STUDIES ON THE MECHANISM OF ACTION OF ANTITUMOUR IMIDAZOTETRAZINONES.

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March 1994.

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Studies on the mechanism of action of antitumour imidazotetrazinones.

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Doctor of Philosophy, March 1994.

The imidazotetrazinones are clinically active antitumour agents, temozolomide currently proving successful in the treatment of melanomas and gliomas. The exact nature of the biological processes underlying response are as yet unclear. This thesis attempts to identify the cellular targets important to the cytotoxicity of imidazotetrazinones, to elucidate the pathways by which this damage leads to cell death, and to identify mechanisms by which tumour cells may circumvent this action.

The levels of the DNA repair enzymes O^6-alkylguanine-DNA-alkyltransferase (O^6-AGAT) and 3-methyladenine-DNA-glycosylase (3MAG) have been examined in a range of murine and human cell lines with differential sensitivity to temozolomide. All the cell lines were proficient in 3MAG despite there being 40-fold difference in sensitivity to temozolomide. This suggests that while 3-methyladenine is a major product of temozolomide alkylation of DNA it is unlikely to be a cytotoxic lesion. In contrast, there was a 20-fold variation in O^6-AGAT levels and the concentration of this repair enzyme correlated with variations in cytotoxicity. Furthermore, depletion of this enzyme in a resistant, O^6-AGAT proficient cell line (Raji), by pretreatment with the free base O^6-methylguanine resulted in 54% sensitisation to the effects of temozolomide. These observations have been extended to 3 glioma cell lines; results that support the view that the cytotoxicity of temozolomide is related to alkylation at the O^6-position of guanine and that resistance to this drug is determined by efficient repair of this lesion. It is clear, however, that other factors may influence tumour response since temozolomide showed little differential activity towards 3 established solid murine tumours *in vivo*, despite different tumour O^6-AGAT levels.

Unlike mitozolomide, temozolomide is incapable of cross-linking DNA and a mechanism by which O^6-methylguanine may exert lethality is unclear. The cytotoxicity of the methyl group may be due to its disruption of DNA-protein interactions, or alternatively cell death may not be a direct result of the alkyl group itself, but manifested by DNA single-strand breaks. Enhanced alkaline elution rates were found for the DNA of Raji cells treated with temozolomide following alkyltransferase depletion, suggesting a relationship between O^6-methylguanine and the induction single-strand breaks. Such breaks can activate poly(ADP-ribose) synthetase (ADPRT) an enzyme capable of rapid and lethal depletion of cellular NAD levels. However, at concentrations of temozolomide relevant *in vivo* little change in adenine nucleotides was detected in cell lines, although this enzyme would appear important in modulating DNA repair since inhibition of ADPRT potentiated temozolomide cytotoxicity in Raji cells but not O^6-AGAT deficient GM892A cells. Cell lines have been reported that are O^6-AGAT deficient yet resistant to methylating agents. Thus, resistance to temozolomide may arise not only by removal of the methyl group from the O^6-position of guanine, but also from another mechanism involving caffeine-sensitive post-replication repair or mismatch repair activity.

A modification of the standard Maxam Gilbert sequencing technique was used to determine the sequence specificity of guanine-N^7 alkylation. Temozolomide preferentially alkylated runs of guanines with the intensity of reaction increasing with the number of adjacent guanines in the DNA sequence. Comparable results were obtained with a polymerase-stop assay, although neither technique elucidates the sequence specificity of O^6-guanine alkylation. The importance of such specificity to cytotoxicity is uncertain, although guanine-rich sequences are common to the promoter regions of oncogenes. Expression of a plasmid reporter gene under the control of the Ha-ras proto-oncogene promoter was inhibited by alkylation with temozolomide when transfected into cancer cell lines. However, this inhibition did not appear to be related to O^6-guanine alkylation and therefore would seem unimportant to the chemotherapeutic activity of temozolomide.

Keywords: alkylating agents, imidazotetrazinones, O^6-methylguanine, temozolomide.

To my parents,
for their continual support.

Acknowledgements

I wish to express my gratitude to Professors Mike Tisdale and Malcolm Stevens for there support and guidance throughout the research undertaken in production of this thesis.

I am also grateful to the following for their generous technical assistance:- Dr. Keith Fox (Southampton University) for the use of his facilities and his technical guidance in the performance of DNA footprinting techniques; Dr. John Hartley (Oncology department, University College London and Middlesex Hospital) for the use of labs and equipment, and his assistance in carrying out both the DNA sequence specificity and alkaline elution studies. In addition, I would like to thank Alan Clark (Aston University, 1991), Bill Thompson (Aston Molecules, Birmingham), Y.Wang, Kevin Farnell and Malcolm Stevens (CRC Laboratories, Nottingham University) for the synthesis and provision of analogues of imidazotetrazinones, Mike Wynter (Aston University) for conducting murine tumour studies, Helen Hussey for her assistance in tissue culture, and Paul Fundak for his technical support.

Finally, I wish to thank all those within the Dept. of Pharmaceutical and Biological Sciences, and especially the Friday footballers, who have made my time at Aston so enjoyable.

I gratefully acknowledge the receipt of a research grant from the Science and Engineering Research Council Total Technology programme.

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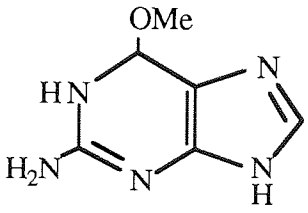
List of Abbreviations

A	Adenine
3-AB	3-Aminobenzamide
ADP	Adenosine 5′-diphosphate
ANOVA	Analysis of variance
AMP	Adenosine 5′-monophosphate
ATP	Adenosine 5′-triphosphate
ACNU	1-(4-Amino-2-methyl-5-pyimidinyl)methyl-3-(2-chloroethyl)-3-nitrosourea
AIC	5-Aminoimidazole-4-carboxamide
BCNU	1,3-Bis (2-chloroethyl)-1-nitrosourea
bp	Base pair
BSA	Bovine serum albumin
C	Cytosine
Ci	Curie
CAT	Chloramphenicol acetyltransferase
cpm	Counts per minute
DFMO	D,L-α-Difluoromethyl ornithine
DMS	Dimethylsulphate
DMSO	Dimethyl sulphoxide
dNTP	Deoxyribonucleotidetriphosphate
DNA	Deoxyribonucleic acid
DNAase	Deoxyribonuclease
dpm	Disintegrations per minute
DTIC	5-(3,3-Dimethyltriazeny-1-yl)imidazole-4-carboxamide
EDTA	Ethylenediaminetetra-acetic acid
EMS	Ethyl methanesulphonate
ENU	N-ethyl-N-nitrosoguanidine
G	Guanine
HEBS	2 x HEPES buffered saline
HEPES	N-[2-hydroxyethyl]piperazine-N′-[2-ethane-sulphonic acid]
HPLC	High performance liquid chromatography
i.p	Intra-peritoneally
3MAG	3-Methyladenine-DNA-glycosylase
Mer+	Methylation repair (O^6-AGAT) proficient
Mer-	Methylation repair (O^6-AGAT) deficient
MCTIC	5-(3-[2-Chloroethyl]-1-triazenyl)imidazole-4-carboxamide
Me^3A	3-Methyladenine

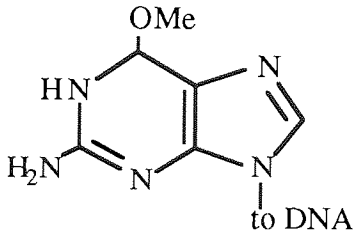
Me^7G	7-Methylguanine
MMS	Methyl methanesulphonate
MNNG	N-methyl-N′-nitro-N-nitrosoguanidine
MNU	N-methyl-N-nitrosoguanidine
MTIC	5-(3-[2-Methyl]-1-triazenyl)imidazole-4-carboxamide
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NCI	National Cancer Institute
n.d	Not determined
n.s	Not significant (P>0.05)
O^6-AGAT	O^6-alkylguanine-DNA alkyltransferase
O.D.	Optical density
O^6MeG	O^6-Methylguanine
PBS	Phosphate buffered saline
Pu	Purine
Py	Pyrimidine
r-oMe^7G	ring-opened 7-methylguanine
RNA	Ribonucleic acid
RPMI	Rosewell Park Memorial Institute (medium)
SD	Standard deviation
SDS	Sodium dodecylsulphate
SEM	Standard error of the mean
T	Thymine
TBE	Tris-borate electrophoresis buffer
TE	Tris EDTA
TEMED	N,N,N′,N′-tetramethylethylenediamine
6-TG	6-Thioguanine
TLC	Thin layer chromatography
Tris	Tris (hydroxymethyl)aminomethane base

Chapter One

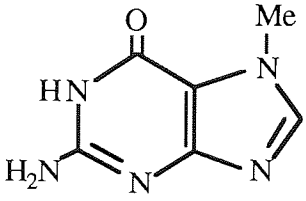
General Introduction



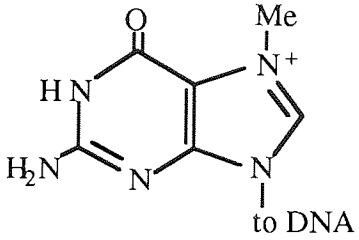
O^6-Methylguanine (O^6MeG)



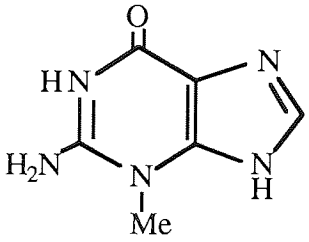
O^6-Methylation of guanine in DNA



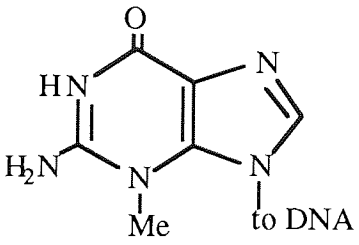
7-Methylguanine (Me^7G)



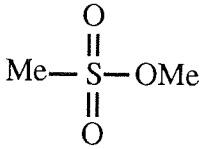
N^7-Methylation of guanine in DNA



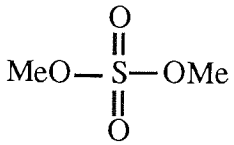
3-Methyladenine (Me^3A)



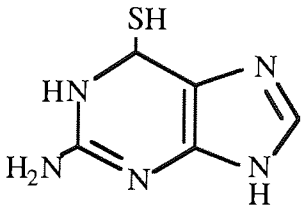
N^3-Methylation of adenine in DNA



Methylmethanesulphonate (MMS)



Dimethylsulphate (DMS)



6-Thioguanine (6TG)

Fig 1.1 Structures and abbreviations of compounds in study.

1.1 Foreword.

Despite a long and intensive search for anti-tumour agents, cancer remains one of the major causes of death in Western society. Progress has been achieved in the treatment of some cancers, such as leukaemias and lymphomas, however, for others, including lung cancers, brain tumours and malignant melanoma, present therapies are largely ineffective.

The search for better drugs continues, the ultimate goal being anticancer agents that selectively destroy cancer cells while possessing no harmful side effects. Unfortunately, the chances of cure by existing clinically used agents are diminished due to the limits on drug doses imposed by their toxicity. These anticancer drugs cause damage to normal rapidly dividing tissues such as the bone-marrow, skin, hair follicles and gastro-intestinal tract since they rely essentially on an anti-proliferative action and lack intrinsic selectivity towards cancerous cells. In an attempt to overcome the problems created by drug toxicity, techniques have been developed that are mainly aimed at combating myelosuppression, however, the selectivity of agents for rapidly dividing cells often causes further complications by allowing quiescent tumour tissue to evade destruction and to proliferate at a later date. These problems aside, the ability of cytotoxic drugs to cure malignant disease is also limited by tumour drug resistance. In many instances tumours, which often consist of a heterogeneous mixture of cells, initially show regression only for resistant sub-populations of cells to re-establish the tumour after destruction of the sensitive population. Consequently, although the use of drugs as adjuvants to, or in combination with, surgery or radiotherapy has recently offered improved therapies, the toxic effects and the knowledge that cure can be achieved in only a few rare cancers have traditionally led to chemotherapy drugs being used as a treatment of last resort when more conventional methods such as surgery and radiotherapy have failed.

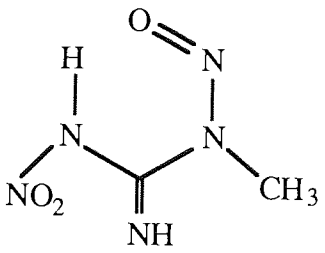
The major hurdle in cancer chemotherapy is the lack of defined biochemical differences between normal and neoplastic cells offering potential for exploitation. It is clear that agents with greater tumour specificity are required, and in order that such drugs may be developed there is a need for an increased understanding of the biochemistry of tumour cells, of the mechanisms of drug resistance, as well as of the mechanisms of action of currently used agents.

1.2 Alkylating Agents.

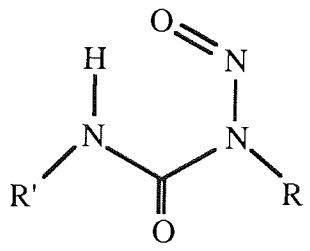
The anti-proliferative effects exhibited by many chemotherapy agents are often achieved by interference with cell division, via inhibition of mitosis or nucleic acid synthesis. Thus, DNA forms the principal target for much cancer chemotherapy. Alkylating agents are electrophiles, or agents that generate electrophiles *in vivo* , which can attack electron rich sites in biological macromolecules, most importantly DNA, adding an alkyl group to nitrogen, sulphur or oxygen atoms, replacing a proton.

Since 1945, when the first studies of cytotoxic therapy in leukaemia established the activity of nitrogen mustard, alkylating agents have played a prominent role in cancer chemotherapy. Of the drugs that have followed the mustard group of agents, and as forerunners of the imidazotetrazinones, it is appropriate to briefly review the nitrosourea and triazene groups of anticancer agents:

1.2.1 Nitrosoureas.



MNNG



	R	R'
MNU	CH_3	H
BCNU	$(CH_2)Cl_2$	$(CH_2)Cl_2$

Fig. 1.2. Structure of nitrosoureas.

In 1956 the first nitrosourea (Fig 1.2), 1-methyl-3-nitro-1-nitrosoguanidine (MNNG), was submitted to the National Cancer Institute for screening and was shown to possess slight activity against L1210 leukaemia (Greene and Greenberg, 1960). It was soon surpassed by 1-methyl-1-nitrosourea (MNU) and significantly this agent was shown to be able to cross the blood brain barrier (Skipper *et al.*, 1961), the CNS having been recognised as a sanctuary for leukaemia cells. This led to the development of a series of lipid soluble chloroethyl derivatives, including 1,3-bis (2-chloroethyl)-1-nitrosourea (BCNU), 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU) and 1-(2-chloroethyl)-3-(4-methylcyclohexyl)-1-nitrosourea (meCCNU). These chloroethyl-

nitrosoureas and chlorozotocin (a variation of streptozotocin, a naturally occurring aminoglucose carrier and MNU based compound) have found clinical use with success in the treatment of gliomas, melanoma, acute lymphoid leukaemia, lymphomas and gastrointestinal cancer (Wasserman *et al.*, 1975).

1.2.2 Triazenes.

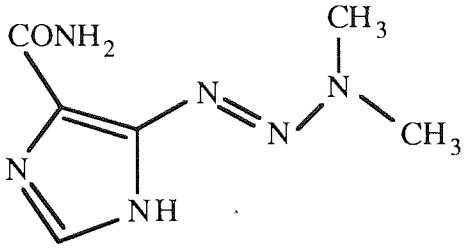


Fig. 1.3. Structure of DTIC.

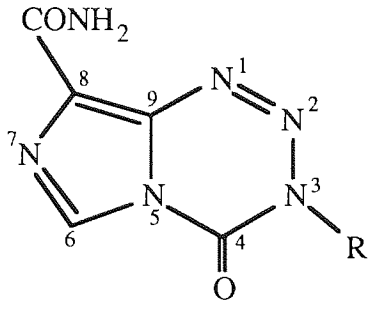
Of the series of triazene agents (5-[3,3-dimethyl-triazen-1-yl]imidazole-4-carboxamide (DTIC) (Fig 1.3) has achieved extensive use in the clinic, being one of the few agents to show activity in the treatment of malignant melanoma. DTIC requires metabolic oxidative N-demethylation to form the proposed active alkylating species 5-(3-methyltriazen-1-yl)imidazole-4-carboxamide (MTIC) (Preussman and Hodenberg, 1970) and substitution of one of the methyl groups of DTIC has no effect on antitumour activity, provided that the replacement group can undergo oxidative dealkylation and is of short chain length (Connors *et al.*, 1976). Apart from the dichloroethyl derivative (BCTIC) (Shealy and Krauth, 1966) di-alkyl derivatives that contain no methyl groups and hence give rise to higher alkylating species are inactive.

1.3 Imidazotetrazinones.

The 3-alkyl substituted imidazotetrazinones (Fig.1.4) are a novel group of antitumour agents whose structural and biological properties reveal a lineage with the two earlier groups of anti-cancer agents; the nitrosoureas and triazenes.

The lead compound in the series, mitozolomide [8-carbamoyl-3-(2-chloroethyl)imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one] was synthesised in 1980 by Stone and Stevens (Stone, 1981, Stevens *et al.*, 1983) and entered Phase I clinical trials in 1983 (Newlands *et al.*, 1985). Strict structural requirements have been indicated for

Fig. 1.4. Structure of imidazotetrazinones.



Mitozolomide	$R = (CH_2)_2Cl$
Temozolomide	$R = CH_3$
Ethazolastone	$R = CH_2CH_3$

the 3-alkyl group in order to exert effective antitumour activity, in that other than a chloroethyl group this position can only be substituted with a methyl group (temozolomide), the 3-ethyl and higher substituents losing selective tumour cytotoxicity both *in vitro* and *in vivo* (Hickman *et al.*, 1985). A comparable relationship is seen with the nitrosoureas (Scudero *et al.*, 1984a) and the triazenes (Connors *et al.*, 1976) and it is no surprise, given their structural similarities, that these agents appear to possess a similar mechanism of action since L1210 leukaemia and TLX5 lymphoma with derived resistance to BCNU and a dimethyltriazene have also displayed cross-resistance to imidazotetrazinones (Hickman *et al.*, 1985). Temozolomide has been developed as a second generation agent, and this compound is currently undergoing Phase II clinical evaluation with promising activity in mycosis fungoides, melanoma and glioma (Newlands *et al.*, 1992, O'Reilly *et al.*, 1993).

