyl-N-nitrosourea resistant HT29 clones, LoVo and HT29 cells with hMSH2 antibody**

Protein extracts from N-methyl-N-nitrosourea resistant HT29 clones 11 to 14 together with the hMSH2 negative cell line LoVo and normal HT29 were electrophoresed on a SDS-polyacrylamide gel, transferred to nitrocellulose membrane and blotted with anti-hMSH2 antibody. Clones 11, 12 and 13 are positive for hMSH2 but clone 14 is negative.



**Figure 3.8 Western blot of hPMS2 in N-methyl-N-nitrosourea-resistant clones**

Protein extracts from the hMLH1 negative clones, 11,12,13 and 14 and normal HT29 were immunoblotted with anti-PMS2 antibody (upper figure). The same nitrocellulose membrane was stained with amido black to check loading (lower figure). This shows that the hMLH1 negative clones are also negative for hPMS2.

**RT-PCR**

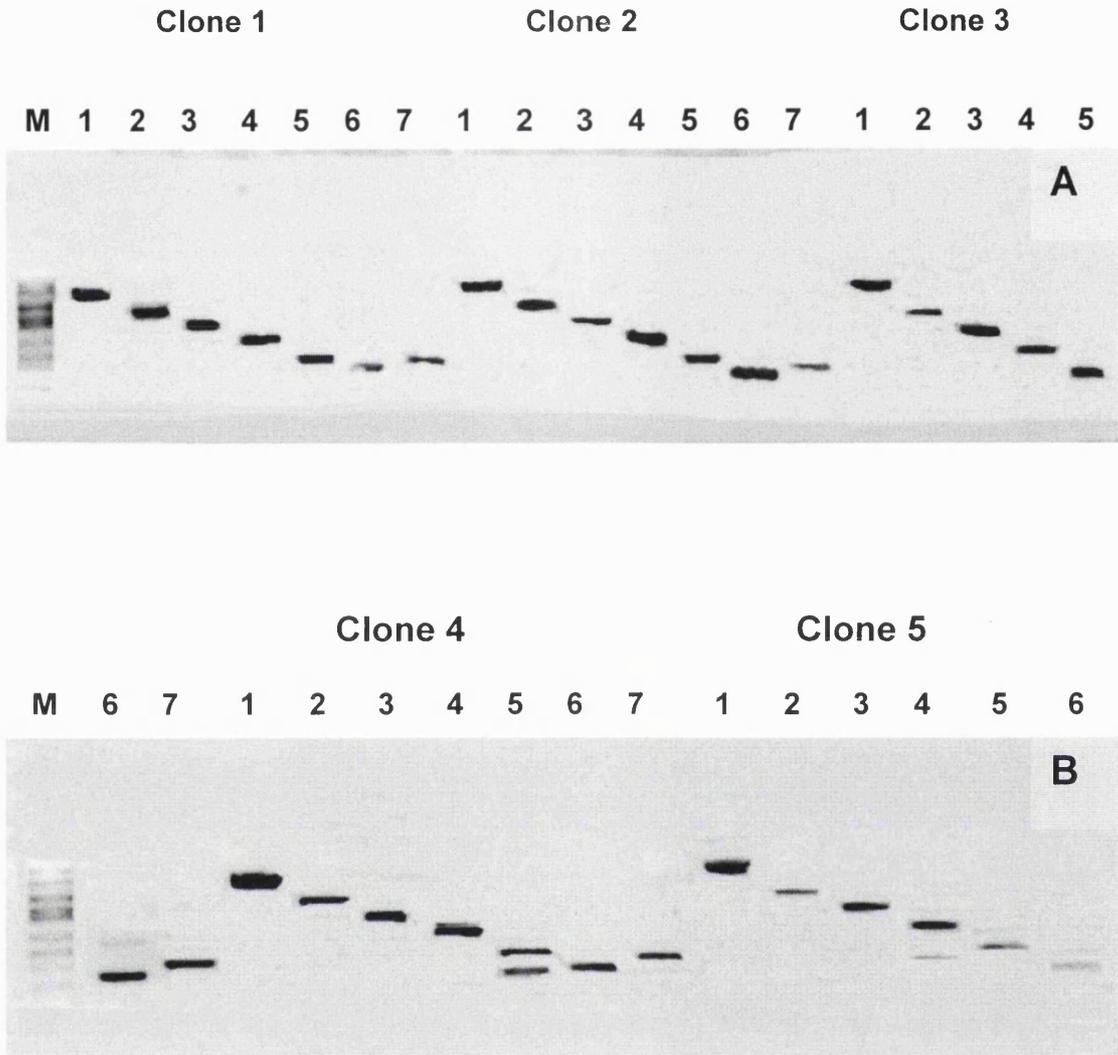
It is unclear from western blot experiments whether the lack of both hMLH1 and hPMS2 in 4 of the N-methyl-N-nitrosourea resistant clones are independent events caused by, for example, mutations in both genes or whether the loss of one partner is the cause of the loss of the other. For this reason, and to investigate further the expression of the mismatch repair genes in the thioguanine resistant clones, a series of RT-PCR experiments were performed.

Primers were designed that amplified the  $\beta$ -actin and mismatch repair genes such that each product had a different base pair length, allowing easy detection of cross contamination between reactions. The  $\beta$ -actin primers, unlike the mismatch repair gene primers, spanned an intron enabling the detection of genomic DNA contamination in the cDNAs. In the absence of contamination a band of 620 bp would result, however if contaminated with genomic DNA then a band of 800 bp would be produced.

The RT-PCR of the mismatch repair genes for clones 1 to 6 is shown in figure 3.9 showing that the mRNAs for all the mismatch repair genes measured are expressed in these clones. This same result was obtained for clones 7, 8, 9 and 10 as shown in figures 3.10. Therefore, the N-methyl-N-nitrosourea resistance does not lie at the level of mismatch repair gene expression for clones 1 to 10.