1.3.1 Decomposition of the imidazotetrazinones.

The imidazotetrazinones were synthesised under the premise that the inherent instability of the ring system would lead to degradation liberating an array of products with potential antitumour activity. Indeed, chemical breakdown of mitozolomide has been shown to occur via two main pathways:

Under thermal and non-aqueous conditions 5-diazoimidazole-4-carboxamide and 2-chloroethylisocyanate are formed by cleavage of the 2,3 and 4,5 bonds.

Under aqueous conditions the stability of the imidazotetrazinones is highly pH dependent, mitozolomide being stable in concentrated sulphuric acid at 60-65 °C. At physiological pH, following nucleophilic attack at C-4, mitozolomide ring opens at the 4,5 bond yielding 5-[3-(2-chloroethyl)-triazen-1-yl]imidazole-4-carboxamide (MCTIC) (Stevens *et al.*, 1984). Further aqueous decomposition ultimately results in the formation

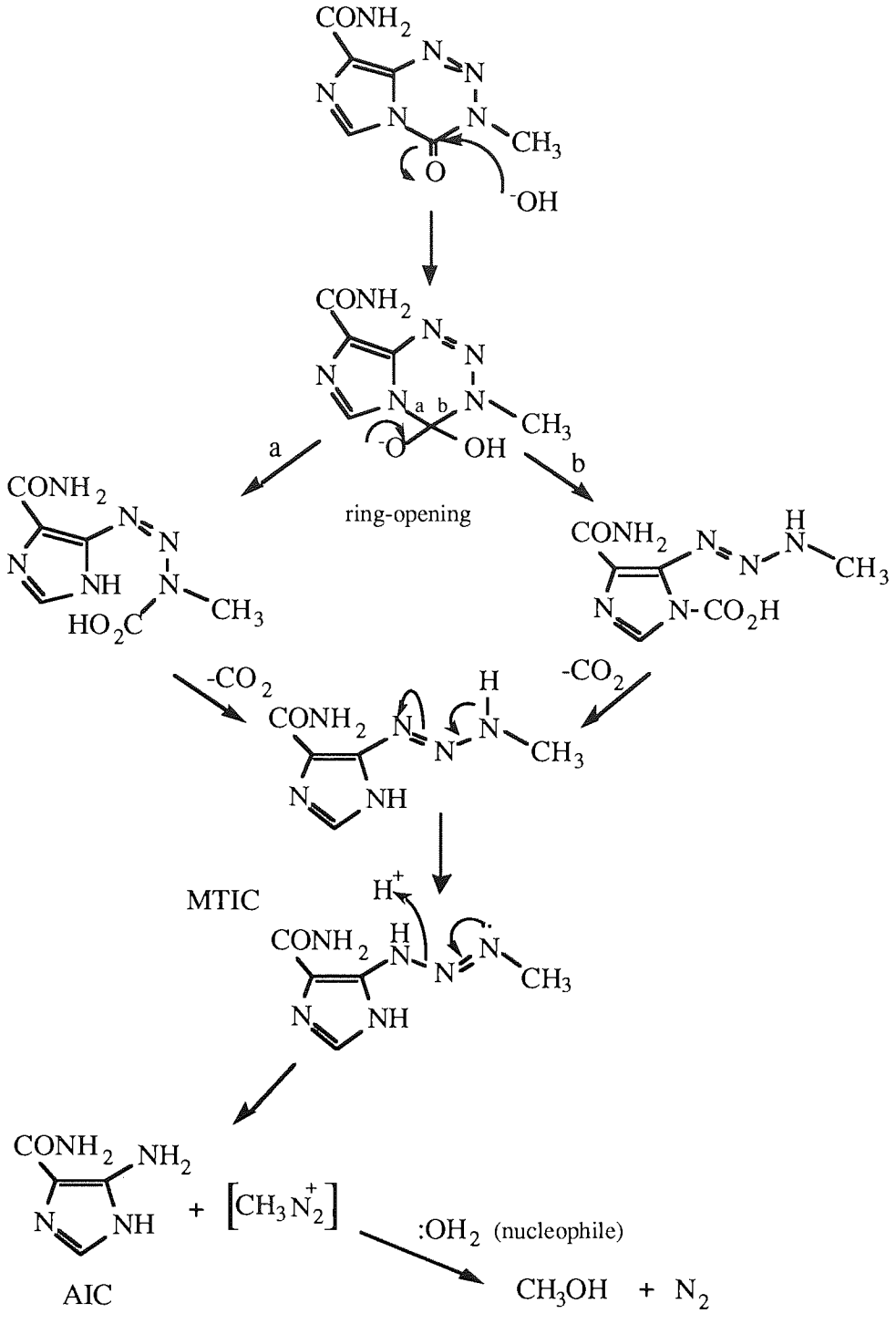


Fig. 1.5. Aqueous decomposition of temozolomide:
Following nucleophilic attack at C-4, temozolomide ring-opens at the 4,5 bond (a) and to a minor extent at the 3,4 bond (b) to yield MTIC. MTIC fragments to give AIC and a highly reactive methyl-diazonium species, which undergoes nucleophilic attack by a water molecule leading to the formation of methanol and nitrogen.

of 5-aminoimidazole-4-carboxamide (AIC), nitrogen and 2-chloroethanol. The isocyanate moiety appears not to be formed, since unlike BCNU and 2-chloroethyl isocyanate, mitozolomide treatment of TLX5 cells does not result in inhibition of cellular glutathione reductase activity (Horgan and Tisdale, 1984). Temozolomide and the 3-ethyl analogue have been shown to undergo a similar cleavage at the 4,5 bond and also to a minor extent at the 3,4 bond to give 5-[3-(2-methyl)-triazen-1-yl]imidazole-4-carboxamide (MTIC) (Baig *et al.*, 1987). MTIC was until recently proposed as the active alkylating species, however, NMR spectra of decomposition products have revealed MTIC to fragment to AIC and a highly reactive methyl-diazonium species which can undergoe nucleophilic attack, alkylating a water molecule and leading to the formation of methanol and nitrogen (Wheelhouse and Stevens, 1993) (Fig.1.5).

1.3.2 Antitumour evaluation of imidazotetrazinones.

1.3.2.1 Antitumour activity of mitozolomide.

In analysis against a range of murine tumour models (Stevens *et al.*, 1983, Hickman *et al.*, 1985) mitozolomide demonstrated curative activity against L1210 and P388 leukaemias, and in colon 38, M5076 sarcoma and ADJ/PC6A plasmocytoma solid tumour types. Comparison against the NCI tumour panel (Table 1.1) indicated it to be equi-potent with the extensively used clinical agents cisplatin, BCNU, adriamycin and cyclophosphamide, whilst proving superior to methotrexate and DTIC (Goldin *et al.*, 1981). Furthermore, activity was maintained against cyclophosphamide-resistant L1210 leukaemia, and although L1210 leukaemia and TLX5 lymphoma with derived resistance to BCNU and DTIC had cross-resistance to mitozolomide, it was apparent that mitozolomide had advantages over these earlier classes of antitumour agent in terms of oral bioavailability and pharmacokinetics (Lunt *et al.*, 1987).These promising murine studies and a pronounced activity against colon, sarcoma and melanoma human tumour xenografts (Fodstad *et al.*, 1985) prompted clinical evaluation of mitozolomide, with Phase I trials being completed in 1985. These established that the drug possessed good oral bioavailability and was in general well-tolerated with only minor nausea and vomiting. However, myelosuppression was found to be the dose limiting toxic effect, manifested by severe and unpredictable thrombocytopenia lasting up to 8 weeks at doses >115mg/m^2 (Newlands *et al.*, 1985). Phase II studies indicated minor antitumour activity

Table 1.1. Comparison of mitozolomide with other agents in the National Cancer Institute murine tumour panel.

From Goldin *et al.* (1981)

%T/C: Treated/Control median survival time or tumour weight inhibition for C38 adenocarcinoma and $CD8F_1$ mammary tumours.

in small cell carcinoma of the lung and in malignant melanoma, but the marked and prolonged thrombocytopenia, experienced by patients despite dosage reduction, precluded its further clinical development (Schornagel *et al.*, 1986, Neijt *et al.*, 1987, Harding *et al.*, 1988, Heriat *et al.*, 1988, Blackledge *et al.*, 1989).

1.3.2.2 Antitumour activity of temozolomide.

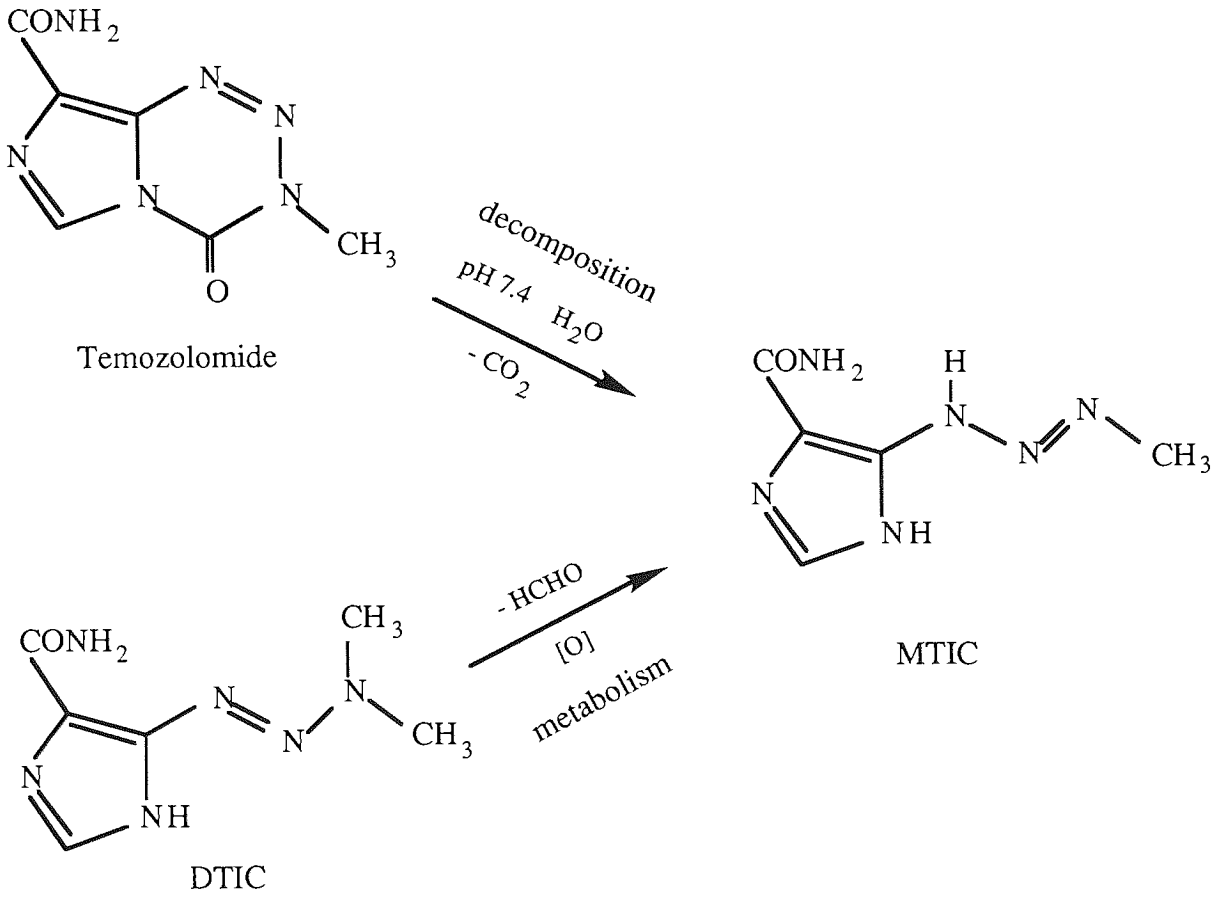
In selection of a second generation agent, all analogues containing chloroethyl substituents were rejected, since, despite showing increased activity in some cases, it was anticipated that these derivatives would display similar toxicity to mitozolomide (Horspool, *et al.*, 1990). Although a large number of imidazotetrazines were synthesised and tested with various substituents in the 3,6 and 8 positions, only the 3-methyl analogue, temozolomide, was found to display similar potency to mitozolomide (Stevens, 1987, Horspool *et al.*, 1990, Lunt *et al.*, 1987) (Table 1.2). A common mechanism of action for temozolomide and the anti-melanoma agent dacarbazine (DTIC) was indicated by cross resistance with DTIC-resistant L1210 leukaemia (Stevens *et al.*, 1987), and indeed MTIC, the product of ring-opening of temozolomide at physiological pH, is also the putative active metabolite of DTIC (Fig 1.6)(Tsang *et al.*, 1991). Comparison of these agents against a range of tumour models has indicated temozolomide to be the superior agent (Table 1.3)(Stevens *et al.*, 1987) and it has been suggested that the lack of requirement for metabolism of temozolomide in generation of MTIC may provide a pharmacokinetic advantage over DTIC.

Consequently, Phase I clinical trials were targeted towards melanoma, and also glioma since temozolomide like mitozolomide is known to cross the blood-brain barrier in mice (Brindley *et al.*, 1986). In contrast to mitozolomide, the anti-tumour activity of temozolomide in mouse tumours had been improved when used in divided doses (Table 1.2)(Stevens *et al.*, 1987) and hence temozolomide was administered on a 5 day schedule. Excellent oral bioavailability was demonstrated and, unlike mitozolomide, the myelosuppression elicited by temozolomide was usually predictable and also reversible. Responses were seen in 4 (17%) out of 23 melanoma patients, a mycosis fungoides patient and in 2 patients with high-grade astrocytomas (Newlands *et al.*, 1992). These promising results have prompted further studies in patients with primary brain tumours where temozolomide has produced a high response rate in gliomas. Improvements in

Table 1.2. Activity of single and multiple dose temozolomide in murine models, including resistant lines.(Stevens, 1987).

a. A
-, T
b. Fe

Fig. 1.6. Temozolomide and DTIC are considered as pro-drugs for MTIC.



Hepatic oxidative N-demethylation is required to activate inert DTIC to MTIC, whilst temozolomide decomposes under aqueous conditions, requiring no metabolic activity to form this active species.

Table 1.3. Comparison of temozolomide and DTIC against murine tumour models. (Stevens, 1987)

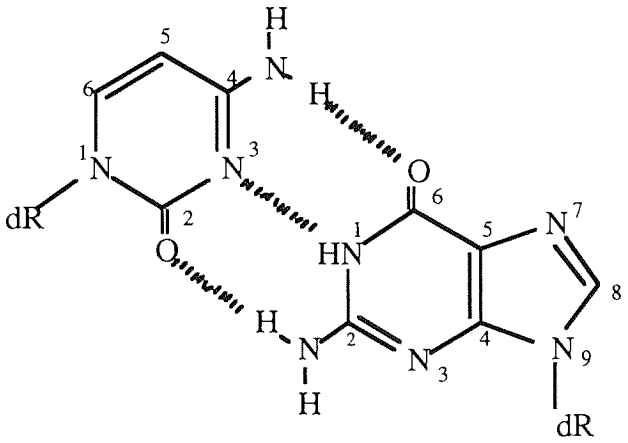
computer tomography (CT) scans were noted in 4/7 patients with newly diagnosed high grade astrocytomas, and in 5/10 patients with astrocytomas recurrent after radiotherapy (O'Reilly *et al.*, 1993) although it remains to be determined whether these responses are translated into improved survival.

1.3.3 Imidazotetrazinone DNA alkylation.

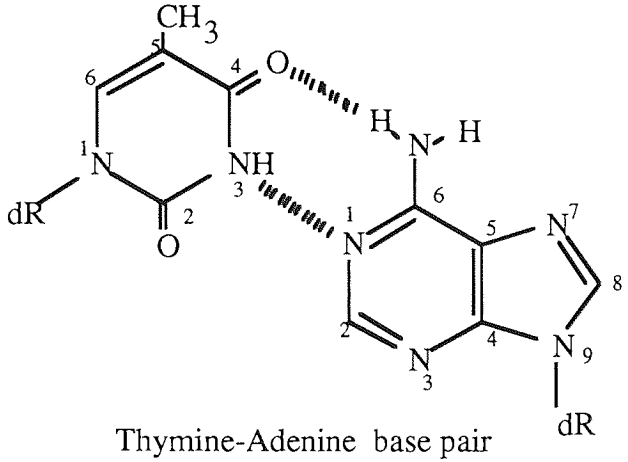
Mitozolomide and temozolomide have been considered as prodrugs of MCTIC and MTIC respectively, which they form by ring-opening under physiological conditions (Stevens *et al.*, 1984, Baig *et al.*, 1987). These unstable triazenes were thought to be the active alkylating moieties, although a methyldiazonium species has been identified as a more proximal alkylating species of temozolomide decomposition (Wheelhouse and Stevens, 1993), covalently linking the 3-substituent alkyl group to C,O,N,S and P containing nucleophiles via an essentially S_N1 type nucleophilic substitution. It is generally accepted that the ultimate target for alkylation is DNA, alkylation of which results in a range of different alkylation product types and amounts dependent on the agent used. Due to its steric situation and the nucleophilicity of the N^7-atom of guanine in double helical DNA, 7-alkylguanine is usually the major product of DNA alkylation. In contrast, there is little pyrimidine alkylation as the N^3 site, the predicted site of electrophilic attack is involved in Watson-Crick base pairing. Hence the major products are N^7-, O^6-alkylguanine, N^3-alkyladenine and alkylphosphotriesters (Table 1.4)(Bull, 1988).

1.4 DNA Repair.

In order to counter the deleterious effects of alkylation cells have evolved specific repair mechanisms. These processes have been widely studied in bacteria, and whilst many equivalent enzyme systems have been identified in mammalian cells, models for repair are still extensively based on prokaryotic studies. Repair mechanisms can be broadly classified as direct, base excision, nucleotide excision and post-replication:



Cytosine-Guanine base pair



Thymine-Adenine base pair

Table 1.4. Products of *in vitro* alkylation of DNA by various agents.

	(% of total DNA alkylation products)			
Lesion	Temozolomide	Ethazolastone	MNNG	MMS
N^7-alkylguanine	70.3	24.0	66.4	81.0
N^3-alkyladenine	9.2	4.9	8.4	11.3
O^6-alkylguanine	5.3	5.4	5.3	0.25
Phosphotriesters	12*	55*	12.1	0.8

Temozolomide and ethazolastone products are as determined by Bull (1988) with * values approximated from MNU and ENU alkylation products. MNNG and MMS are taken from Beranek *et al.* (1988).

1.4.1 Direct repair

Direct repair involves the simple reversal of DNA alkylation, without excision and replacement of the damaged base. Repair of the O^6-alkylguanine lesion is mediated by the DNA repair protein O^6-alkylguanine-DNA-alkyltransferase (O^6-AGAT) which reacts stoichiometricaly with O^6-alkylguanine residues in DNA, transferring the alkyl group to a cysteine-thiol group on the protein, resulting in regeneration of the intact guanine residue, and also in inactivation of the protein (review Pegg and Byers, 1992). In double stranded DNA the preferred substrate for the enzyme is O^6-methylguanine, although higher alkyl groups can be removed, albeit at a decreasing rate for a series of methyl to n-butyl groups, with O^6-ethyl guanine being repaired three times more slowly than O^6-methylguanine. In addition, O^6-AGAT has been shown to be capable of repairing 2-hydroxyethyl and 2-chloroethyl groups (Pegg and Singer, 1984).

The kinetics of repair indicate a rapid removal of the O^6-alkyl group which is followed by a saturation of the repair process due to the suicide nature of the protein. The point of saturation is dependent on cellular alkyltransferase content, which varies widely between cell types. All normal cells and cell lines have been found to be proficient in O^6-AGAT (Harris *et al*, 1983, Yarosh *et al* ,1983) however, approximately 20-30% tumour cell lines appear to lack the enzyme, and are designated Mex- (Sklar and Straus, 1981).

Day and co-workers found that some cell lines were unable to support the growth of adenovirus which had been alkylated with MNNG. This phenotype was described as Mer- (methylation repair) and it was subsequently shown that these cells were incapable of removing O^6-alkylguanine lesions from DNA (review Day *et al.*, 1987) (The Mer- and Mex- phenotype are both attributable to the lack of O^6-AGAT and are thus referred to in this thesis as Mer-).

It appears that the O^6-AGAT gene is normally retained in Mer- cell lines without significant rearrangement and is probably transcriptionally silent since the phenotype has been related to the methylation state of the promoter region of the gene which contains clustered CpG sequences that are characteristic of promoters of house keeping genes (review Karran and Bignami, 1992). It has been suggested that the origin of this

phenotype may be due to loss of O^6-AGAT activity in tissue culture conditions, since most human tumours examined have shown some alkyltransferase activity, and furthermore, Mer+ fibroblasts tumour cells from patients have been observed to give rise to Mer- cell lines (review Day,1987).

1.4.2 Base and nucleotide excision repair.

The base excision of 3-methyladenine and 7-methylguanine from the DNA of fibroblasts treated with MNU has been demonstrated by Medcalf and Lawley (1981). Release of the N-methylated purine as a free base occurs either through the action of specific alkyl-N-purine-DNA glycosylases or through the spontaneous hydrolysis of the N-glycosyl bond (Lawley, 1976). The resulting apurinic sites are then excised, along with adjoining nucleotides, by a 5′-acting endonuclease and a 5′→3′ exonuclease, whereupon the resulting gaps can be filled in by DNA polymerase-directed repair synthesis and DNA ligase-mediated strand rejoining.

An absolute distinction between base and nucleotide excision is not always applicable, although in general nucleotide excision is used in the repair of more bulky lesions such as those produced by cisplatin, or ultraviolet light induced pyrimidine dimers (review Burt *et al.*, 1991). A number of human nucleotide excision repair genes have been identified, termed ERCC genes (Excision Repair Complementing Chinese hamster). These genes reverse nucleotide excision repair deficiency when transfected into repair deficient CHO cells, and show homology with bacterial repair enzymes, although an equivalent to the *E.Coli* SOS response-induced ABC excinuclease genes, has not been identified in mammalian cells (review Burt *et al.*,1991).

1.4.3 Post-replication repair.

Whilst direct and excision repair are mechanisms of repair before replication, post-replication repair (also known as recombinational or daughter strand gap repair) is a mechanism of correcting damaged DNA after replication of the parental DNA strands. In this process, replication proceeds discontinuously, with DNA polymerase 'skipping' damaged bases, leaving gaps in the daughter strand. These gaps can then be filled in by recombination with undamaged DNA from the opposite parental (or sister) strand. This would appear to allow mitosis to proceed without the necessity for absolute repair of

damaged DNA, although the role of post-replication in chemotherapeutic resistance is poorly understood.

1.4.4 Induction of repair.

The ability to greatly increase the expression of repair genes is an important facet of bacterial response to alkylation damage. In contrast, the regulation of eukaryotic repair is more complex and remains unclear. No counterparts to the bacterial adaptive or SOS responses have been identified, although low-level damage dependent induction of repair enzymes has been described for O^6-AGAT, excision repair and recombinational activity (review Burt *et al.*, 1991). Observations of O^6-AGAT induction by DNA damage have been mainly confined to rat hepatocytes (Engelse *et al* .,1986, Laval, 1991), although more recently induction of O^6-AGAT and N^3-methyladenine-DNA-glycosylase has been reported in a human hepatoma cell line (LICH cells) and a human glioblastoma cell line, whilst no enhancement was seen in human fibroblasts (Lefebvre *et al.*, 1993). Typical alkyltransferase induction was 2-3 fold for a wide range of DNA damaging agent treatments including γ-rays and alkylating agents.

Numerous additional mRNA transcripts have been shown to increase following DNA damage (review Burt *et al.*, 1991, Karran and Bignami, 1992). These include transcripts of Gadd (growth arrest and DNA inducible genes) and β-polymerase genes, which appear to be directly involved in DNA repair. β-Polymerase is a DNA repair polymerase that fills in the gaps following excision of damaged nucleotides, whilst Gadd genes appear to positively regulate arrest of the cell cycle following DNA damage, G_2 arrest appearing to allow the cells the opportunity for repair before cell cycle progression to mitosis. The role of other damage induced transcripts, for example: c-Fos, c-jun, c-myc, metallothionein and ubiquitin, is uncertain, although given the lack of specificity of signals required for O^6-AGAT induction it is possible that the activation of transcription factors results in transcription of many genes, as part of a general stress response (Fritz and Kaina, 1992).

1.4.5 Alternative mechanisms of resistance.

DNA repair is not the sole determinant of drug resistance. Other mechanisms effect resistance, such as increased rates of drug efflux, as determined by levels of P-glycoprotein (Endicott and Ling, 1989), or increased drug inactivation by glutathione (Kramer *et al*.,1988) or metalothionein (Kelley *et al*., 1988). In addition, the efficiency of DNA repair has been shown to be gene specific (Russev and Boulikas, 1992) and strand specific (Basiczaninovic *et al*., 1992), and consequently lack of correlation between drug resistance and DNA repair at the genomic level, may relate to enzymes involved in control of these processes.

1.5 DNA Damage and Cell Death.

Although DNA damage and cytotoxicity have been closely correlated, the precise mechanism by which alkylation leads to cell lethality remains unclear. Cytotoxicity may be as a result of the presence on DNA of an alkyl group itself, or alternatively alkylation can lead to single-stranded DNA breaks, inter- or intra-strand DNA crosslinks or DNA-protein crosslinks. Conventionally it is these structural changes that have been considered to be the main causes of cytotoxicity of agents, whilst the presence of alkyl groups themselves have been more associated with mutagenesis and carcinogenesis, although alkyl groups may have a direct role in cytotoxicity by interfering with DNA-protein interactions (e.g DNA polymerase, RNA polymerase, transcription factors).

1.5.1 DNA single-stranded breaks.

Strand breaks may result as a direct consequence of chemical effects on nucleic acids, as a result of slow spontaneous hydrolysis of alkylated bases, or due to enzymic excision-repair processes. Alkylation labilises the glycosidic bond between the purine and the sugar causing slow spontaneous depurination (Lawley *et al*., 1976), which along with the activities of glycosylase enzymes, results in breaks in the DNA which may be of significance if not rapidly filled in by DNA polymerase and re-ligated by DNA ligase.

This type of lesion frequently accompanies other types of damage and may not be the primary cytotoxic lesion, although it appears to be in the case of streptonigrin (Cone *et al*, 1976) and bleomycin (Umezawa, 1975). Single-stranded breaks may cause transient inhibition of the initiation of DNA replication (Painter and Young, 1976),

however, a role in the cytotoxic action of alkylating agents remains uncertain. Various anticancer agents including BCNU (Sariban *et al.*, 1987) and aryl triazenes have been shown to produce strand breaks in DNA, although this effect may be related to carbamoylation, since Gibson *et al.* (1984a) have found that the agents mitozolomide, MCTIC and CNU cause negligible single-strand breaks.

A relationship between DNA single-strand breaks, NAD^+ metabolism and cell viability has been demonstrated in quiescent human lymphocytes (review Carson *et al*, 1986). The balanced excision repair process that occurs continually in these cells is accompanied by consumption of NAD^+ for poly(ADP-ribose) synthesis which may then be used in post-translational modification of diverse nuclear proteins. It has been proposed that the nuclear enzyme responsible for this, the ubiquitous poly(ADP-ribose) polymerase, has a role in control of chromatin structural changes and DNA repair (Boulikas, 1993), and also in the programmed removal of cells with extensively damaged DNA (Berger, 1985, Carson *et al.*, 1986, Marks and Fox, 1991). The enzyme is activated by single-stranded DNA breaks, and thus DNA damage is linked to metabolic activity and an increase in strand-breaks due to alkylation could precipitate lethal NAD^+, and hence ATP, depletion in a form of 'suicide response'.

1.5.2 DNA inter- and intra-strand cross-linking

Since the early cancer success of nitrogen mustards it has been surmised that antitumour alkylating agents produce toxic effects due to formation of covalent cross-links. Bifunctional alkylating agents can react with two nucleoside residues resulting in either inter or intra-strand crosslinking, depending upon the relative positions of the two bases. Two types of cross-linking have been demonstrated with BCNU (Tong *et al*, 1982) and monofunctional agents mitozolomide and MCTIC (Gibson *et al*, 1984a):

(1) an ethano bridge between two guanine N^7-positions, which is probably restricted to intra-strand linking between adjacent residues and is of unknown biological significance,

(2) an ethano bridge between a N^1-guanine and N^3-cytosine. A mechanism has been proposed by which this forms following initial chloroethylation at the guanine-O^6-position. Chloroethylation at this position causes

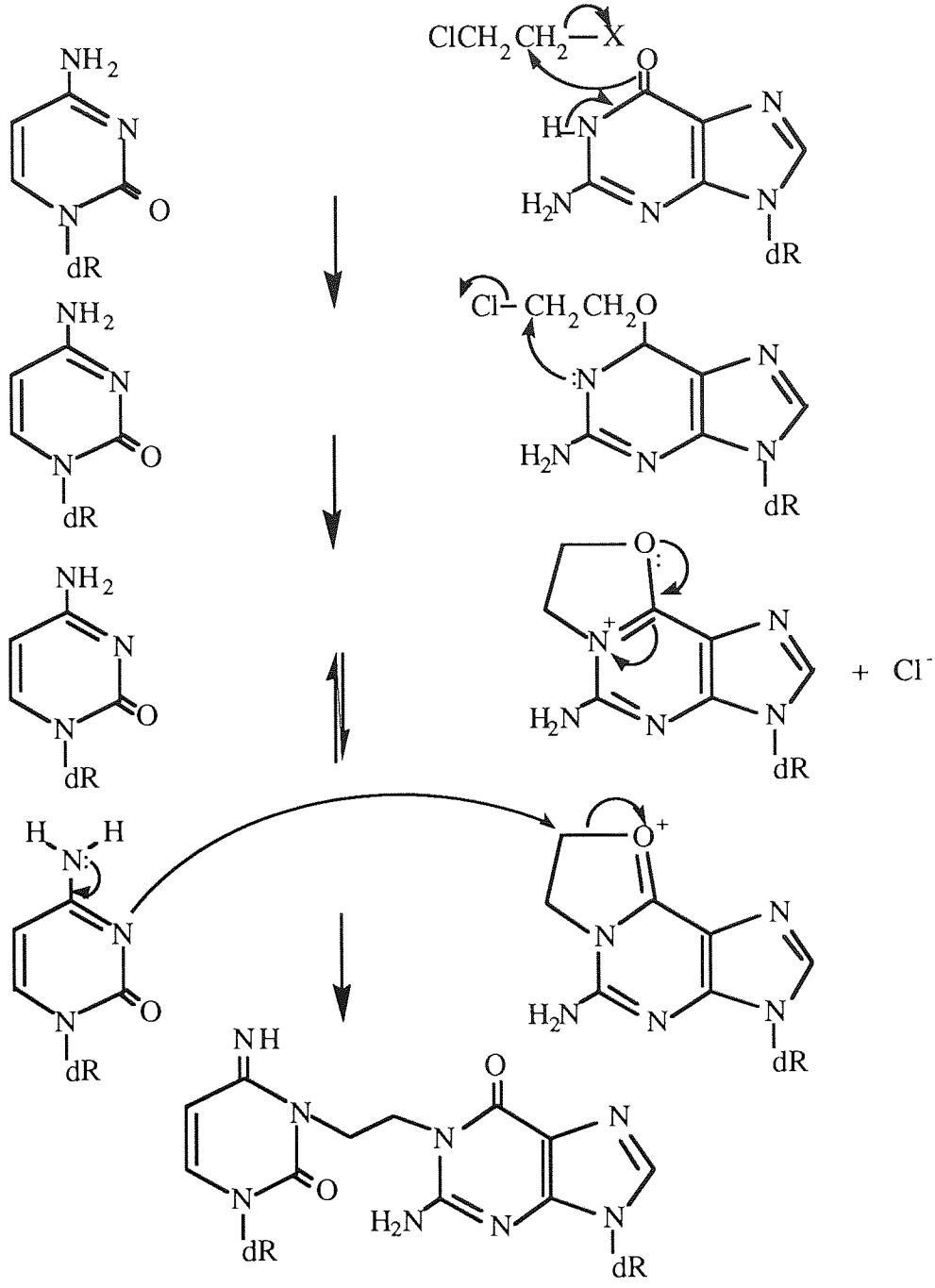


Fig. 1.7. Mechanism for the formation of cross-linked DNA:
chloroethylation at the O^6-position of a guanine residue results in disruption in the hydrogen-bonding of the guanine-cytosine base pair, leaving the adjacent N^1-position of guanine free to undertake intramolecular nucleophilic attack, with subsequent loss of the chloride ion generating resonance-stabilised N^1,O^6-ethanoguanine. Nucleophilic attack by the N^3-position of the cytosine in the opposite strand results in cleavage of the ethanoguanine moiety and formation of an N^1-guanine-N^3-cytosine interstrand cross-link.
(dR = deoxyribose and phosphate backbone of DNA).

disruption of the GC base-pair hydrogen-bonding, allowing access for reaction of the chloroethyl group with the guanine-N^1 position. This results in formation of an unstable intermediate, N^1,O^6-ethanoguanine, which interacts with the N^3-position of cytosine in the complementary DNA strand, leading to the formation of an interstrand cross-link (Fig.1.7). It is important to note that this is a relatively slow process and that both intermediates are subject to repair by O^6-AGAT prior to the formation of the cross-link (Hayakawa *et al.*, 1990). Furthermore, the cross-link itself may be subject to an excision-repair type mechanism since wild-type CHO cells have been reported to be 20-fold more resistant to the cytotoxic effects of 1-(2-chloroethyl)-1-nitrosourea (CNU) than excision repair deficient isogenic CHO UV41 cells (Wu *et al.*, 1992). If unrepaired it is easy to envisage how these cross-links could disrupt the cell-cycle since semi-conservative replication of DNA requires strand separation and it is unlikely that DNA polymerase, and hence replication, could proceed past an interstrand cross-link, with consequent lethality.

1.5.3 DNA-protein cross-links.

Cross-linking between DNA and protein is a prominent feature of the exposure of cells to the chloroethylating agent MCTIC (Gibson *et al.*, 1986). However, this type of DNA damage is not thought to be highly lethal since formaldehyde, an agent that produces DNA-protein cross-links as the predominant DNA lesion, has low cytotoxicity towards L1210 cells (Ross and Shipley, 1980). This appears to be due to efficient repair of these lesions, since the accumulation of DNA single-stranded breaks has been observed following induction of DNA-protein cross-links in the presence of DNA polymerase inhibitors, suggesting that mammalian cells possess an excision-repair mechanism for DNA-protein cross-link removal (Fornace *et al* ., 1982).

1.5.4 Cell death.

The steps between DNA damage and cell death are poorly understood and although many cellular events have been identified following DNA damage it remains difficult to ascribe these to cause or consequence of cytotoxicity. Although hypothetical, the intracellular events that follow DNA damage, resulting in cell death are summarized

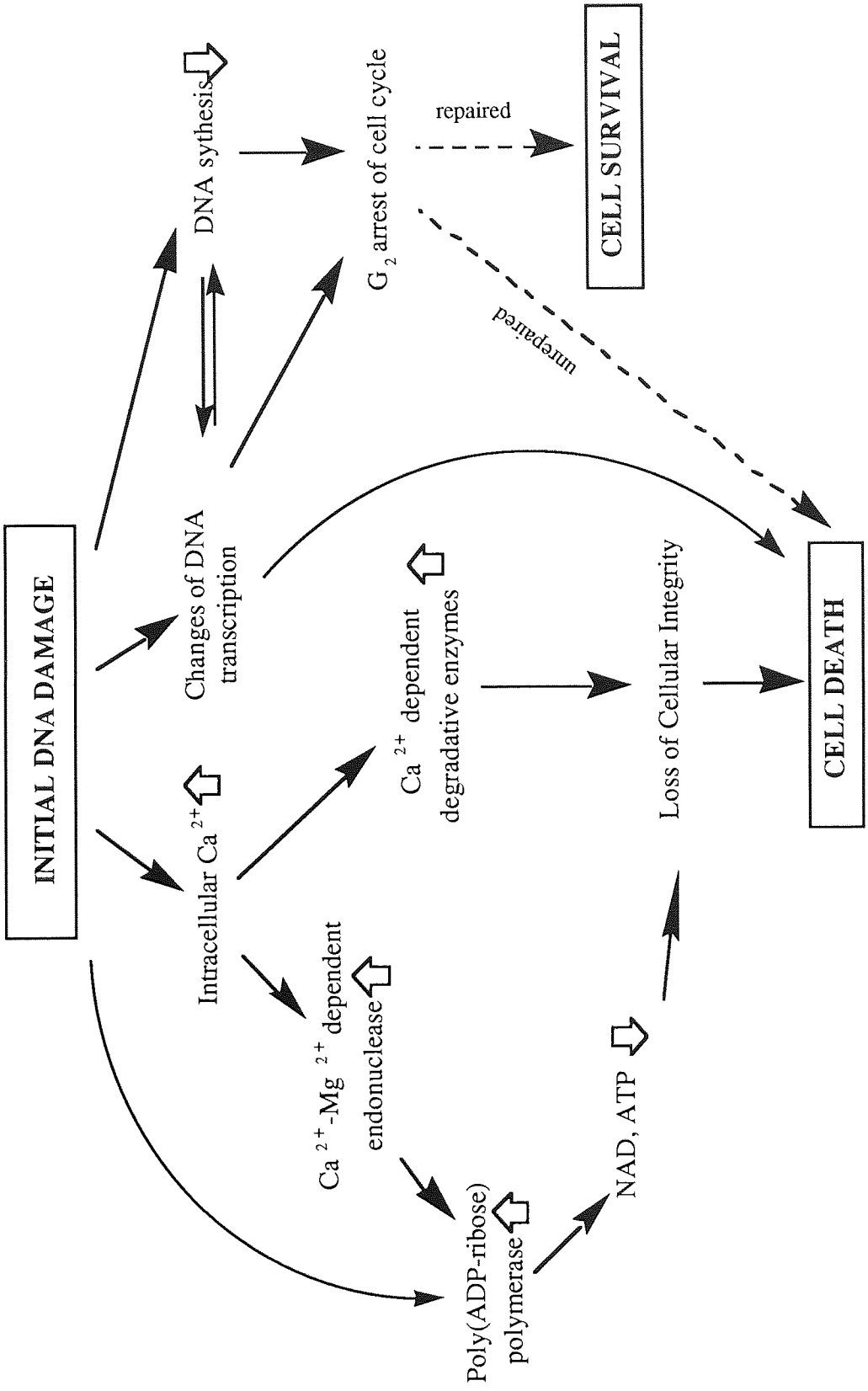


Fig. 1.8. Intracellular events following DNA damage that may result in cell death.

in Fig.1.8 (based on scheme suggested by Kubota, 1991). Cell death may be as a result of alkylation damage directly inhibiting DNA polymerase and hence replication, or perhaps due to interference with proteins involved in transcription, eventually leading to cell death via disruption of pathways that are vital for cell viability.