In contrast, figure 3.11 shows that clones 11, 12, 13 and 14 do not express hMLH1 mRNA, suggesting that lack of hMLH1 is caused by lack of transcription of the gene. Interestingly, the hPMS2 mRNA is present in these 4 clones leading to the conclusion that hPMS2 protein was not detected by western blot because it is short lived in the absence of hMLH1. Also worth noting is the absence of hMSH2

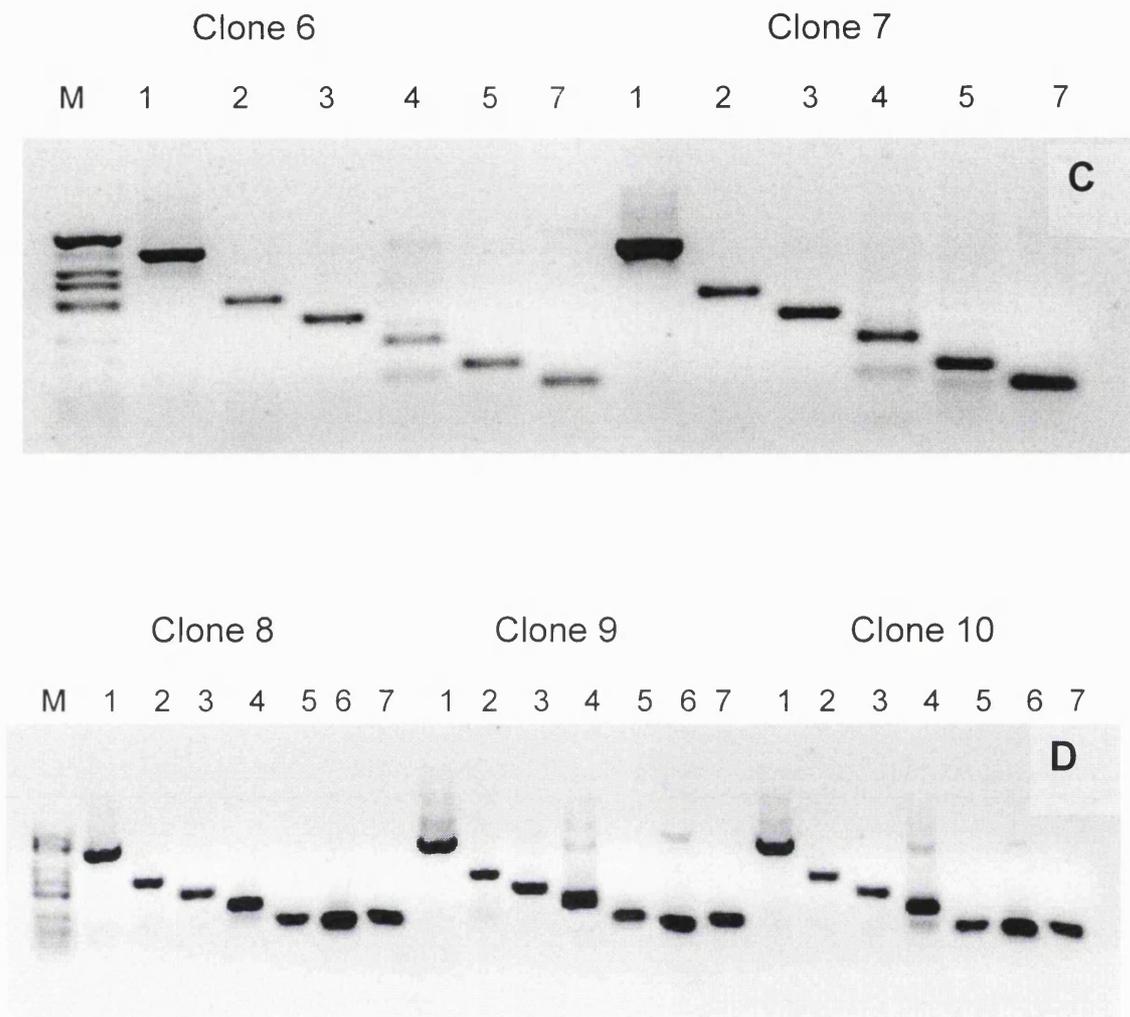
mRNA expression in clone 14, the same clone that lacked hMSH2 protein in western blot experiments. This clone is unique in this experiment since it is the only one isolated that fails to express mRNA for both hMLH1 and hMSH2.



**Figure 3.9 Assessment of the expression of mismatch repair gene mRNA in N-methyl-N-nitrosourea resistant HT29 clones**

M -  $\phi$ x174 markers:1,078 827 603 310 276 234 194

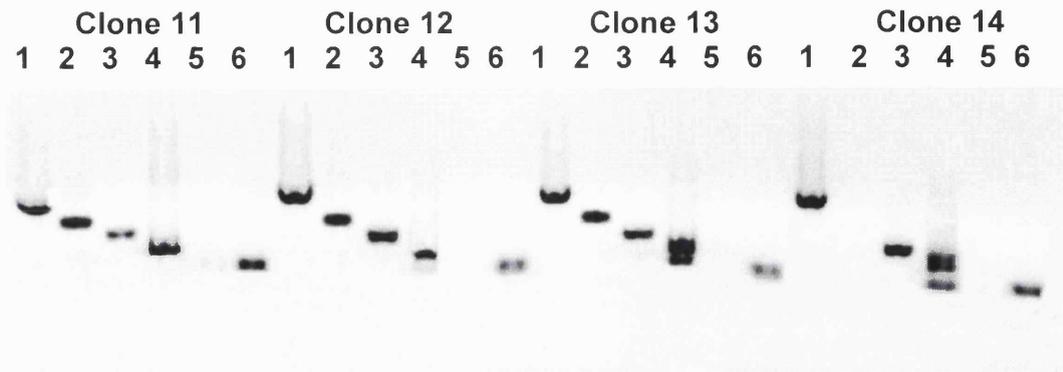
- 1 -  $\beta$  actin
- 2 - hMSH2
- 3 - hPMS2
- 4.-.hMSH6
- 5.- hMLH1
- 6.-.hMSH3
- 7.- hPMS1



**Figure 3.10 Assessment of the expression of mismatch repair gene mRNA in N-methyl-N-nitrosourea resistant HT29 clones**