Alternatively, cell death may be a positively-regulated process. As discussed previously (section 1.4.4) many genes are activated in response to DNA damage, of which several appear to be directly involved in DNA repair. Although it had been originally thought that G_2 arrest following DNA damage was a consequence of inhibition of transcription of genes essential for passage to mitosis, it appears that entrance into mitosis is a positively-regulated process. It has been proposed that this is to allow increased time for DNA repair. Post-replication repair would appear to allow tolerance of a certain amount of damage, and once repair is sufficient mitosis may be allowed to proceed. Conversely, if cells remain extensively damaged cell cycle progression may eventually occur to produce inoperative daughter cells, or alternatively be prevented and the cells 'allowed' to die in order to prevent mutation and carcinogenesis, or perhaps even positively eliminated preventing the release of toxic cell debris.

The activation of poly(ADP-ribose) polymerase has been discussed as one potential mechanism by which cells may be eliminated. A second process, 'apoptosis' or 'programmed cell death' has been identified and shown to be characterised by internucleosomal DNA cleavage, chromatin condensing and morphological change (review Wylie, 1993). Fragmentation of DNA appears to be one of the general steps leading to cell death and has been shown to involve protein synthesis-dependent activation of a Ca^{2+}-Mg^{2+}-dependent endonuclease. Elevation of intracellular Ca^{2+} levels appears to precede the appearance of DNA fragmentation, a feature that has also been related to the activation of several other degradative enzymes which result in loss of cellular integrity (Mckonkey *et al.*, 1989, Nicotera *et al.*, 1986). Although apoptotic cell death has been reported following treatment of cells with etoposide, mafosfamide and cisplatin, cell necrosis also accounts for a large proportion of cell death following exposure to these drugs (Marks and Fox, 1991, Eastman, 1990).

1.6 Aims:

The aims of this project are three fold: to identify the targets important to imidazotetrazinone-induced lethality; to ascertain the mechanisms by which this damage causes cell death; and to clarify the mechanisms by which tumour cells may circumvent this action. It is envisaged that through the knowledge provided by such studies, the rational design of more tumour specific agents may be realised.

Chapter Two

Materials and General Methods

2.1 Purchased Chemicals, Reagents and Materials.

Aldrich Chemical Company, Poole, Dorset

Adenosine 5′ diphosphate
Formamide
Dimethylsulphate (DMS)
3-Methyladenine
Piperidine

Amersham International plc, Amersham, Bucks.

Deoxyribonuclease I (DNAase I)
N-[^{3}H] Methyl-N-nitrosourea (17.4 Ci/mmol)
Deoxyadenosine-5′-triphosphate, [γ-^{32}P] (5000 Ci/mmole)
Hyperfilm-MP
Acrylamide / bis-acrylamide (29:1)

BDH Chemicals Ltd., Poole, Dorset

2-Ethoxyethanol
Ethylenediaminetetraacetic acid, disodium salt (EDTA)
2-Mercaptoethanol
Sodium acetate
Sodium cacodylate
Sodium dihydrogen orthophosphate (analar)
Sodium dodecyl sulphate (Biochemical grade)
Trichloroacetic acid

Bio-rad Laboratories Ltd., Watford, Herts.

Bradford dye reagent
N,N,N′,N′-tetramethylethylenediamine (TEMED)
Ammonium persulphate
Acrylamide/bis-acrylamide (29:1)

Boehringer Corporation Ltd., Lewes, Sussex

Transfection-reagent (DOTAP)

Charles River UK Ltd.

BDF female mice

Fisons Scientific Equipment, Loughborough, Leics.

Acetonitrile (HPLC grade)
Hydrochloric acid (analytical grade)
Methanol (HPLC grade)
Optiphase 'Hisafe III' scintillation fluid
Perchloric acid (analytical grade)
Sodium chloride
Sodium Hydroxide
Urea
Water (HPLC grade)

Gibco, Paisley, Scotland

Bam HI restriction endonuclease
Foetal calf serum
L-Glutamine (200mM)
Hams F10 culture medium
Hams F12 culture medium
Penicillin (10,000 IU/ml) and streptomycin (10,000 UG/ml)
Phosphate buffered saline (PBS)
Pyruvate (100mM) (100x)
RPMI 1640 (with 25 mM HEPES and L-glutamine)
Trypsin-EDTA solution (10x)

ICN Pharmaceuticals Inc., Irvine, California, USA

Deoxyadenosine-5′-triphosphate, [γ-^{32}P] (3000 Ci/mmole)

MERK, W.Germany

C-18 LichroCART 5-µM (25 x 0.5 cm) HPLC column
Silica TLC plates

New England Nuclear, Du Pont (UK) Ltd., Southamton, Hants.

Chloramphenicol, D-threo-[dichloroacetyl-1,2-^{14}C]-
(CAT assay grade, 50.1 mCi/mmol)
Dimethyl sulfate, [methyl-^{3}H]- (1.2 Ci/mmol)

Northumbria Biological Limited

BamHI restriction endonuclease
DNA, pBR322
Sal I restriction endonucleases
T4 polynucleotide kinase

Perkin Elmer Cetus

T.aq DNA polymerase, Amplitaq recombinant

Pharmacia LKB Biotechnology, Milton Keynes, Bucks.

Agarose -M

Reverse transcriptase, Moloney murine leukaemia virus

Sigma Chemical Company Ltd., Poole, Dorset

Acetyl CoA

Adenosine 5′-diphosphate (ADP)

Adenosine 5′-monophosphate (AMP)

Adenosine 5′-triphosphate (ATP)

Albumin, bovine serum

3-Aminobenzamide

Ammonium acetate

Ampicillin

p-Aminosalicylic acid

Ava I restriction enzyme endonuclease

Bioluminescence ATP assay kit

Bromophenol blue

Caffeine

Calcium Chloride

Dacarbazine (DTIC)

DEAE-sephadex A

2′-Deoxyadenosine 5′-triphosphate (dATP)

2′-Deoxycytidine 5′-triphosphate (dCTP)

2′-Deoxyguanosine 5′-triphosphate (dGTP)

D-19 Developer (Kodak)

Dithiothreitol

Dimethysulphoxide (DMSO)

DNA, calf thymus

DNA, *M.lysodeikticus*

DNA polymerase I Klenow fragment

Ethidium bromide

Ethylmethanesulphonate (EMS)

Glycerol

N-(2-Hydroxyethyl)piperazine-N′-(2-ethanesulphonic acid)(HEPES)(Mol.Biol. grade)

N-(2-Hydroxyethyl)piperazine-N′-3-propanesulphonic acid (EPPS)

Kodafix
Lysozyme
7-Methylguanine
Methyl methane sulphonate (MMS)
N-Methyl-N′-nitro-N-nitrosoguanidine (MNNG)
Nicotinamide adenine dinucleotide phosphate
Nicotinamide adenine dinucleotide
N-Nitroso-N-methylurea (MNU)
Non-essential amino acids (100x)
Nonidet-NP40
Phenol / chloroform / *iso*amyl alcohol (25:24:1)
Phenylmethylsulphonyl fluoride (PMSF)
Potassium hydroxide
RNAase, pancreatic (DNAase free)
Sarcosy(N-lauryl sarcosil) (molecular biology grade)
Sodium chloride
Spermidine hydrochloride
N,N,N′,N′-tetramethylethylenediamine (TEMED)
6-Thioguanine (2-amino-6-mercaptopurine)
Thymidine 5′-triphosphate (dTTP)
Tris (hydroxymethyl) aminomethane (Tris), base
Trypan blue

Whatman Labsales Ltd., Croydon, Surrey
Cellulose nitrate filters (0.45μM)
Whatman 3MM chromatography paper

2.2 Synthesised and Donated Chemicals and Reagents.

Temozolomide was synthesised and donated by J.Slack and M.F.G. Stevens, Aston Molecules, Birmingham.

Ethazolastone, mitozolomide, 8-Carbomyl-3-[^{14}C]-methylimidazo [5,1-d]-1,2,3,5-tetrazin-4(3H)-one (26.3 mCi/mmol) and 8-Carbomyl-3-[^{14}C]-ethylimidazo [5,1-d]-1,2,3,5-tetrazin-4(3H)-one (16.0 mCi/mmol) were donated by May and Baker (now Rhone Poulenc), Dagenham, England.

5-(3-Methyltriazen-1-yl)imidazole-4-carboxamide (MTIC) was prepared by G.Baig, Aston University, Birmingham.

Spermidine-temozolomide conjugate was synthesised by Richard T.Wheelhouse, CRC Laboratories, University of Nottingham.

3-Substituted imidazotetrazinone compounds (**1**-**5**) were synthesised by Bill Thompson, Aston Molecules, Birmingham, and (**6**-**9**) by Y.Wang, CRC Laboratories, University of Nottingham. The 3-substituted pyrazolotetrazinones (**10**,**11**) were synthesised by Kevin Farnell, CRC Laboratories, University of Nottingham, and the benzotriazinone (**12**,**13**) and pyrazolotriazinone (**14**) analogues of temozolomide were synthesised by Alan S.Clark (Aston University, PhD thesis 1991), under the direction of M.F.G.Stevens, CRC Laboratories, Nottingham University.

D,L-α-Difluoromethyl ornithine (DFMO) was donated by the Marion Merrell Dow Research Institute, Cincinnati, USA.

O^6-Methylguanine was prepared by P.A.Hepburn (Aston University, PhD thesis 1991) by the method of Demple *et al.* (1983).

pKMΔ-98 DNA was a gift of Dr Keith R.Fox, Dept. of Physiology and Pharmacology, Southampton.

pHrasCAT DNA was kindly provided by Jean-Numa Lapeyre, Dept. of Molecular Pathology, the University of Texas, Houston, USA.

2.3 Stock Solutions and Buffers.

Alkaline elution solution

Tetra-ethyl ammonium	40 ml
EDTA	5.84 g

EDTA was dissolved in alkali first, then made up to 1l with water and adjusted to pH 12.1.

Cell lysis buffer

Tris base	10mM
EDTA, disodium salt	10mM
Nonidet NP-40	5.0 ml/l

Adjusted to pH 7.5 with conc. HCl

DNAase1 buffer

NaCl	20 mM
$MgCl_2$	2 mM
$MnCl_2$	2 mM

DNAase1 stop buffer

Tris borate (pH 8.3)	50 mM
formamide	80% (v/v)
bromophenol blue	0.1% (w/v)

Ethidium Bromide Reagent

Tris base	10 mM
EDTA	1 mM
Ethidium bromide	5.0 mg/l

Adjusted to pH 7.6 with conc. HCl

Formamide loading buffer

Formamide	98% (v/v)
EDTA (pH 8.0)	10 mM
Xylene cyanol FF	0.025% (w/v)
Bromophenol blue	0.025% (w/v)

Gel-loading buffer (6x)

Bromophenol blue	0.25% (w/v)
Xylene cylanol FF	0.25% (w/v)
sucrose in water	40.0% (w/v)

G- stop

sodium acetate (pH 7.0)	1.5 M
mercaptoethanol	1.0 M

2 x HEPES- buffered saline (HeBS)

NaCl	16.4 g
HEPES acid	11.9 g
Na_2HPO_4	0.21 g

The solution was made up to 1.0 l, adjusted to pH 7.05 with 5.0M NaOH and filter sterilised using a 0.2 μm Acrodisc filter (Gelman Sciences, UK).

LB medium (Luria Bertani medium)

Bacto-tryptone	10 g
Bacto-Yeast	5 g
NaCl	10 g

Made up to 1l with deionized water , adjusted to pH 7.0 with 5M NaOH and autoclaved.

O^6-Methylguanine stock solution

O^6-methylguanine was dissolved in HCl (0.1 N) to a concentration of approximately 1M, as determined spectrophotometrically, and was stored at -20°C. A_{280} = 7.9 for 1 mM O^6-methylguanine in a 1cm pathlength.

Sarcosyl lysis solution

Sarcosy(N-lauryl sarcosine)	0.2% (w/v)
NaCl	2.0 M
EDTA, disodium	0.04 M

Adjusted to pH 10

Solution I

Potassium acetate (5M)	60 ml
glacial acetic acid	11.5 ml
Water	28.5 ml

The resulting solution is 3 M with respect to potassium and 5M with respect to acetate.

STE buffer

NaCl	0.1 M
Tris-Cl (pH 8.0)	10 mM
EDTA (pH 8.0)	1 mM

Tris-borate electrophoresis buffer (TBE)(5x concentration)

Tris base	54.0 g/l
Boric acid	27.5 g/l
EDTA, disodium salt	3.7 g/l

Adjusted to pH. 8.0

Tris-EDTA , pH 8.0 (TE)

Tris base	10 mM
EDTA, disodium salt	1 mM

Adjusted to pH 8.0 with conc. HCl

TEOA buffer

EDTA	1 mM
triethanolamine	25 mM

Adjusted to pH7.2 with HCl

Trypan-blue solution

Trypan-blue	0.1 % (w/v) in PBS

Trypsin / versene

Versene stock (10x)	10 ml
Trypsin / EDTA (10x)	10 ml
Water (sterile)	80 ml

Versene stock solution

PBS tablets	10
EDTA, disodium	371 mg
Phenol red	50 mg

Made up to 100 ml, adjusted to pH 7.4 with 1M NaOH. Autoclaved and stored at -4°C.

2.4 General Techniques.

2.4.1 Determination of protein concentration by the Bradford method.

Protein assays were performed based on the method of Bradford (1976). Protein samples were diluted to 0.8 ml with water and 0.2 ml of Bradford reagent (Bio-rad) was added. Following vortexing, solutions were read on a spectrophotometer at 595 nm against a water blank. Protein concentrations were determined from a standard calibration curve, 0.2-25 μg/ml, prepared by dilution of a 0.1 mg/ml BSA stock solution.

2.4.2 Determination of DNA concentration by the ethidium bromide-fluorimetric assay.

DNA samples were diluted to 100 μl with water and vortexed with ethidium bromide reagent in a final volume of 1 ml. The solution was transferred to a Hellma quartz Suprasil cell with a path length of 10mm, and then read against a blank containing 0.1 ml water and 0.9 ml ethidium bromide reagent, on on a Perkin Elmer LS-5 luminescence spectrometer. The following settings were used- excitation wavelength of 360nm, emission wavelength of 590nm, excitation slit at 10 and emission slit at 5. DNA concentrations were determined from a standard concentration curve (0-25 μg/ml) prepared by dilution of calf thymus DNA stock solution (0.5 mg/ml).

2.4.3 Denaturing polyacrylamide gel (6%) preparation.

Urea	50.4 g	(7M final conc.)
40% acrylamide / bisacrylamide (29:1) in TBE (1x)	18 ml	(warm to dissolve)
TBE (5x)	24 ml	
Made upto 120 ml with water		

The solution was filtered and de-gassed in a sealed flask under vacuum, and stored on ice until use. The bottom of the sequencing gel plate was sealed by rapid polymerisation of 10 ml of the solution by mixing with 50μl of TEMED and 70μl of freshly prepared ammonium persulphate (25%). The remainder of the acrylamide/urea solution was mixed with 337 μl ammonium persulphate and 39 μl TEMED and poured into the plate. The gel was allowed to polymerise for 1hr before use.

2.4.4 Determination of [^{14}C]imidazotetrazinone purity.

The purity of [^{14}C]temozolomide and [^{14}C]ethazolastone was determined by chromatographic analysis on silica TLC plates eluted by a mobile phase of chloroform / acetonitrile / glacial acetic acid (80:30:5). Spots corresponding to imidazotetrazinone markers were excised, the radiolabel counted and expressed as % total TLC plate radioactivity.

2.5 Tissue Culture Methods.

2.5.1 Cell lines used.

GM892A: The GM892A cell line is a human lymphoblastoid line derived from a normal individual, which is deficient in O^6-alkylguanine-DNA alkyltransferase and classified as Mer- (Harris *et al.*, 1983). Obtained from Dr. P. Karran, Mill Hill Laboratories, London.

Raji: The Raji cell line is a human lymphoma line derived from a Burkitt lymphoma patient, which is proficient in the repair of O^6-alkylguanine and has been classified as Mer+ (Harris *et al.*, 1983).Obtained from Dr. P. Karran, Mill Hill Laboratories, London.

Glioma (Gl 7, Gl 16, Gl 18): The glioma cell lines were derived from a human Glioma patient, the cells having been isolated and cloned from a single brain tumour biopsie. The cell lines were kindly provided by J.Beith and J.Hartley, Dept. of Oncology, University College and Middlesex School of Medicine.

A549 cells: Caucasian male lung adenocarcinoma.

MCF7: Human breast adenocarcinoma.

K562 cells: Human chronic myelocytic leukemia.

MAC13: A murine colon adenocarcinoma line derived from a dimethylhydrazine induced solid murine tumour (Double and Ball, 1975).

MAC16: Murine colon adenocarcinoma.

CHO: Chinese hamster ovary. Purchased from European Collection of Animal Cell Cultures (ECACC), Porton Down, Wiltshire.

2.5.2 Maintenance of cell lines.

GM892A, Raji and K562 cell lines were cultured as suspensions in RPMI 1640 medium (with 25mM HEPES and L-glutamine) supplemented with 10% foetal calf serum and 1% L-glutamine, and were maintained in exponential growth at a density of between 0.8 and 10 x 10^5 cells/ml.

The MAC16 cell line was maintained under similar conditions (RPMI 1640, 10% FCS, 1% L-glutamine), at a concentration of 0.2-2 x 10^5 cells/ml. For dilution or counting of cells the semi-adherent MAC16 cells were dislodged from the plastic surface of the flask by shaking.

The A549, CHO, MCF7, MAC13 and Glioma cell lines were grown as monolayers attached to plastic, and were maintained in exponential growth at 0.1 - 2.5 x 10^6 cells/T25 flask in 10 ml of media. MAC13 cells were grown in RPMI 1640 medium, A549 and CHO cells in HAM's F12, Glioma cell lines in HAM's F10, and MCF7 in DMEM with added pyruvate (1mM) and non-essential amino-acids (sigma-100x). The media were supplemented with 10% FCS and 1% glutamine (Gibco-100x), and A549 and MCF7 cells were cultured in the presence of penicillin (100 IU) and streptomycin (100 UG).

For routine subculturing or cell counting, monolayer cells were detached from the plastic by trypsinisation. Following aspiration of the medium from cell cultures, A549, MCF7 and Glioma cells were detached by the direct addition of trypsin/versene solution and incubation at 37°C for approximately 5 min. The MAC13 and CHO cell lines were detached by incubation at 37°C with trypsin solution, after having first been washed with 10ml of Mg^{2+}/Ca^{2+} free PBS and rinsed quickly with trypsin solution. Following detachment of the cells 10 vol of media was added to the cell suspensions in order to inactivate the trypsin and trypsin/versene solutions.

For subculture of the cell lines, cell numbers were determined and fractions of cell suspensions were reseeded in fresh medium. All the cell lines were incubated at 37°C under an atmosphere of 5% CO2, and under the above conditions approximate doubling times for the cell lines were as follows:-

MAC13 -14 hrs, CHO - 16 hrs, Raji- 20 hrs, GM892A, A549, K562, MCF7, Gl 7 and Gl 16 - 24hrs, Gl 18 and MAC16 - 36 hrs.

2.5.3 Cell counting.

Cell counting was carried out using a coulter counter (model ZM, coulter counter electronics, Bedfordshire), using the following settings:-

cell line	Current (I)	lower threshold(T_L)	Attenuation
A549	130	12	16
GM892A	200	15	16
Raji	200	12	8
K562	200	20	16
CHO	200	12	16
Glioma	200	12	16
MCF7	160	13	16
MAC13	120	15	8
MAC16	200	18	16

An upper threshold (Tu) setting of 99.9 was used for all the cell lines.

2.5.4 Storage of cell lines in liquid nitrogen.

Exponentially growing cells were pelletted and re-suspended at a density of 1 x 10^6/ml in media containing 10% DMSO. Cell suspensions, contained in cryogenic vials, were cooled to -70°C and then immersed in liquid nitrogen for storage. Cell lines were re-surrected after rapid thawing of vials, by washing the cells with media and re-suspending and incubating cells in media containing 20% FCS.

2.5.5 Trypan-blue exclusion assay.

The viability of cell cultures was determined by mixing 100µl cell suspension with an equal volume of trypan-blue solution (0.1% w/v in PBS). Cellular integrity was examined by microscope for a minimum of 250 cells.

Chapter Three

Imidazotetrazinone-Induced DNA Lesions and their Importance to Cytotoxicity

3.1 Introduction.

The alkylating agent temozolomide reacts with the oxygen and nitrogen atoms of heterocyclic bases to form assorted products in DNA, notably N^7-alkylguanine (70.3 %), N^3-alkyladenine (9.2%), 0^6-alkylguanine (5.3%) and alkylphosphotriesters (approximately 12 %), and in addition a range of minor products (Bull, 1988). It is not surprising that cells are endowed with mechanisms for DNA repair, however if unrepaired, these lesions may result in mutation, carcinogenesis and, most importantly to the action of anticancer agents, in disruption of cellular function and cell death.

Day and co-workers (1984) have determined that one lethal hit to an MNNG treated adenovirus population correlated with production of 2.3 O^6-methylguanine, 1.4 3-methyladenine, 16 7-methylguanine and about 5 methylphosphotriesters per viral genome, whilst, all other lesions produced were too few to present one per genome for one lethal hit per virion. It would appear, therefore, that the lesion/lesions important to this cytotoxicity are among these four major alkylation products, and that although the O-alkylated pyrimidines, O^2-alkylcytosine, O^2-alkylthymine and O^4-alkylthymine, have been shown to exhibit strong miscoding properties, and appear to contribute to mutagenisis and carcinogenesis (Swenberg *et al.*, 1984, Singer *et al.*, 1986, Dosanjh *et al.*, 1993), the low instance of these lesions (Beranek *et al*, 1980), would suggest them to be of little importance in the cytotoxic action of alkylating agents.

Although, as for many alkylating agents, 7-methylguanine is the major product of temozolomide alkylation, this lesion is apparently harmless since it is tolerated by the cell for generations. The lesion is repaired, although only slowly with a half life of 30 hours (Medcalf and Lawley, 1981), and it is uncertain whether lack of this repair would be significant. Methyl and ethyl adducts do not appear to change the coding specificity of the guanine base or to interfere with DNA polymerase (Karran and Lindahl, 1985), although spontaneous ring-opening of the imidazole moiety, to give formamido-pyrimidine, is known to inhibit DNA chain elongation (Boiteux and Laval, 1983, Tudek *et al.*, 1992). Indeed, a lethal role for this lesion cannot be ruled out since DNA methylases appear unable to bind to an N^7-alkylated polymer (Drahovsky and Morris, 1972), while in addition, it is possible that excision of the lesion, producing single-stranded breaks, may

be of cytotoxic significance.

In contrast, considerable experimental data has been presented correlating the ability to repair the relatively minor lesion O^6-alkylguanine with the cytotoxicity of the imidazotetrazinones (Tisdale, 1987), triazenes (Gibson *et al.*, 1986) and nitrosoureas (Scuderio *et al.*, 1984a &b). Indeed, O^6-alkylguanine has been suggested by some to be the lesion of major importance in cell killing, mutagenesis and carcinogenesis.

It has been shown that adenoviruses treated with MMS survive independently of the ability of the infected cells to repair O^6-methylguanine, whereas MNNG or MTIC treated adenoviruses only survive in Mer+ strains (Scudiero *et al.*, 1984a). It would seem likely therefore, that a lesion produced by MNNG (O^6-methylguanine = 5.4% of total alkylation) but not by MMS (0.25 %) is a lesion lethal to the virus, and a lesion which is not repaired in Mer- strains. In agreement with this, expression of the *E.Coli ada+* gene in CHO cells (Mer-) has been demonstrated to afford substantial protection against the cytotoxic and mutagenic effects of MNNG (Kataoka *et al.*, 1986). In addition to its O^6-methylguanine-DNA methyltransferase activity, the Ada protein is also active towards methylphosphotriesters and the minor lesion O^4-methylthymidine, properties not shared by its mammalian counterpart (Hall and Karrran, 1986). However, expression in CHO cells of a truncated version of the *ada+* gene encoding only the methylphosphotriester repair domain conferred no protection at drug concentrations which resulted in >10% cell survival (Kataoka *et al.*, 1986). Thus, methylphosphotriester repair does not appear to be a major determinant of the elevated MNNG resistance (Hall *et al.*, 1988), and the possibility that increased resistance is due to the ability to repair O^4-methylthymidine can be discounted following the report that transfection of a plasmid expressing the mammalian repair enzyme O^6-AGAT into an alkyltransferase deficient human cell line elicits resistance to the cytotoxic effects of MNNG, CNU and ACNU (Hayakawa *et al.*, 1990, Wu *et al.*, 1991).

In addition to these observations, further evidence for a correlation between the ability to repair alkylation at the O^6-position of guanine and cytotoxicity have been provided by experiments in which cellular O^6-AGAT levels have been depleted using the free base O^6-methylguanine, a weak substrate for the enzyme (Karran and Williams,

1985), which has been shown to result in sensitisation of Mer+ cell lines to the effects of CNU (Yarosh *et al*, 1986), mitozolomide and temozolomide, but not the 3-ethylimidazotetrazinone (Tisdale, 1987). More recently, as an improved substrate for the mammalian alkyltransferase enzyme (Dolan *et al.*, 1990), O^6-benzylguanine has provided definitive results for depletion of alkyltransferase and the potentiation of the cytotoxicity of BCNU (Chen *et al.*, 1993), CCNU and temozolomide (Baer *et al.*, 1993). Consequently, the potential utility of O^6-benzylguanine as an adjuvant in combination chemotherapy with such agents is currently under investigation *in vivo*.

The participation of the O^6-position of guanine in the initial formation of cytotoxic DNA interstrand cross-links would account for the differential effects demonstrated by CNU, mitozolomide and MCTIC towards Mer+ and Mer- cells (section 1.5.2)(Erickson *et al,* 1980, Gibson *et al,* 1984b, Gibson *et al*, 1986), however, temozolomide is chemically incapable of cross-linking DNA suggesting that alkylation at the O^6-position may in itself be sufficient to cause cell death. It is more difficult to see why O^6-methylguanine should be a cytotoxic lesion, although it can clearly be mutagenic (Newbold *et al.*, 1980), since mispairing of O^6-methylguanine occurs upon DNA replication as the alkylated base appears to pair better with thymine than cytosine (Singer *et al.*, 1989). Thus, while production of O^6-methylguanine in DNA correlates well with mutation induction a mechanism by which the lesion may exert lethality is uncertain. Whilst, some workers have shown a correlation between unrepaired O^6-methylguanine and cytotoxicity, others have not, and it has been suggested that Mer- cells may possess defects other than lack of O^6-AGAT which are responsible for their sensitivity (Schwartz *et al.*, 1989)(Karran and Williams, 1985). Indeed, the very existence of cell lines that are O^6-AGAT deficient yet MNU and MNNG resistant (designated Mer-rem+) must cast doubt on the role of the O^6-lesion in cell killing (Day *et al.*, 1980b).

Studies with mutant bacterial strains have identified N^3-methylpurines as lesions important to the lethal effects of simple alkylating agents (Lindahl and Karran, 1983). However, little evidence has been presented in mammalian cells to implicate 3-methyladenine in cell killing, since the lesion appears to be removed from DNA efficiently with a half life of approximately 2 hours (Medcalf and Lawley, 1981),

although an ability to repair the lesion has been related to recovery from the cytotoxic action of simple alkylating agents (Harris *et al.*, 1981). In contrast to 7-methylguanine, unrepaired 3-methyladenine would appear to be potentially cytotoxic, since the alkyl group protrudes into the minor groove of the DNA helix and could possibly act as a block to DNA polymerases (Karran and Lindahl, 1985). Indeed, a potential role in cytotoxicity has been implied by human glioma cell lines where a mutant resistant to the cytotoxic effects of haloethylnitrosoureas had levels of 3-methyladenine-DNA-glycosylase (3MAG) 2-3 fold higher than a sensitive strain. In addition, the resistant line possessed elevated levels of O^6-AGAT and it was suggested that the increased level of the two enzymatic activities was coordinated in some way (Matejasevic *et al.*, 1991).

In view of these possibilities, a range of experiments have been undertaken in order to further establish the relationship between DNA base-alkylations, repair of specific DNA lesions, and the cytotoxicity of the imidazotetrazinones. Alkylating agents, including the imidazotetrazinones, triazenes and nitrosoureas, and also a range of new imidazotetrazinone analogues of previously undetermined activity, have been compared against an alkyltransferase proficient (Raji) and a deficient (GM892A) cell line. In order to further investigate the cytotoxicity of the lesions produced by temozolomide, an assessment has been made of the response, and of the repair capabilities of a range of human and murine cell lines. In addition, the effect on cytotoxicity of depletion of O^6-AGAT, by the free base O^6-methylguanine, has been examined. In order to more closely relate the findings to the clinical situation, the effect of temozolomide dose scheduling on the sensitivities of a Mer+ and a Mer- cell line has been examined, and cytotoxicity and alkyltransferase studies have been repeated in a range of glioma cell lines and in *in vivo* established murine solid tumours.

3.2 Materials and Methods.

3.2.1 *In vitro* growth inhibition studies.

Raji and GM892A cells growing in exponential phase were seeded at 0.8 x 10^5 cells/ml in Nunc 24 well plates and treated with a range of drug concentrations (drugs were dissolved in DMSO and added so that DMSO concentration did not exceed 0.5%). A minimum of 3 cell populations were treated for each drug concentration and cell numbers were duplicate counted following a 72 hour incubation period. The growth of the treated cell population was compared to the growth of a solvent treated control cell population, after subtraction of the seed number from the final cell counts. Results were plotted as % of control population growth, from which the concentration of drug required to cause 50% inhibition (the ID_{50} value) of growth was calculated.

3.2.2 Effect of O^6-AGAT depletion on drug cytotoxicity.

Raji and GM892A cells were seeded at 0.8 x 10^5 cells/ml in T25 flasks and incubated for 16 hr with 0.5mM O^6-methylguanine (from a stock solution dissolved in 0.1 N HCl at a concentration of approx. 0.1 M (as determined spectrophotometrically in section 2.3), or with a solvent control. After pelleting and re-suspension at 0.8 x 10^5 cells/ml the cells were aliquoted into Nunc 24 well plates, and were treated with a range of concentrations of temozolomide, MMS or EMS. After 72 hr, the cells were counted and population growth was compared for drug treated and solvent treated controls, and the effect of pre-incubation with O^6-methylguanine on drug toxicity was determined .

3.2.3 Sensitivity to temozolomide and cell line O^6-AGAT levels.

The cellular levels of O^6-alkylguanine-DNA alkyltransferase have been compared for a range of cell lines with differing sensitivity to temozolomide. GM892A and Raji O^6-AGAT levels were determined from the ability of cell extracts to excise methylated bases from a [^{3}H]methyl-DNA substrate, as described by Tisdale (1987).

3.2.3.1 Sensitivity of cell lines to temozolomide.

The ID_{50} concentrations for temozolomide were determined for cell lines as described in section 3.2.1. GM892A and Raji cells were seeded at 0.8 x 10^5 cells/ml and treated with a range of concentrations of temozolomide and the inhibition of cell population growth was determined. In the studies of Tisdale (1987) the sensitivity of K562 cells was determined by similar growth inhibition studies, whilst MAC13, MAC16, and A549 cytotoxicity was determined from the loss of colony-forming ability by an *in situ* assay in which cells were treated at cloning densities.

3.2.3.2 Preparation of [^{3}H]methyl-MNU-alkylated DNA substrate.

[^{3}H]Methyl-DNA was prepared by reaction of 20 mg of *M.Lysodeicticus* DNA (2.5 mg/ml) with 1.0 mCi of N-[^{3}H]methyl-N-nitrosourea (23 Ci/mmol) in 0.2 M EPPS buffer (pH 8.6) for 2 hr at 37°C. After incubation the reaction was chilled on ice and the DNA precipitated by the addition of 0.1 vol of 5M NaCl followed by 2 volumes of cold absolute ethanol. The DNA was wound onto a glass rod, washed twice with 80% ethanol and then dissolved in 6.0 ml of 10mM Tris-HCl (pH 7.5)- 1mM EDTA. The alkylated DNA was dialysed overnight at 4°C against 2 changes of 0.1 M NaCl -10 mM sodium citrate -10 mM potassium phosphate (pH 7.4) and then heated in a sealed glass ampoule for 18 hrs at 80 °C to remove the majority of the unstable alkylated purines, whilst retaining heat stable O^6-methylguanine. The depurinated DNA was again extensively dialysed first against 1M NaCl -10 mM Tris-HCl -1mM EDTA (pH 8.0) and then against 10 mM Tris-HCl -1mM EDTA (pH 8.0).

The alkylated DNA had a specific activity of approximately 2,800 dpm/µg DNA, and hydrolysis of a sample by incubation in 0.1 N HCl for 18 hr followed by HPLC analysis of the base products (section 3.2.3.4) indicated 69 % of the label to be O^6MeG, and 28 % Me^7G and Me^3A.

3.2.3.3 Preparation of cell extracts.

Approximately 1.5 x 10^7 exponentially growing cells were harvested by low speed centrifugation (adherent cells having first been scraped from the flask) and washed twice with 0.9% NaCl. The cells were resuspended in 150 µl of 10 mM Tris/HCl (pH

7.8) -1mM DTT -1mM EDTA -5% glycerol and sonicated twice for 5 s on ice. A supernatant fraction for enzyme assay was produced by centrifugation for 5 min at 4°C in an Eppendorf microcentrifuge and the protein content of the cell extract was determined by the Bradford method (section 2.4.1).

3.2.3.4 O^6-AGAT enzyme assay.

Portions of cell extracts were incubated in 100 µl assay mixtures containing 70mM Hepes/KOH (pH 7.8) -1mM DTT -1mM EDTA -50µM spermidine -5% glycerol and [^{3}H]methyl-DNA (1x10^4 dpm [^{3}H]O^6MeG) for 45 min at 37°C. The reaction was terminated by the addition of 30 µl of ice-cold 50% trichloroacetic acid, and after 10 min at 0°C the acid-insoluble precipitate formed was pelleted by centrifugation at 4°C in an Eppendorf microcentrifuge and the supernatant was discarded. The DNA was then hydrolysed by heating at 70°C for 45 min with 50 µl 0.1N HCl. The O^6MeGua released was separated from the other alkylated bases present in the hydrolysate using HPLC: The acid supernatant was analysed on a 5 µm endcapped C-18 LichroCART column (0.5 x 25 cm) eluted by an isocratic mobile phase of 10 mM $NH_4H_2PO_4$ / 16% (v/v) methanol at a flow rate of 1ml/min. The retention times for Me^3A, Me^7G,and O^6MeG were 2.7 min, 4 min and 9 min respectively. Fractions corresponding to the O^6MeG peak were collected, mixed with 10 vol of Hisafe III scintillation fluid and counted. The O^6MeG removed from the substrate by the cell extract was calculated from the difference in counts released in control (enzyme extract omitted) fractions and those in the corresponding fractions of the experimental sample. O^6-AGAT levels ,which equal the quantities of O^6MeG released, were determined from the specific activity of the [^{3}H]methylnitrosourea and expressed per mg of protein present in the enzyme assay.