M -  $\phi$ x174 markers: 1,078 827 603 310 276 234 194

- 1 -  $\beta$  actin
- 2 - hMSH2
- 3 - hPMS2
- 4.-hMSH6
- 5.- hMLH1
- 6.-.hMSH3
- 7.- hPMS1



**Figure 3.11** Assessment of the expression of mismatch repair gene mRNA in N-methyl-N-nitrosourea resistant HT29 clones

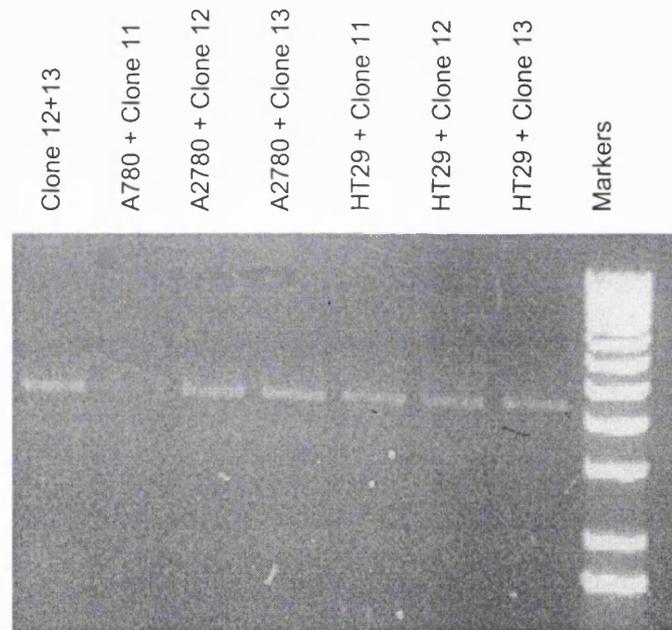
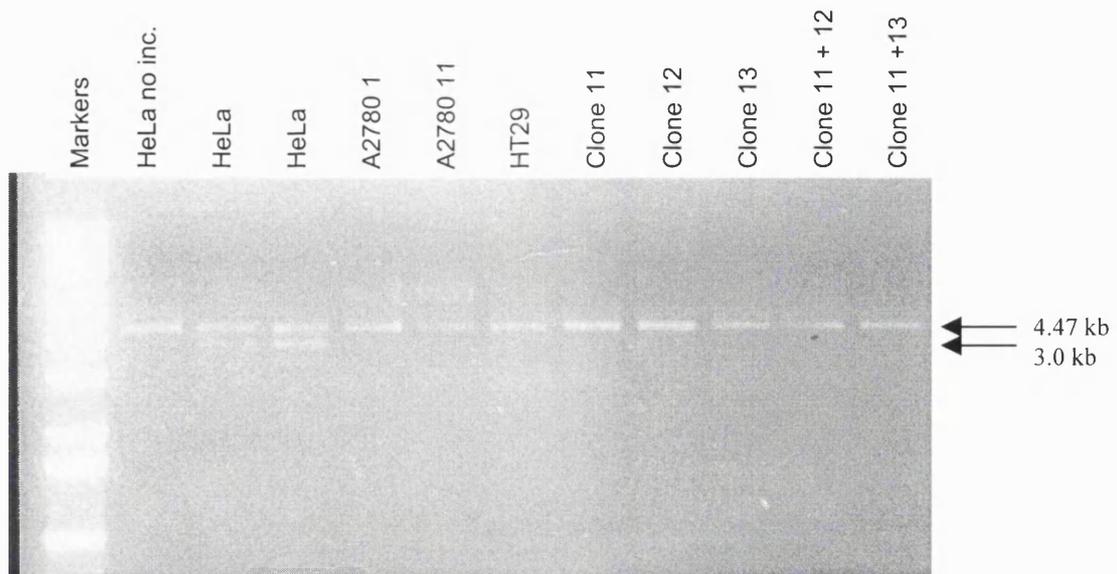
- 1 -  $\beta$  actin
- 2 - hMSH2
- 3 - hPMS2
- 4.-hMSH6
- 5.- hMLH1
- 6.-.hMSH3
- 7.- hPMS1

**In vitro mismatch repair assay**

An attempt was made to measure the ability of a protein extract to carry out repair of a mismatch using an *in vitro* mismatch repair assay (Hampson *et al.*, 1997). The basic procedure of this assay involves the incubation of total protein extract with a DNA plasmid substrate containing a single mismatch in a restriction site. In addition to the mismatch, the substrate contains a single stranded nick, which serves as a strand discrimination signal for the mismatch repair machinery. Repair of the mismatch recreates the restriction site and allows the DNA to be cut by the restriction enzyme. Digestion of the substrate with the appropriate restriction enzyme can then be used for diagnosis of repair activity. This assay was used to measure the repair activity of the thioguanine treated, N-methyl-N-nitrosourea resistant HT29 clones 11, 12, 13 and 14 that had been determined hMLH1 negative by western blot and RT-PCR. Clone 14 is also negative for hMSH2. Cell lines that lack either hMLH1 or hMSH2 are mismatch repair deficient and have been shown to be negative in the mismatch repair assay (Hampson *et al.*, 1997). Therefore, clones 11 to 14 would also be expected to be negative for repair.

A reaction containing HeLa extract that was left on ice instead of incubation at 37°C gave the expected negative result of 1 band of 4.47 kbp (fig 3.12). Duplicate reactions that had undergone incubation with the HeLa extract at 37°C for 20 mins resulted in 2 bands of the size expected after repair of the TC mismatch in the Mlu I site (4.47 and 3.9 kbp). A2780 clone 1 is MLH1 negative and A2780 clone 11 is MSH6 negative therefore both are repair deficient. The parental HT29 cell line would be expected to be repair proficient, however there is no 3.9 kbp band visible. Therefore, no conclusions can be drawn from the repair negative result obtained for the thioguanine resistant HT29 clones tested here. There may have been a problem with the HT29 cell extracts so fresh extracts were prepared that were used

for 2 further attempts at this assay. However, no positive result could be obtained with the parental HT29 cell line. For some unknown reason, this cell line is not compatible with the *in vitro* repair assay. Not only does it not give a positive result, there appears to be some contaminant in the extract that degrades DNA as illustrated in figure 3.12, (lane for A2780 + clone 11), a feature seen each time HT29 extract was used in repeat experiments.