3.2.4 Determination of 3-methyladenine-DNA glycosylase activity.

3.2.4.1 Preparation of [^{3}H]methyl-DMS-alkylated DNA substrate.

[^{3}H]Methyl-DNA was prepared as described by Matejasevic *et al.*(1991). 0.8 ml of calf thymus DNA (5 mg/ml) in 50mM sodium cacodylate buffer, pH 7.5, was alkylated by incubation for 2 hr at 37°C with 2 mCi [^{3}H]dimethylsulphate (1.2

Ci/mmole). The DNA was ethanol precipitated by addition of 1 volume of 3M NaCl followed by 2.5 volumes of ethanol, redissolved in water and reprecipitated until constant activity (5000 cpm/μg)was obtained. The products of the alkylation were determined by HPLC analysis following depurination of the DNA in 0.1 N HCl at 37°C for 16 hr. The principal alkylation products were determined to be 7-methylguanine (81%) and 3-methyladenine (19 %) , with insignificant O^6-methylguanine being formed.

3.2.4.2 Preparation of cell extracts.

The 3-methyladenine-DNA glycosylase activities of GM892A, Raji, A549, K562, MAC16 and MAC13 cell lines were determined by the method of Matejasevic *et al.*(1991). Each cell line was grown to confluence, or to the maximum density obtainable in exponential cell growth, and approximately 400 mg of cells were harvested following the scraping of adherent cells. All steps in the preparation of cell extracts were carried out on ice. The cells were washed with 0.9% NaCl, centrifuged and resuspended in 50mM Tris buffer, pH 8, containing 1mM EDTA, 100 mM NaCl, 0.1 mM phenylmethylsulphonyl fluoride and 0.03 unit/ml of aprotinin. The NaCl concentration was increased to 700mM by the addition of 5M NaCl and the cells were disrupted by sonication for 5 sec x 3. The NaCl concentration was then adjusted to 300mM by the addition of 50mM Tris buffer (pH 8), containing 1mM EDTA , 2mM 2-mercaptoethanol, and 0.1mM phenylmethylsulphonyl fluoride. The solutions were stirred for 2 hr at 4°C and then centrifuged at 17,200 x g for 2.5 hr and the supernatents aliquoted and stored at -70 °C.

3.2.4.3 Enzyme assay.

Cell extracts containing 0-150 μg of protein were incubated with approximately 3 μg (15,000 cpm) of DNA substrate in a buffer containing 20mM Tris (pH 8), 2mM 2-mercaptoethanol and 60mM NaCl in a total volume of 100μl. After incubation at 37°C for 30 min, the reactions were stopped by cooling on ice and the DNA was precipitated by the addition of NaCl-ethanol for 30 min at -70 °C followed by centrifugation at 4°C for 20 min at 10,000 x g. The radioactivity in the supernatant was determined directly in Optiphase scintillation fluid and the radioactivity released by boiled extracts was subtracted from these figures.

3.2.3.4 Identification of excised bases by HPLC.

In order to identify radioactive bases released by cell extracts, the incubation mixture was scaled up 20-fold and the release by extracts containing 700 μg of protein was determined. After incubation the mixture was passed through a DEAE-Sephadex A-25 column (10 x1 cm) to remove oligonucleotides, lyophilised, dissolved in HPLC buffer (0.5 ml), and 100 μl was analysed on a preparative Lichrosorb C-8 column. The column was eluted at 1ml/min by 100 mM ammonium acetate (pH 4.25) and 0.25% acetonitrile, and 1ml fractions were collected and the radioactivity determined. Under these conditions retention times for markers, ring-opened 7-methylguanine (r-oMe^{7}G), 3-methyladenine, 7-methylguanine and O^6-methylguanine (following an increase in acetonitrile to 50% after 20 minutes) were 7.0, 10.5, 20.8 and 35 min, respectively. Profiles of release for the various cell extracts were compared with those of albumin and boiled cell extracts.

3.2.5 Characterisation of glioma cell lines.

3.2.5.1 Effect of temozolomide on cell population growth.

The sensitivity to alkylation of three cell lines (Gl 7, Gl 18, Gl 16) which are derived from a single human glioma tumour was determined by growth inhibition studies as described in section 3.2.1. Following seeding of the cells at 0.1 x 10^5 cells/ ml in 24 well plates, the cells were treated with a range of concentrations of drug and were counted, after incubation for 72 hr at 37 °C.

3.2.5.2 O^6-AGAT levels and the effect of O^6-AGAT depletion on temozolomide cytotoxicity.

Cell line O^{6}AGAT levels were determined as described in section 3.2.3 , and the effects of depletion of this enzyme on the sensitivity of the cell lines to temozolomide was examined following pretreatment for 16 hr with 0.5 mM O^6-methylguanine (section 3.2.2).

3.2.6 Effect of a divided dose schedule upon temozolomide cytotoxicity *in vitro.*

GM892A and Raji cells were seeded at 0.8 x10^5 cells/ml and treated with 5 split doses of temozolomide at 6 or 12 hr intervals. Each temozolomide dose was at 20% of the ID_{50} concentration, the drug having been dissolved in 20% DMSO to give a maximum concentration of DMSO in the medium of 0.5% after 5 treatments (treatment times on 6 hr schedule = 0, 6, 12, 24, 30 hr, and on 12 hr schedule = 0, 12, 24, 36, 48 hr). Equal concentrations of DMSO were given to control cells over 5 doses, and after 72 hr the cells were counted and the population growth compared with that of the temozolomide treated cells. The level of inhibition observed was compared with that for cells which were treated with a single initial dose of temozolomide at the ID_{50} concentration (10μM for GM892A, 180μM for Raji, in 20% DMSO) and four subsequent solvent only doses.

3.2.7 *In Vivo* Murine Tumour Studies.

3.2.7.1 Effect of temozolomide on tumour volume increase in established murine tumours.

Female BDF mice (6-8 week old) were transplanted with M5076 (murine reticulum sarcoma) tumour cells by intramuscular injection of 1 x 10^6 cells in the hind leg. MAC13 or MAC26 (murine colon adenocarcinoma) tumour fragments were implanted subcutaneously in NMRI mice. Tumours were allowed to establish until palpable (approx. 14 days) and mice were selected with five mice for each drug dose and each group possessing approximately the same average initial tumour volume. Mice were injected on a daily schedule *i.p* with 5 doses of temozolomide (0-80 mg/Kg) dissolved in 200 μl 20% DMSO, and tumour volumes were measured daily using callipers.

3.2.7.2 Tumour O^6-AGAT levels.

M5076, MAC13 and MAC26 tumour cells were implanted and solid tumours allowed to establish. Tumours were then excised and following homogenisation and sonication in ice-cold 10 mM Tris/HCl (pH 7.8) -1mM DTT -1mM EDTA -5% glycerol cell extracts were prepared and assayed for O^6-AGAT (section 3.2.2).

3.2.7.3 MAC13 tumour O^6-AGAT levels following temozolomide treatment.

MAC13 tumours were excised 24 hr after the final temozolomide dose (day 6) as treated in section 3.2.7.1.Tumour extracts were then prepared and assayed for O^6-AGAT (section 3.2.7.2) to determine whether treatment has an effect on MAC13 tumour alkyltransferase levels.

The results presented in this chapter represent the mean of at least 3 separate determinations (±SEM) unless otherwise stated. Differences have been analysed statistically using t-test.

3.3 Results.

3.3.1 *In Vitro* growth inhibition studies in Mer+ and Mer- cell lines.

Drug cytotoxicity has been determined in two cell lines of differing capabilities for repair of the O^6-alkylguanine lesion; Raji (Mer+) and GM892A (Mer-)(Harris *et al.*, 1983). From growth inhibition assays the concentration required to cause 50% inhibition of the population growth of the cell lines (ID_{50} value) have been determined for a range of compounds (Table 3.1).

Growth inhibition curves for the imidazotetrazinones (Fig. 3.1) indicate the order of potency for these agents to be mitozolomide> temozolomide> ethazolastone; the ethyl analogue possessing 23-fold less activity towards the alkyltransferase deficient GM892A cell line than temozolomide and 37.5 fold less than mitozolomide. The GM892A (Mer-) cell line was 8-and 23-fold more sensitive to mitozolomide and temozolomide, respectively, than the Raji alkyltransferase proficient cell line. In contrast, ethazolastone showed little differential between the two cell lines; GM892A being only 2.4- fold more sensitive than the Raji cell line. In addition, the inhibition studies show a shoulder type curve for the Mer+ cell line by comparison with the Mer- line, with the exception of the 3-ethyl analogue which exhibits this feature in both cell lines.

Similar differential effects to those found for temozolomide were seen for the triazene product of temozolomide ring-opening, MTIC, (20-fold differential activity) and to a lesser extent for the antimelanoma agent DTIC (9-fold), and for the nitrosourea agents, MNU (7-fold) and MNNG (10-fold), whilst MMS was only 3.8-fold more active towards the Mer- cell line.

Of a range of 3-substituted tetrazinones tested only the pyrazole analogue of temozolomide proved to be of impressive differential potency (51-fold).

Table 3.1. ID_{50} drug concentrations in GM892A (Mer-) and Raji (Mer+) cell lines.

Agent	GM892A ID_{50} concentration (μM ± sem)	Raji	Ratio of ID_{50}'s (Raji/GM892A)
Mitozolomide	4.8 ± 0.4	38.8 ± 6.25	8.1
Temozolomide	7.6 ± 1.5	175 ± 7.2	23.0
Ethazolastone	180 ± 12.1	435 ± 33	2.42
MTIC	4.9 ± 2.1	101 ± 15.7	20.6
DTIC	14.2 ± 6.1	128.1 ± 10.4	9.0
MNU	34.8 ± 9.3	259 ± 28	7.44
MNNG	0.2 ± 0.07	2.0 ± 0.4	10.0
MMS	43.5 ± 6.7	164.6 ± 29.2	3.78
Other 3-subtituted imidazotetrazinones: (3-substituent)			
(**1**) $-CH_2Ph$	>500[a]	360	<1
(**2**) $-CHPh_2$	70	>100[b]	<2
(**3**) $-CH_2C_6H_4.OCH_3$(P)	>500[c]	230	<0.5
(**4**) structure withheld	25	179.4 ± 21.5	9.0
(**5**) $-CH_2O(CH_2)_2Si(CH_3)_3$	85	65	0.7
(**6**) $-CH_2CO_2H$	860	270	0.31
(**7**) $-CH_2CO_2CH_2CH_3$	115	80	0.7
(**8**) $-CH_2(CO_2)_2CH_2CH(CH_3)_2$	150	65	0.43
(**9**) $-CH_2Cl$	20	40	2
3-substituted pyrazolotetrazinones: (3-substituent)			
(**10**) $-CH_3$	5.25 ± 0.75	270 ± 15	51.4
(**11**) $-CH_2Ph$	325 ± 28	405 ± 41	1.25

ID_{50} determination was prevented by the poor aqueous solubility of compounds **1-3**. (a)At 500μM % control population growth = 70%; (b) at 100 μM = 55%; and (c) at 500 μM = 60%. Values for compounds **1-9** are the average of 2 determinations. Other figures are the mean ± sem for at least 3 determinations.

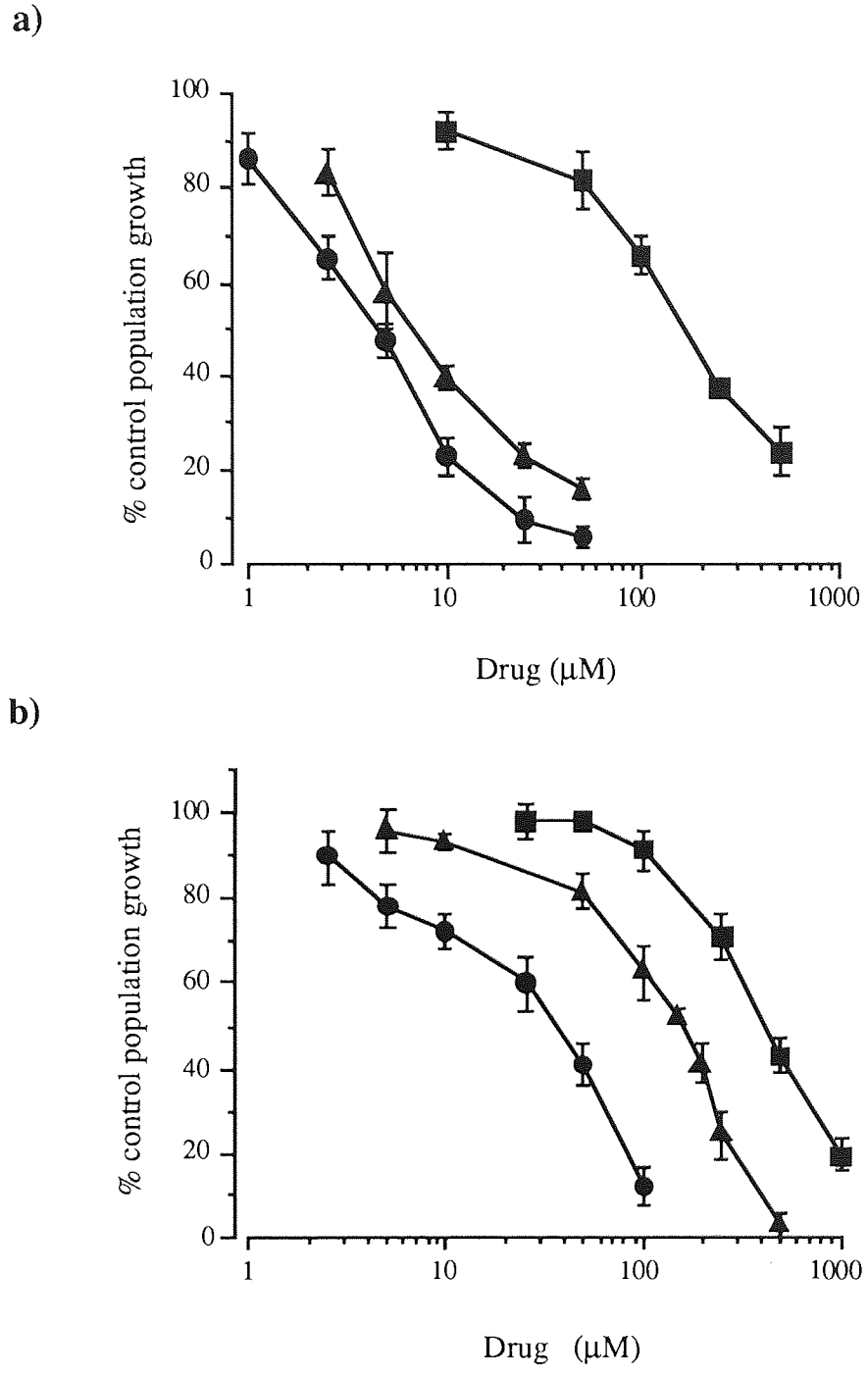


Fig 3.1. Effect of imidazotetrazinones on cell population growth.
(**a**) GM892A (Mer-) and (**b**) Raji (Mer+) cells were treated with various concentrations of mitozolomide (●), temozolomide (▲) or ethazolastone (■) and cell population growth was determined relative to untreated control cells. Values are the mean ± SEM for at least 3 determinations.

3.3.2 Effect of O^6-AGAT depletion on drug cytotoxicity.

The activity of O^6-AGAT in Raji cells has been shown to be reduced by the inclusion of the free base O^6MeG in the culture medium (Karran and Williams, 1985). The effect of overnight incubation with 0.5mM O^6MeG on the sensitivity of Raji and GM892A cells to temozolomide is shown in Fig 3.2. The toxicity of temozolomide was increased in O^6MeG pretreated Raji cells, an effect that correlated with a reduction in the O^6-alkylguanine repair capability of cell extracts (Table 3.2). In contrast, the alkyltransferase deficient GM892A cell line showed no sensitisation to temozolomide. Furthermore, Raji cells were insignificantly sensitised to MMS, an agent which produces few O^6-methylguanine lesions, indicating the effects of O^6MeG pretreatment to be specific to repair of the O^6-lesion.

Incubation with O^6MeG also appeared to sensitise Raji cells to EMS, although the toxicity of ethazolastone has been reported as not being enhanced following alkyltransferase depletion (Tisdale, 1987).

3.3.3 Sensitivity to temozolomide and cell line O^6-AGAT levels.

The O^6-AGAT actvity has been compared with the 3-methyladenine-DNA glycosylase acitivity for cell lines shown to be sensitive and resistant to temozolomide (Tisdale, 1987). The toxicity of temozolomide against a range of human and murine tumour cell lines is shown in table 3.3. The cell lines possessed wide variation in sensitivity to temozolomide and sensitivity correlated with the cellular level of the repair enzyme O^6-AGAT (Fig. 3.3). Thus cell lines of low O^6-AGAT repair capability such as GM892A showed greatest sensitivity, whilst cell lines with high alkyltransferase levels were resistant to temozolomide (Tisdale, 1987).