**Figure 3.12 Assessment of the repair competence of the hMLH1 negative clones using the *in vitro* mismatch repair assay**

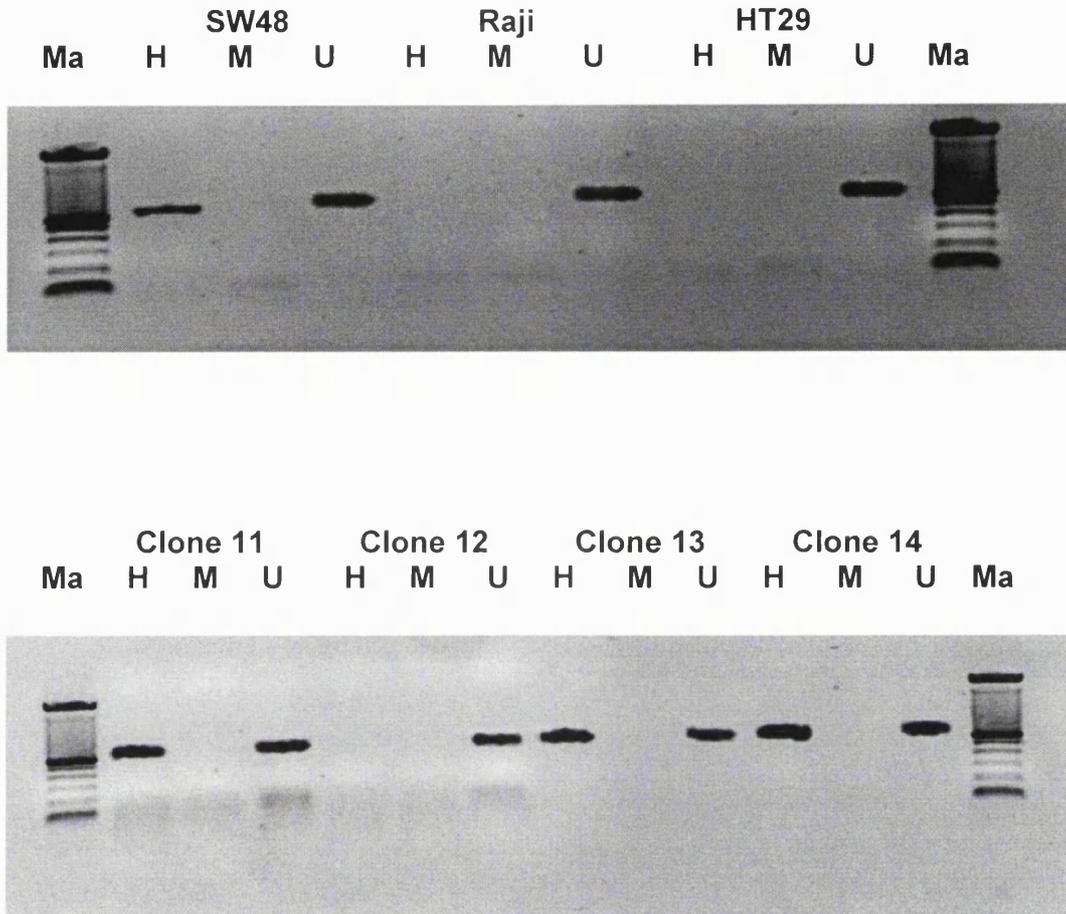
Substrates containing a TC mispair were incubated with protein extracts from the HT29 clones and various control cell lines. DNA was recovered, digested with Mlu I followed by agarose gel electrophoresis. If DNA repair occurs a 3.9 kbp band is produced. This band can be seen clearly in the HeLa controls in the upper figure but in none of the other samples including that from untreated HT29 cells which should have been repair competent.

The absence of hMLH1 in sporadic cancers is commonly caused by methylation of its promoter (Brown *et al.*, 1997) (Fleisher *et al.*, 1999; Herman *et al.*, 1998; Kane *et al.*, 1997; Leung *et al.*, 1999; Plumb *et al.*, 2000; Simpkins *et al.*, 1999; Veigl *et al.*, 1998; Wheeler *et al.*, 1999). To investigate whether the lack of hMLH1 mRNA expression observed here is due to such promoter silencing, the methylation pattern in the hMLH1 negative clones was investigated. The approach used for the analysis of hMLH1 promoter methylation was based on that of Kane *et al.* (1997). Simpkins *et al.* (1999), Veigl *et al.* (1998) and Leung *et al.* (1999) have also recently used this approach for promoter methylation analysis of the same gene. Kane *et al.* (1997) analysed the structure of the hMLH1 promoter region by sequencing the -1 to -1554 region upstream of the ATG of hMLH1 (GenBank accession number U83845). The sequence from -1 to -1295 was analysed for the presence of transcription factor binding sequences revealing the presence of numerous such sites. They did not determine which, if any of these sites function in the regulation of transcription but their presence in this upstream region suggested that this region is important for the regulation of transcription of hMLH1.

The procedure used for methylation analysis of the promoter region of hMLH1 involves extraction of genomic DNA, digestion with Msp I or Hpa II followed by PCR using primers designed to amplify -670 to -67 of the hMLH1 promoter. This region is a CpG island (Ito *et al.*, 1999; Kane *et al.*, 1997). HpaII recognition sites are at positions -567, -527, -347 and -341 (Kane *et al.*, 1997). Both Msp I and Hpa II cut at CCGG sites but only Msp I cuts when the internal cytosine is methylated, therefore no PCR product will be obtained following PCR of DNA containing methylated promoter that has been digested with Msp I. In addition to the PCR reactions for the Msp I and Hpa II digestions, a third PCR reaction with uncut

DNA was included. Figure 3.13 shows the end result of promoter methylation analysis for clones 11, 12, 13 and 14 together with controls, SW48, Raji and HT29. The SW48 cell line contains a methylated hMLH1 promoter, while Raji does not.

The result shows that the parental HT29 cell line, as expected does not contain a methylated hMLH1 promoter in its genome, however the hMLH1 promoter in clones 11, 13 and 14 is methylated, explaining the lack of mRNA expression for this gene. However this assay suggests that the fourth hMLH1 negative clone, clone 12, does not contain a methylated promoter for this gene, therefore the reason for the lack of expression in this clone is unknown.



**Figure 3.13 Assessment of the degree of methylation of the hMLH1 promoter of clones 11 to 14 with SW48, Raji and HT29 controls**

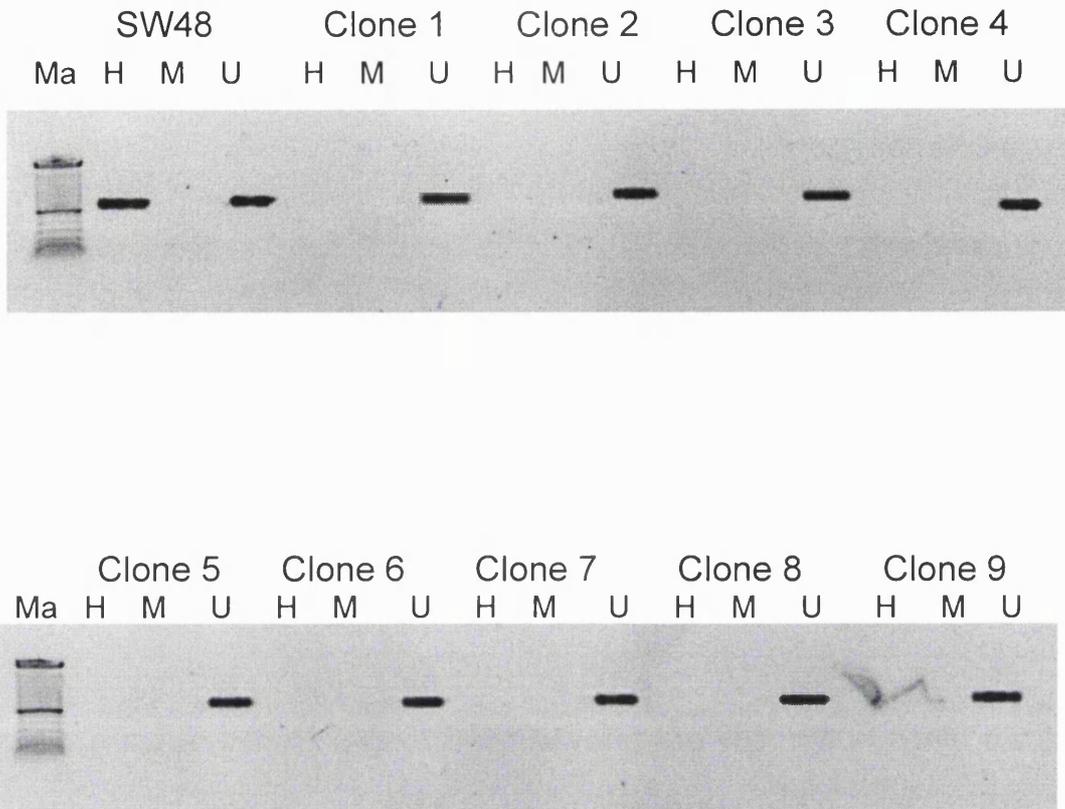
Ma – 100 bp ladder

H – Hpa II

M – Msp I

U – Uncut control DNA

DNA samples from SW48, Raji, HT29 and from N-methyl-N-nitrosourea resistant clones, 11, 12, 13 and 14 were separately digested with Hpa II and Msp I then a 603 bp segment was amplified by PCR. A PCR product was obtained only if the DNA had not been cut with the restriction enzymes



**Figure 3.14 Assessment of the degree of methylation of the hMLH1 promoter of clones 1 to 9 with SW48 control**

Ma – 100 bp ladder

H – Hpa II

M – Msp I

U – Uncut control DNA

DNA samples from SW48, Raji, HT29 and from N-methyl-N-nitrosourea resistant clones, 11, 12, 13 and 14 were separately digested with Hpa II and Msp I then a 603 bp segment was amplified by PCR. A PCR product was obtained only if the DNA had not been cut with the restriction enzymes

## Discussion

The aim of the experiments described in this chapter was to determine whether treatment of cells with thioguanine could result in the production of mismatch repair defective derivatives. The approach was to treat the human colon carcinoma cell line HT29 with a subtoxic concentration of thioguanine for an extended period of time and then select for mismatch repair defective clones with N-methyl-N-nitrosourea and O<sup>6</sup>-benzylguanine. When normal HT29 cells were treated with O<sup>6</sup>-benzylguanine and N-methyl-N-nitrosourea (fig 3.2), 10 µg/ml N-methyl-N-nitrosourea was sufficient to kill 100% of the cells. This is comparable with the result of Aquilina *et al* (1998). As expected, there were no surviving clones after treatment of normal HT29 cells with single doses of 210 µg/ml (2mM) N-methyl-N-nitrosourea and 25 mM O<sup>6</sup>-benzylguanine. However, treatment of the thioguanine pretreated cells with 210 µg/ml (2mM) N-methyl-N-nitrosourea and 25 mM O<sup>6</sup>-benzylguanine resulted in the isolation of 14 resistant clones. We could therefore be reasonably certain that pretreatment with thioguanine was responsible for the resistance to N-methyl-N-nitrosourea and O<sup>6</sup>-benzylguanine in the 14 selected clones. Clones 1 to 10 were from one experiment and clones 11 to 14 were from a second. Each clone was selected from separate tissue culture plates to ensure that they were not all descendants of the same cell.