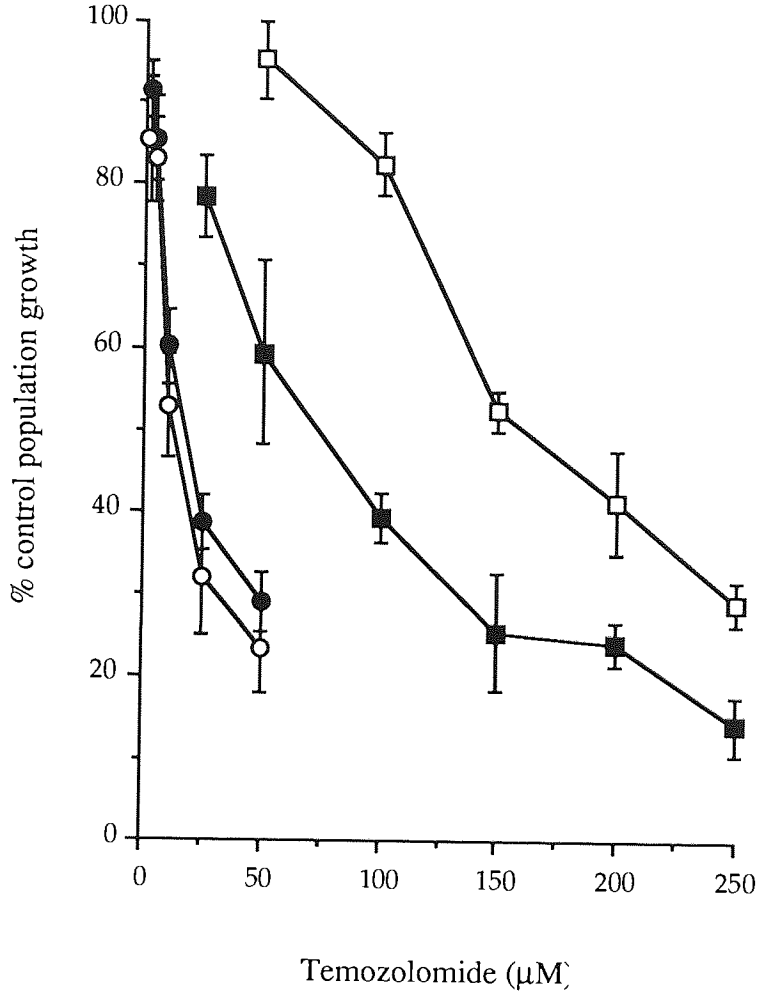


Fig. 3.2 Effect of alkyltransferase depletion on sensitivity to temozolomide *in vitro*. The growth inhibitory effects of temozolomide towards the Raji (□ ,■) and GM892A (○ , ●) cell lines were determined for control cells (open symbols) and following pretreatment of cells with 0.5 mM O^6-methylguanine for 16 hr (closed symbols). Values are the mean ± SEM for 3 separate determinations.

Table 3.2. Effect of alkyltransferase depletion on drug cytotoxicity.

	GM892A		Raji		
	A	B	A	B	% (B/A) Raji
			O^6-AGAT (fmol/mg protein)		
	22.8 ± 2.8	-	372.1 ± 67.7	35.9 ± 8.1	10.4 %
			ID_{50} μM (±SEM)		
Temozolomide	12.5 ± 2.8	17.4 ± 3.2	161.1 ± 7.2	73.6 ± 9.9	45.7 % (P<0.01)
MMS	43.5 ± 6.7	-	164.6 ± 16.9	132.3 ± 7.2	80.4 % (P>0.05)
EMS	-	-	1050 ± 89.9	450 ± 43	42.8 %*
(4)	25.0	-	179.4 ± 21.5	92.5 ± 10.5	50.1 %*

The alkyltransferase levels and sensitivity to various agents are compared for control solvent treated cells (A) and cells pretreated with 0.5 mM O^6-methylguanine for 16 hr (B). Values are the mean ± SEM for 3 experiments, or * the mean of 2 separate determinations. Differences were analysed statistically by t-test.

Table 3.3. Sensitivity to temozolomide, level of O^6-AGAT and release of alkylated bases by cell extracts.

Cell line	ID_{50} temozolomide (μM)	O^6-AGAT (fmol/mg protein)	pmoles [^{3}H]methyl released	
			r-oMe^7G + Me^7G	Me^3A
Albumin	-	-	17.2 ± 0.5	13.1 ± 0.1
Raji	175± 7.2	372± 67.7	17.7 ± 1.0	26.6 ± 0.4
Raji (boiled extract)	-	-	17.1 ± 1.4	13.2 ± 0.4
GM892A	7.6 ± 1.5	22.8± 2.8	18.1 ± 0.8	21.0 ± 0.7
A549	299± 30[a]	391 ± 60	18.0 ± 0.9	23.7 ± 0.5
MAC13	77± 10[b]	44 ± 4[b]	17.1 ± 0.5	22.9 ± 1.5
MAC16	245± 8[a]	320 ± 75[a]	19.1 ± 0.4	18.8 ± 0.4
K562	15± 5[a]	87 ± 40[a]	17.7 ± 0.3	21.3 ± 1.2

Results are expressed as the mean ± SEM for at least 3 separate determinations for individual values.

a. From Tisdale (1987)

b. From Hepburn and Tisdale (1991)

3-Methyladenine (Me^3A); 7-methylguanine (Me^7G); ring-opened 7-methylguanine (r-o^{7}MeG).

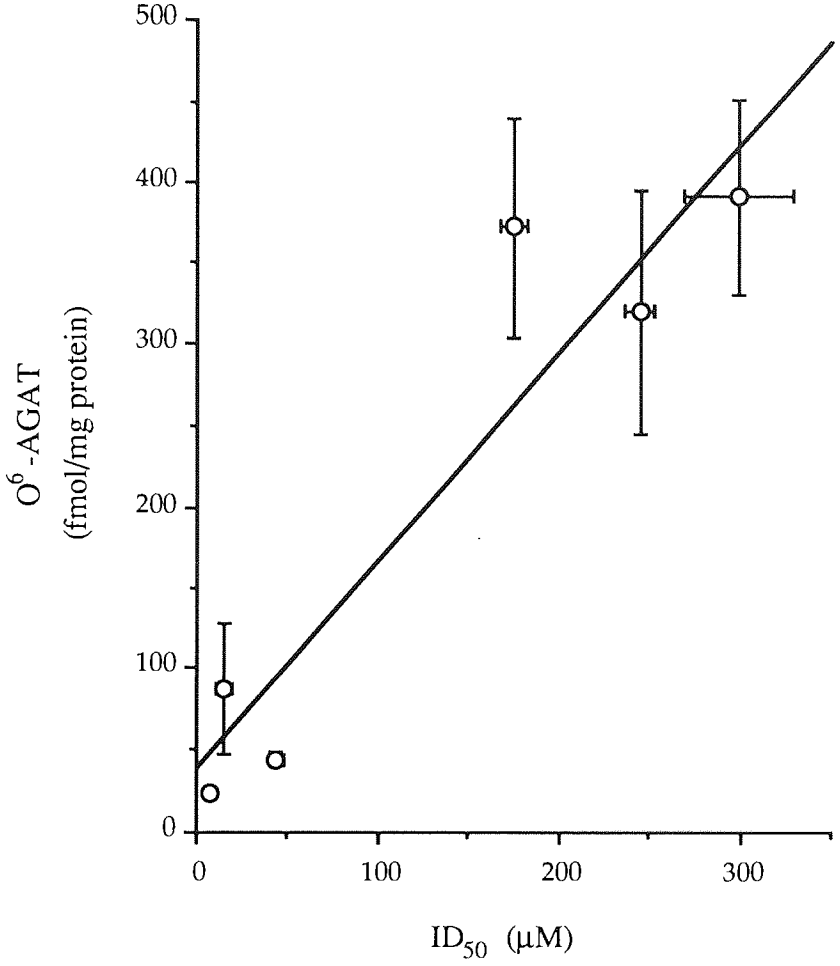


Fig. 3.3. Correlation of O^6-AGAT levels with the ID_{50} of temozolomide towards six cell lines. Values are the mean ± SEM for at least 3 separate determinations for Raji, GM892A, A549, MAC13, MAC16, and K562 cell lines. (Correlation coefficient 0.88)

3.3.4 3-Methyladenine-DNA glycosylase (3MAG) activity.

The ability of extracts from each of the cell lines to excise labelled bases from a [^{3}H]DMS-DNA substrate was determined. The protein-dependent release of radioactivity after subtraction of the counts released by boiled extracts is shown in Fig 3.4. Extracts from all the cell lines showed release in a protein-dependent manner and there appeared not to be any major differences in activity between the various cell extracts despite there being a 20-fold difference in the O^6-AGAT activity.

Since the [^{3}H]DMS-DNA substrate contained approximately 4-times as much 7-methylguanine as 3-methyladenine, evidence that the release of radioactivity was the result of 3MAG came from comparison of the distribution of bases released by extracts with that released spontaneously in the presence of albumin or inactivated extracts. The distribution of radioactivity released by Raji and GM892A cell extracts in comparison with those of control experiments is shown in Fig 3.5. In these HPLC profiles the first peak represents ring-opened 7-methylguanine, the second 3-methyladenine and the third 7-methylguanine. The profiles show a constant release of 7-methylguanine by controls and cell extracts and confirming that the variation of radioactivity released is attributable entirely to the 3-methyladenine peak. These results are quantitated in table 3.3.

Comparison of the radioactivity released by 700 μg of protein extract with the ID_{50} values for each cell line showed that there was no correlation between 3MAG and the cytotoxicity of temozolomide (Fig. 3.6). Thus, the Raji cell line with low sensitivity to temozolomide and high levels of O^6-AGAT showed only slightly higher 3MAG activity than the GM892A cell line which is highly sensitive to temozolomide and possesses only low levels of O^6-AGAT, and the MAC16 cell line with the highest resistance to temozolomide also appeared to have the lowest glycosylase activity.

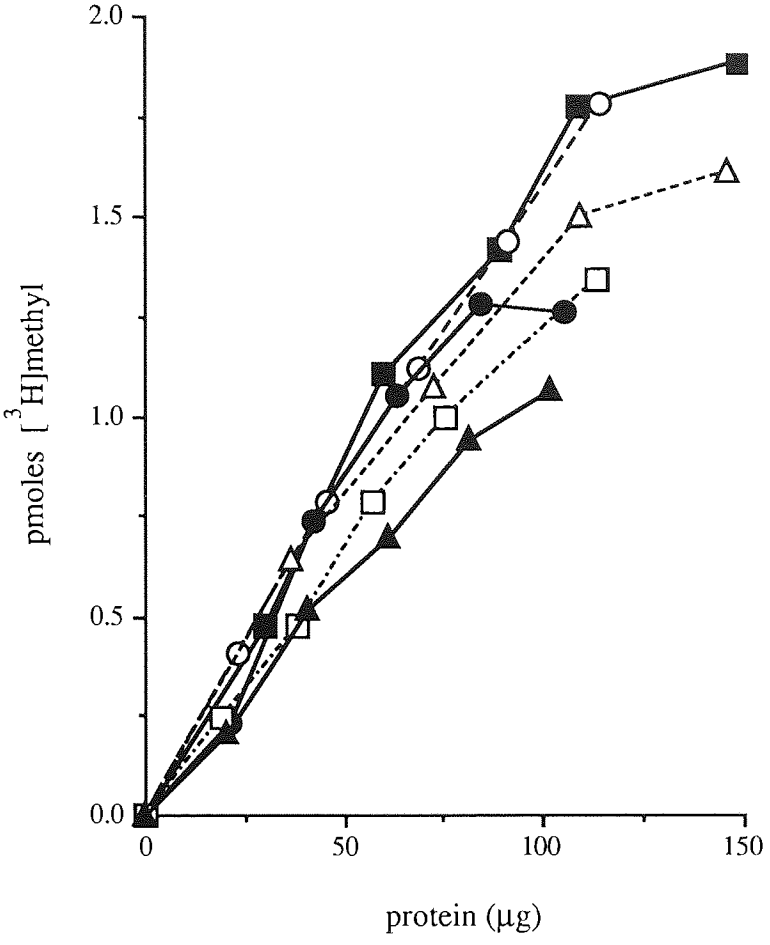


Fig. 3.4. Protein-dependent release of radioactivity from [^{3}H]DMS-DNA by cell extracts. Values are the mean for 3 separate determinations, for Raji (■), GM892A (△), K562 (□), MAC13 (○), MAC16 (▲) and A549 (●) cell lines. All standard errors (SEM) are less than 12 %.

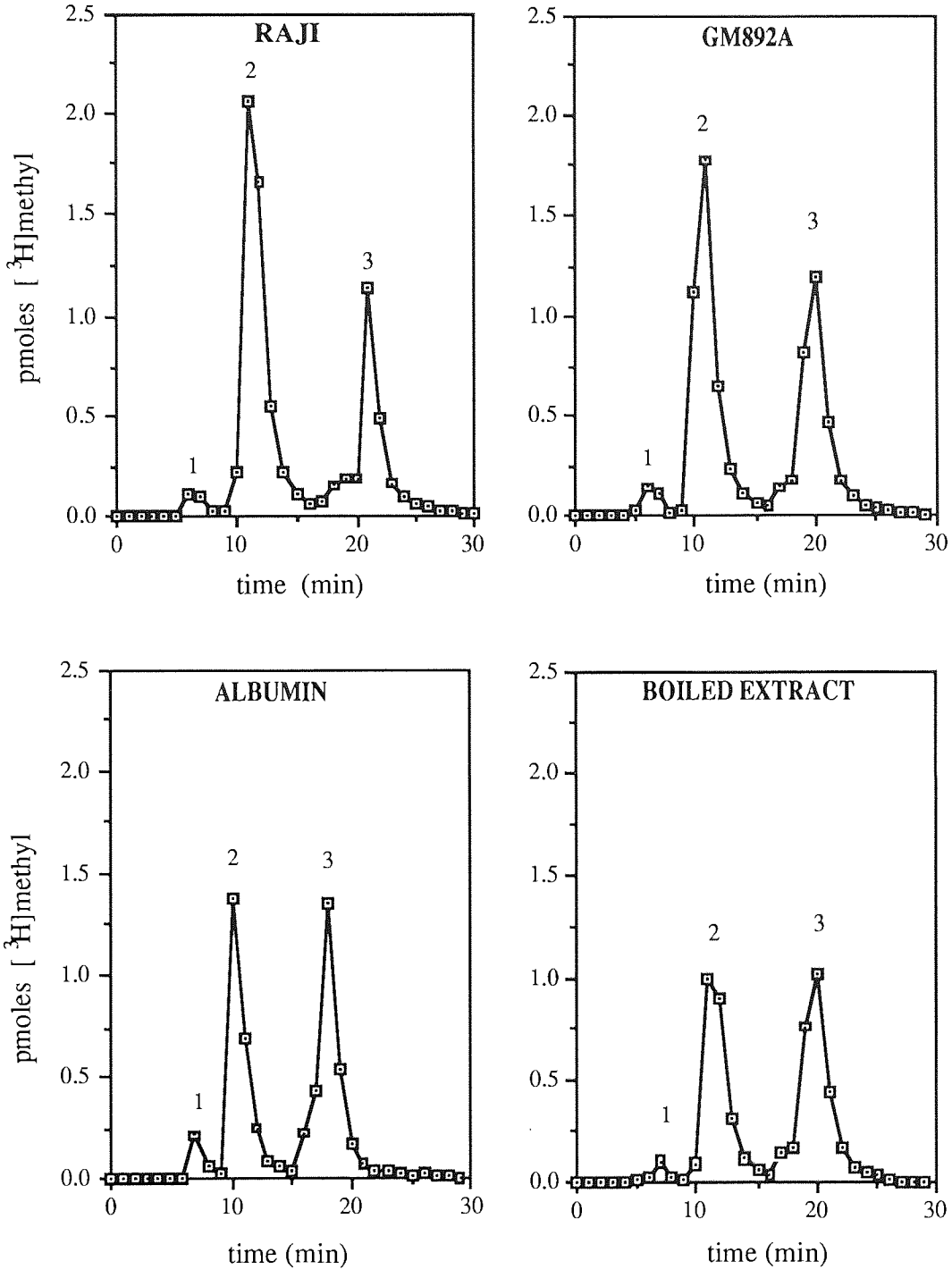


Fig.3.5. HPLC profiles of bases released from [^{3}H]DMS-DNA.
Peak 1, r-oMe^{7}G; peak 2, Me^{3}A; and peak 3, Me^7 G. The results refer to the total pmoles methyl groups released by 700 µg of cell extract after incubation with 60 µg [^{3}H]DMS-DNA for 30 min at 37°C.

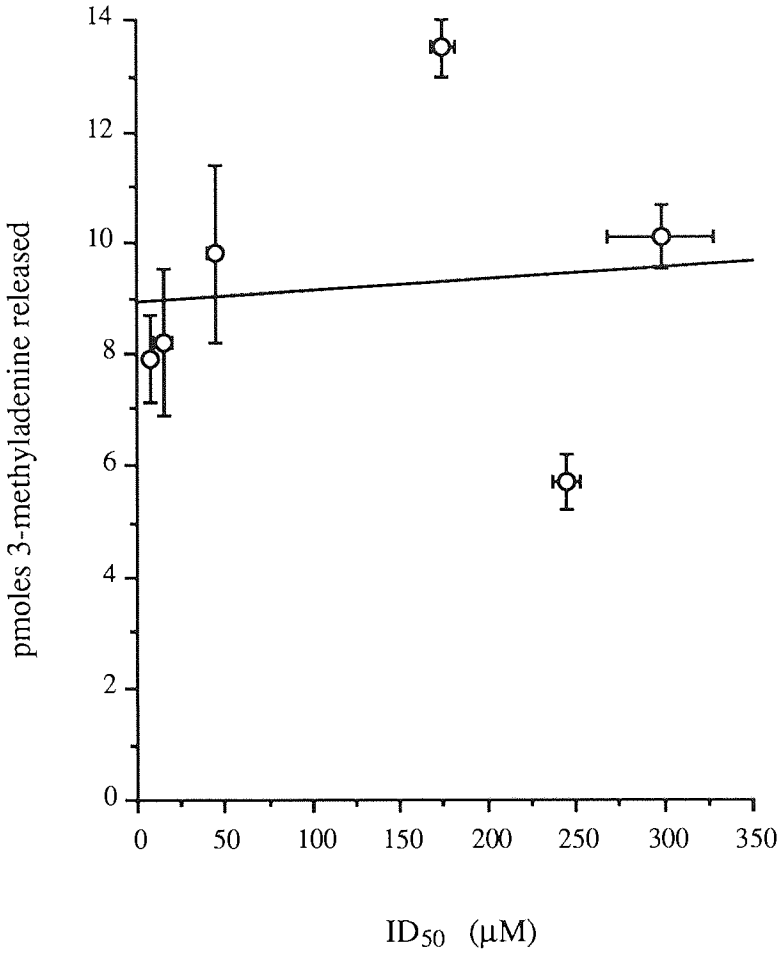


Fig 3.6. Comparison of the 3-methyladenine excision capability of cell lines with sensitivity to temozolomide. Values are the mean ± SEM for Raji, GM892A, MAC13, MAC16, A549 and K562 cell lines. (Correlation coefficient = 0.01).

3.3.5 Characterisation of glioma cell lines.

The O^6-AGAT repair enzyme levels and drug sensitivities of three cell lines isolated from a single glioma tumour are shown in table 3.4. The cell lines exhibited different repair capabilities, a feature which appears to reflect the heterogeneous nature of the cell population within a tumour, and whilst GL16 cells showed a high O^6-AGAT level, GL18 possessed medium, and GL7 only low levels of the alkyltransferase repair enzyme.