Western blots of gels loaded with an equal amount of protein from the resistant clones from the parental HT29 cell line showed that 4 of the 14 surviving clones were negative for hMLH1 (fig 3.4). One of the clones, clone 11 was investigated further and shown to contain at least 6 times less hMLH1 than normal HT29 cells (fig 3.5). These four clones had undetectable levels of hMLH1 mRNA (fig 3.11). In human cells hMLH1 functions in a heteroduplex with hPMS2 (Bronner *et al.*, 1994). HCT116 cells are negative for the hMLH1 protein because they contain a

nonsense mutation in the coding region of the gene and have a marked reduction in the level of hPMS2 protein (Shin *et al.*, 1998). Thus, it was not unexpected to find that clones 11, 12, 13 and 14, in addition to being negative for hMLH1 were also negative for hPMS2 by western blot (fig 3.8) However, as in the HCT116 cells, there was undetectable expression of *bMLH1* mRNA but apparently normal expression of *bPMS2* mRNA (fig 3.11).

Cells that lack the hMLH1 protein would be expected to be defective in post-replicative mismatch repair, as is the case for HCT116 cells. To confirm that the clones negative for hMLH1 are mismatch repair deficient, an *in vitro* repair assay developed in Dr Peter Karran's laboratory was used (Hampson *et al.*, 1997). This assay uses a DNA substrate containing a single mismatch in a restriction endonuclease recognition sequence. Repair of the mismatch restores the restriction endonuclease Mlu I recognition sequence. The experiments using the *in vitro* repair assay were carried out with Dr Peter MacPherson in Dr Peter Karran's laboratory. Unfortunately, they did not go well. Protein extracts from HeLa cells were used as a positive control for this assay incubation of HeLa cell protein extract with the substrate followed by purification and digestion of the substrate with Mlu I resulted in the production of DNA fragments consistent with repair. It would be expected that the parental HT29 cell line be post-replicative mismatch repair proficient, however protein extracts prepared from untreated HT29 cells did not give a positive result for repair activity with this assay. The reason for this is not known. Therefore, no conclusions can be drawn for the negative result obtained for clones 11, 12 and 13.

The importance of defective mismatch repair in human cancer was first demonstrated with the discovery of mutations in mismatch genes in patients with

hereditary non-polyposis colorectal, however more recently cases of sporadic colon cancers have been shown to have defects in the post-replicative mismatch repair pathway. Unlike the hereditary form of colon cancer there are no mutations in any of the mismatch repair genes in sporadic colon cancer, however *hMLH1* is commonly silenced by methylation of the promoter region of the gene (Herman *et al.*, 1998; Toyota *et al.*, 1999; Veigl *et al.*, 1998). Epigenetic silencing of genes by changes in the methylation pattern of the promoter region is a common event in carcinogenesis and the connection between DNA methylation and gene expression has been known for over 20 years (for review see (Robertson and Jones, 2000). The only naturally occurring modification of DNA in higher eukaryotes is the methylation of the 5-position of cytosine leading to the formation of 5-methylcytosine. The reaction is catalysed by the enzyme (cytosine-5)-DNA-methyltransferase. The enzyme first binds to its target sequence, moves the target cytosine into its catalytic site and covalently attaches to the C6 position of cytosine (Zingg and Jones, 1997). This generates a covalently bound enzyme-cytosine intermediate with the cytosine activated at C5, which can then accept the methyl group from the cofactor S-adenosylmethionine. Methylation occurs in mammals almost exclusively at the dinucleotide CpG, which are clustered in certain regions of the genome, commonly on the 5' side of genes. These promoter regions have been termed CpG islands. Methylation of cytosine changes the structural characteristics of DNA in several ways. The methyl group of cytosine sterically extends into the hydrophilic major groove of B DNA and introduces hydrophobicity, two changes that may be responsible for the altered specificity of proteins interacting with DNA (Zingg and Jones, 1997). Three different alterations are common in human cancer. Firstly there is global hypomethylation often seen within the coding region of genes. Secondly there is a 'disregulation' of the enzymes involved in maintaining methylation patterns and thirdly there is regional

hypermethylation in normally unmethylated CpG islands (Jones and Laird, 1999). Work in recent years has shown that the silencing of tumour suppressor genes associated with promoter hypermethylation is a common feature in human cancer, and serves as an alternative mechanism for loss of tumour suppressor gene function.

Sporadic cancers with microsatellite instability have a high frequency of promoter region hypermethylation of *hMLH1*. In fact about half of tumour suppressor genes that have been shown to be mutated in the germ line of patients with familial cancer syndromes have also been shown to have hypermethylation in a proportion of sporadic cancers (Baylin *et al.*, 1998; Jones and Laird, 1999). Loss of functional post-replicative mismatch repair due to silencing rather than mutation of the *hMLH1* gene is a common event in both cell lines and tumours (Esteller *et al.*, 1998; Herman *et al.*, 1998; Kane *et al.*, 1997; Plumb *et al.*, 2000; Strathdee *et al.*, 1999). The silencing was found to be the cause and not a consequence of microsatellite instability (Herman *et al.*, 1998). Microsatellite instability and promoter methylation of *hMLH1* has also been reported in endometrial cancers (Simpkins *et al.*, 1999). For reasons that are not understood, the *hMLH1* gene appears to be the only mismatch repair gene that is silenced in sporadic cancer. There has however been a recent report of *hMSH6* gene repression by hypermethylation in Raji cells *in vitro* following treatment with N-methyl-N-nitrosourea (Bearzatto *et al.*, 2000).

MLH1 promoter methylation has also been reported in tumours in response to chemotherapy. In ovarian cancer, a higher frequency of *hMLH1* promoter methylation is observed in cisplatin treated tumours compared with pre-chemotherapy tumours (Strathdee *et al.*, 1999). The majority of cisplatin resistant derivatives of the ovarian tumour cell line A2780 lack hMLH1 expression because of methylation of the *hMLH1* promoter (Strathdee *et al.*, 1999). Therefore, since

the thioguanine treated HT29 cells that lacked the hMLH1 protein did not express hMLH1 mRNA, it seemed possible that these cells could have similar promoter methylation of *hMLH1*. Untreated HT29 cells were found to have an unmethylated *hMLH1* promoter and 3 of the 4 hMLH1 negative clones were found to have a methylated *hMLH1* promoter. The remaining N-methyl-N-nitrosourea resistant clones did not contain a methylated *hMLH1* promoter.

The method used for analysis of the methylation of the promoter region of hMLH1 was based on that of Kane *et al* (1999) and has also been used by Leung *et al* 1999, Simpkins *et al* 1999 and Veigl *et al* 1998. This method relies on there being Hpa II sequences (CCGG) in the promoter region to be analyzed. The region of the hMLH1 promoter examined here contained 4 Hpa II sites at positions -567, -527, -347 and -341. Therefore, when using this assay to analyse promoter methylation, the assumption is made that methylation of these CCGG sites is representative of the general pattern of methylation throughout the hMLH1 promoter at other CpG sequences that are not contained within Hpa II recognition sequences. An alternative method for promoter methylation analysis is methylation-specific PCR (Herman *et al.*, 1998). Extracted DNA is treated with sodium bisulphite, which converts cytosine to uracil but 5-methylcytosine will remain intact. PCR primers are designed to span regions containing CpG sequences. Primers will anneal to sequences containing uracil but not 5-methylcytosine therefore, the presence of a PCR product indicated that the region to which the primers were designed to anneal to was unmethylated. The absence of a PCR product indicates that this same region was methylated. This assay is more sensitive than the method of Kane *et al* since it is able to detect the methylation of cytosines that are not in Hpa II sequences. However, the method of Kane *et al* was chosen because it a less problematic method and methylation of Hpa II sites has

been shown to be representative of the general level of CpG methylation in the promoter region of hMLH1 (Kane *et al.*, 1997), (Leung *et al.*, 1999; Simpkins *et al.*, 1999; Veigl *et al.*, 1998). If time had permitted, it would have been desirable to examine sequence of the entire hMLH1 gene in clones 11, 13 and 14 to ensure that it did not contain any mutations. In addition, immunohistochemical analysis of clones 11, 12 and 13 using hMLH1 antibodies could have been used to confirm that the cells did not contain any hMLH1 protein.

The hypermethylation is a reversible phenomenon as demonstrated by treatment of colon cell lines containing a hypermethylated *hMLH1* gene with 5-aza-2'-deoxycytidine resulting in re-expression of *hMLH1* and restoration of MMR activity (Herman *et al.*, 1998). This phenomenon has recently been demonstrated *in vivo* by treating a repair deficient xenograft with 5-aza-2'-deoxycytidine, restoring the xenograft sensitivity to cisplatin, carboplatin, temozolomide and epirubicin (Plumb *et al.*, 2000). This raises the possibility that post-replicative mismatch repair-related resistance to certain cytotoxic drugs could be overcome clinically.

The results obtained in this thesis provide evidence that exposure to thioguanine for extended periods can also result in the production of repair deficient cells by *hMLH1* promoter silencing. This raises the possibility that a similar downregulation of mismatch repair genes occurs in people treated with thioguanine, or one of its precursors such as azathioprine, and that this downregulation might play a part in the aetiology of skin cancer. As discussed in the introduction to this chapter, a number of mechanisms have been proposed to be involved in the development of skin cancer in organ transplant recipients such as UV radiation, immune suppression, human papillomavirus and Epstein-Barr virus. The results of this chapter suggest that azathioprine through its metabolite

thioguanine might cause the down-regulation of mismatch repair genes in patients who are receiving azathioprine treatment. However, since these tumours appear mainly on the sun-exposed surfaces of the skin, it is likely that any downregulation of mismatch repair is not the only factor contributing to the development of skin cancers in these patients.

# **Chapter 4**

## **General Discussion**

## Chapter 4 General Discussion

The aim of this thesis was to answer two questions relating to thioguanine toxicity. These questions arose from a proposal by our laboratory that the delayed cytotoxicity of thioguanine is the result of the formation of S<sup>6</sup>-methylthioguanine in DNA by the chemical methylation of thioguanine residues in the DNA by S-adenosylmethionine (Swann *et al.*, 1996). Thus the action of thioguanine was seen as being analogous to that of S<sub>N</sub>1 methylating agents such as N-methyl-N-nitrosourea and temozolomide whose cytotoxicity is caused by the methylation of guanine in DNA to form O<sup>6</sup>-methylguanine (for review see Pegg *et al.*, 1990). So the first question was, is the combination of thioguanine and methylating agent more cytotoxic than thioguanine alone? In order to answer the first question a number of *in vivo* and *in vitro* experiments were performed. The methylating agent chosen for these studies was temozolomide which, at the time these studies began, had just been licensed for the treatment of brain tumours and end-stage melanoma. These clinical applications were initially suggested by the successful treatment of glioblastoma and melanoma xenografts in nude mice (Wedge *et al.*, 1996a; Wedge *et al.* 1996b) so the combined thioguanine-temozolomide treatment was tested for its effect on the same xenografts. Xenograft experiments are difficult, time consuming, expensive and use animals, therefore the study of synergism was continued using the same glioblastoma and melanoma cells in culture. It is perhaps fortunate that the xenograft experiments were done first since the predicted strong synergism was seen in both the glioblastoma (fig 2.4) and the melanoma (fig 2.5) xenografts but the synergism was not seen when the cells were treated *in vitro*.

Possible reasons for the failure to reproduce the synergy between thioguanine and temozolomide *in vitro* have been discussed in chapter 2. Since we do not know the relative effectiveness of O<sup>6</sup>-methylguanine compared with S<sup>6</sup>-methylthioguanine in inducing cell death the ratio of O<sup>6</sup>-methylguanine to S<sup>6</sup>-methylthioguanine produced by thioguanine-methyl methanesulphonate versus thioguanine-temozolomide could be crucial. There will be almost no O<sup>6</sup>-methylguanine produced by methyl methansulphonate, whereas temozolomide will produce a much higher ratio of O<sup>6</sup>-methylguanine to S<sup>6</sup>-methylthioguanine. It could be proposed that because S<sup>6</sup>-methylthioguanine is not efficiently repaired by MGMT or any other known repair protein, it has a higher level of toxicity than O<sup>6</sup>-methylguanine. The amount of S<sup>6</sup>-methylthioguanine and O<sup>6</sup>-methylguanine produced after treatment with thioguanine and temozolomide *in vitro* may be below the threshold required to obtain synergistic cytotoxicity. This again comes back to the problem of why there was synergism between thioguanine and temozolomide *in vivo* but no synergism *in vitro* and again suggests that there must be some effect of this treatment on cells of the tumour blood supply as well as on the tumour cells themselves.

Until the steps following binding of MutS $\alpha$  in mismatch repair mediated cell death are determined, the precise involvement of this system in the cytotoxicity of S<sup>6</sup>-methylthioguanine and O<sup>6</sup>-methylguanine will remain controversial. An alternative mechanism of O<sup>6</sup>-methylguanine mediated cell death involving topoisomerase I has recently been proposed (Pourquier *et al.*, 2001). Topoisomerase I regulates DNA topology during crucial processes such as DNA replication, transcription, chromosome condensation and segregation during mitosis (Wang, 1996). The enzyme reversibly cleaves one strand of duplex DNA by forming a covalent tyrosyl-phosphodiester bond with the 3' end of the broken

DNA. This intermediate is referred to as the topoisomerase I cleavable complex. The study of Pourquier *et al* (2001) shows that duplexes containing both O<sup>6</sup>-methylguanine paired with cytosine and O<sup>6</sup>-methylguanine paired with thymine are able to enhance topoisomerase I cleavable complexes. Conversely, a duplex containing a 6-thioguanine residue paired with cytosine did not affect topoisomerase I cleavage complex formation. Furthermore they show that yeast cells overexpressing human topoisomerase I are significantly more sensitive to N-methyl-N'-nitro-N-nitrosoguanidine than the corresponding wild type cells. Additional evidence for a possible role of topoisomerase I in mediating methylating agent cytotoxicity is the observation that O<sup>6</sup>-benzylguanine is able to sensitise cells to various topoisomerase I inhibitors (Sekikawa *et al.*, 2000). Therefore it seems that our understanding of the mechanism of cytotoxicity in response to 6-methylated guanines in DNA is poorly understood. The relative contribution of the mismatch repair system and topoisomerase I cleavable complexes remains to be determined together with the subsequent steps in the cytotoxic pathway(s). The involvement of topoisomerase I in O<sup>6</sup>-methylguanine is a relatively recent proposal and it is conceivable that there are further undiscovered systems involved in the cytotoxicity of thioguanine and methylating agents.

It is not clear whether the synergy observed between thioguanine and temozolomide will be clinically relevant. Temozolomide in combination with O<sup>6</sup>-benzylguanine is currently being used clinically for the treatment of high-grade gliomas both in adults and in children (Middlemas *et al.*, 2000). This causes concern, especially for the treatment of children. Temozolomide will itself be carcinogenic because it produces exactly the same lesion in DNA, O<sup>6</sup>-methylguanine as do the potent carcinogens, N-methyl-N-nitrosourea (MNU) and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG). MNU has been shown to

produce tumours in all organs of rats and is carcinogenic in a wide range of other animals including rabbits, mice, guinea pig, mini-pig, boxer dog and non-human primates (Kliehues *et al.*, 1970; Kliehues *et al.*, 1973; Denkinger *et al.*, 1978; Pinsky *et al.*, 1980; Thorgiersson *et al.*, 1994; Harris *et al.*, 1994). Reducing the alkyltransferase activity in these tumours using O<sup>6</sup>-benzylguanine as a means of increasing the toxicity of temozolomide will also reduce the alkyltransferase activity systemically. This will increase the risk of cancer developing in other tissues (Becker *et al.*, 1997; Mace *et al.*, 1996). It is therefore of great concern treating children with drugs that could greatly increase their risk of developing other tumours. It is conceivable that treatment with thioguanine and temozolomide will be a better option for children, because as discussed in chapter 2, thioguanine is not likely to deplete alkyltransferase levels but is still able to increase the toxicity of temozolomide to levels comparable to that achieved with O<sup>6</sup>-benzylguanine.

The second question relating to thioguanine cytotoxicity that was posed in this thesis was whether treatment with thioguanine and other thiopurine analogues such as mercaptopurine and azathioprine will lead to the production of mismatch repair defective cells and therefore increase the risk of cancer. This is of particular clinical relevance for organ transplant recipients, especially those with kidney transplants who are receiving azathioprine as an immune suppressant to prevent organ rejection. Such patients have a 27-40% risk of developing either a basal cell or squamous cell carcinoma within 20 years of transplantation (McGregor and Proby, 1995). The general consensus is that the tumours are a result of immune suppression. However, heart transplant patients usually receive cyclosporin therapy, a much stronger immune suppressant and there are no reports of an increase in skin cancer incidence in heart transplant patients. Harwood *et al* (2001)

reports the presence of microsatellite instability in sebaceous carcinomas of 4 post transplant patients who have received azathioprine therapy. This microsatellite instability was associated with the loss of MSH2. One of the N-methyl-N-nitrosourea resistant HT29 clones isolated in this thesis was found to be deficient in the MSH2 protein (fig 3.7) and it will be interesting to see if the same is true of other tumours isolated from a larger number of organ-transplant patients, particularly those receiving azathioprine therapy. However, the loss of functional mismatch repair as a result of exposure to thioguanine cannot be the sole explanation for the development of tumours in these patients because they develop the majority of the tumours on the light exposed surfaces of the skin. A recent report using a thiopyrimidine base may help to explain the localised nature of these tumours. It was shown that 4-thiothymidine is incorporated into DNA and interacts synergistically with UVA light and dramatically sensitises cultured human cells to very low non-lethal UVA doses (Massey *et al.*, 2001). The same could be true of thioguanine in DNA thus exerting a strong selective pressure for cells to avoid UVA related toxicity.

The observation presented in chapter 3 of this thesis that chronic treatment of cells in culture with thioguanine results in down-regulation of one of the genes of the mismatch repair system requires further study. The down-regulation of MLH1 appears to be caused by methylation of its promoter region but the mechanism for the loss of the MSH2 in one of the clones was not determined. Perhaps there is a progressive loss of mismatch repair gene expression as a result of general genome instability. When this work began it was intended that skin tumours collected from kidney transplant patients would be examined unfortunately time did not permit. The loss of mismatch repair in these tumours could be a late stage effect, with other factors such being more important during early tumour development

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