Temozolomide was differentially active towards the cell lines (Fig. 3.7); the ID_{50} values determined from these growth inhibition studies indicating the GL7 cell line to be 1.8-fold and 3.8-fold more sensitive than GL18 and GL16, respectively. Thus, resistance to temozolomide, and also to mitozolomide, MNNG and the clinically used anti-glioma agent BCNU (Table 3.4), was again correlated with O^6-AGAT repair capability (Fig. 3.8). In addition, depletion of O^6AGAT with free O^6MeG resulted in sensitisation to the cytotoxic effects of temozolomide of the GL18 and GL16 Mer+ cell lines, and also of the GL7 cell line, despite its already low O^6-methylguanine repair capability.

3.3.6 Effect of a divided dose schedule on temozolomide toxicity *in vitro*.

The responses of GM892A and Raji cell lines to treatment with ID_{50} concentrations of temozolomide in a single or divided divided dose are shown in Fig 3.9. Scheduling treatments over 6 or 12 hr made no improvement on the toxicity attained by a single dose, although while a similar activity was maintained in GM892A cells, dividing the dose in the Mer+ Raji cell line resulted in decreased efficacy in a 12 hr schedule.

Table 3.4. O^6-Alkylguanine-DNA alkyltransferase activity and sensitivity of glioma cell lines to various alkylating agents, and to temozolomide following alkyltransferase depletion.

Cell Line	O^6-AGAT fmol/mg protein (±SEM)	ID_{50} (μM ± SEM)				
		MNNG	BCNU	Mitozolomide	Temozolomide	Temozolomide [+ 0.5mM O^6MeG]
GL7	18.9 ± 11	0.58 ± 0.25	4.2 ± 1.3	7.0 ± 1.3	78.5 ± 16.3	42.5 ± 8.2
GL18	126.5 ± 67.8	2.5 ± 0.8	-	-	142.1 ± 16.1	85.0 ± 11.1
GL16	306.8 ± 38.5	4.25 ± 1.5	15.9 ± 3.7	47.5 ± 9.1	299.3 ± 40.5	94.5 ± 12.3
ratio GL7:18:16	1: 6.7: 16.2	1: 4.3: 7.3	1: -: 3.8	1: -: 6.8	1: 1.81: 3.8	1: 2: 2.2

Values are the mean ± SEM for at least 3 determinations.

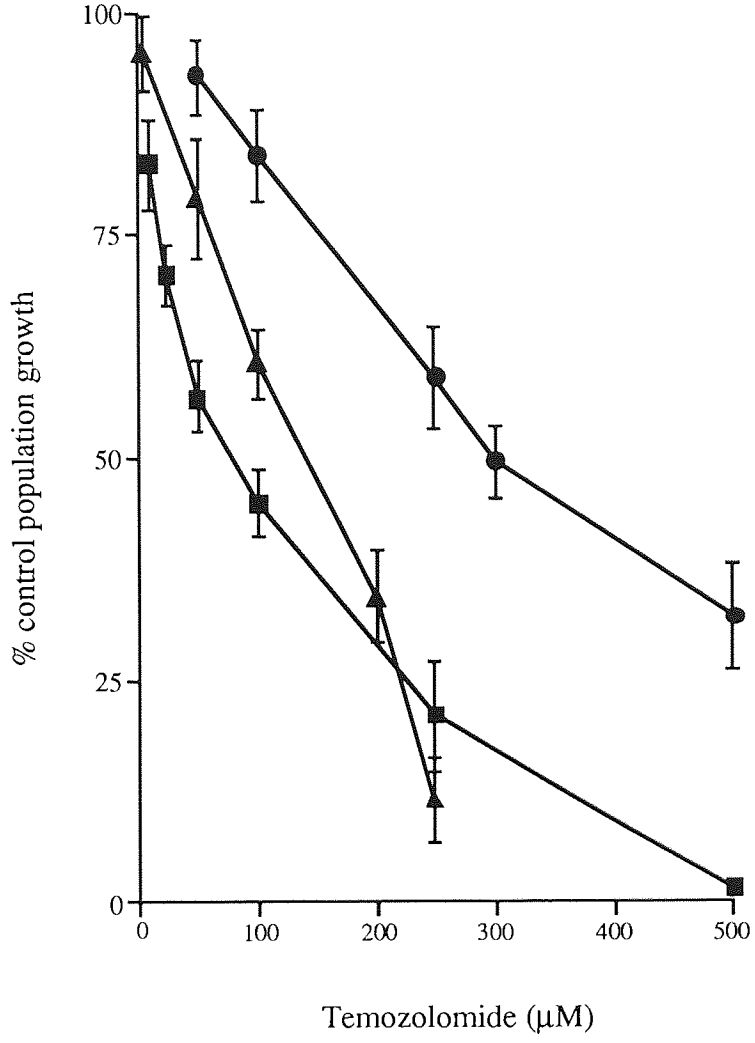


Fig. 3.7. Sensitivity of glioma cell lines to temozolomide.
The effect of temozolomide on GL 7 (■), GL 18 (▲), and GL 16 (●) cell population growth was determined relative to untreated cell populations. Values are the mean ± SEM for at least 3 separate determinations.

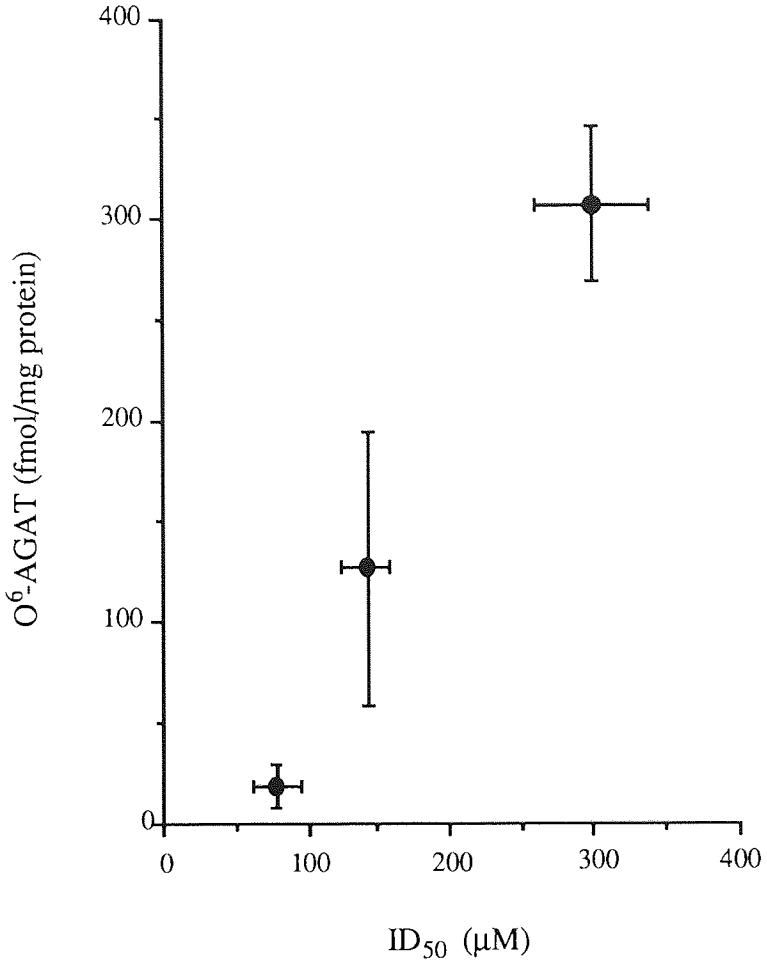


Fig 3.9. Correlation of O^6-AGAT with the ID_{50} of temozolomide towards glioma cell lines. Values are the mean ± SEM for 3 separate determinations for GL 7, GL 18 and GL 16 cell lines.

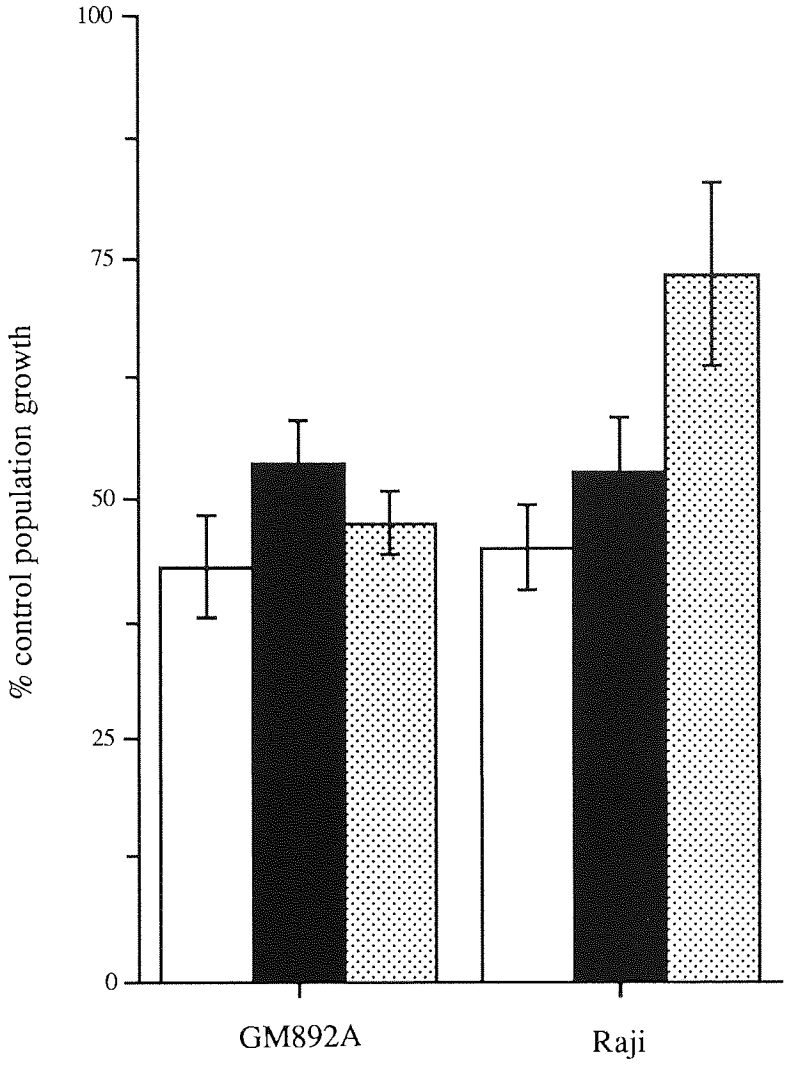


Fig. 3.9 Effect of a divided dose schedule on growth inhibition by temozolomide *in vitro*.
GM892A and Raji cells were exposed to ID_{50} concentrations of temozolomide (10 μM and 180 μM respectively) as a single initial dose (□), or divided over 5 treatments on a 6 hr schedule (■, treatment times = 0, 6, 12, 24, 30 hr) or a 12 hr schedule (⊡, treated at 0, 12, 24, 36, 48 hr). Population growth was determined relative to appropriate solvent dosed cells. Values are the mean ± SEM for 3 separate determinations.

3.3.7 *In Vivo* Murine Tumour Studies.

The effects of treatment with temozolomide on the increase in initial volume (tumour vol/ initial vol) of M5076, MAC13 and MAC26 established solid tumours are shown in figures 3.10, 3.11 and 3.12 and are quantitated as a % of control increase on day 6 and 8 after commencement of treatment in table 3.5. Whilst activity was seen against MAC13, and to a slightly lesser extent against MAC26 tumours (with an optimal dose of 50-60 mg/Kg temozolomide, and toxic side effects apparent at 80 mg/Kg), M5076 tumours responded only poorly to temozolomide, although experiments with this tumour did not proceed beyond 24 hours after the final treatment (Day 6).

The O^6-AGAT levels of extracts prepared from tumours are shown in figure 3.13. Despite MAC26 tumours possessing significantly higher levels of the methyltransferase (278 ± 7.1 fmol O^6-AGAT/mg protein ±SEM) than MAC13 tumours (10.7 ± 7.1 fmol/mg), only a minor differential response to temozolomide was seen for the tumours, and although the M5076 tumour had 4.5-fold lower O^6-AGAT levels (60.4 ± 4.3 fmol/mg protein), the MAC26 tumour was slightly more sensitive. Thus, although the alkyltransferase deficient MAC13 tumour showed the greatest response, the correlation between O^6-AGAT levels and the response of these solid murine tumours to temozolomide appears marginal.

It would appear, therefore, that other factors play a role in determining tumour sensitivity. Since, one potential mechanism for tumour resistance could involve induction of repair in response to DNA damage, the alkyltransferase repair capabilities have been determined following exposure of MAC13 tumours to temozolomide (Fig.3.14). At 3 of the 4 temozolomide concentrations used tumour O^6-AGAT levels appeared elevated in comparison with the tumours from solvent treated mice. As a trend alkyltransferase increased with temozolomide dose, with O^6-AGAT levels approximately 1.6-fold higher than controls at 24 hours after treatment with 5 daily doses of 30mg/Kg (n.s $P>0.05$) temozolomide, 2.3-fold (n.s $P>0.05$) following 40 mg/Kg and 2.7-fold after 60mg/Kg ($P<0.05$), however, after 50 mg /Kg O^6-AGAT levels were only 64% of control levels and as such may cast doubt on the significance of these results.

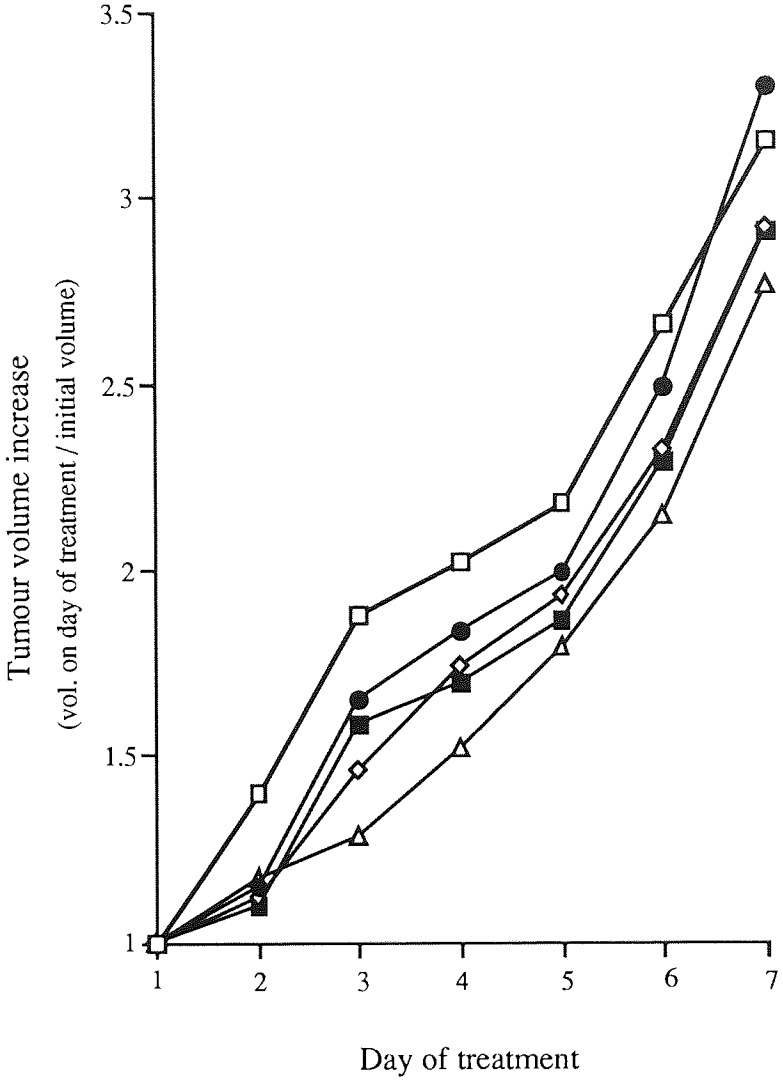


Fig. 3.10 Effect of temozolomide on M5076 murine tumour volume increase. Mice possessing established M5076 tumours were injected on a daily schedule *i.p* with 5 doses at 10 (●), 20 (△), 40 (◇), and 80 mg/Kg (■) t emozolomide and the increase in tumour volume monitored and compared to that for solvent treated control mice (□). Values are the mean for at least 3 tumours. Standard errors did not exceed 14 %.

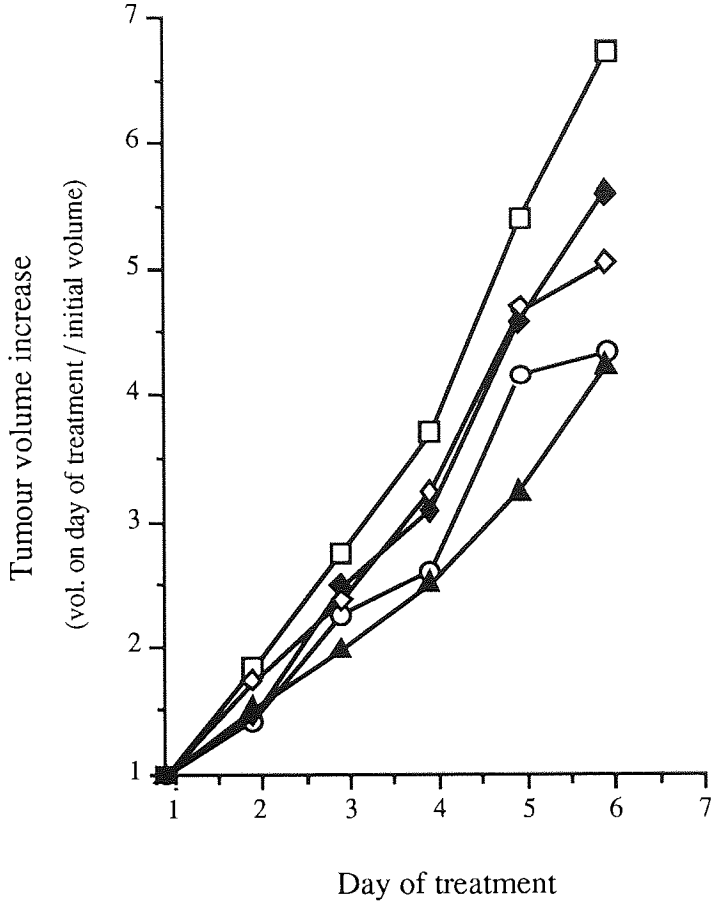


Fig. 3.11. Effect of temozolomide on MAC13 murine tumour volume increase. Mice possessing established MAC13 tumours were injected on a daily schedule (at days 1-5) *i.p* with doses of 30 (◆), 40 (◇), 50 (▲), and 60 mg/Kg (○) temozolomide. The increase in tumour volume was monitored and compared to that for the tumours of solvent treated control mice (□). Values are the mean for at least 3 tumours. Standard errors did not exceed 20 %.

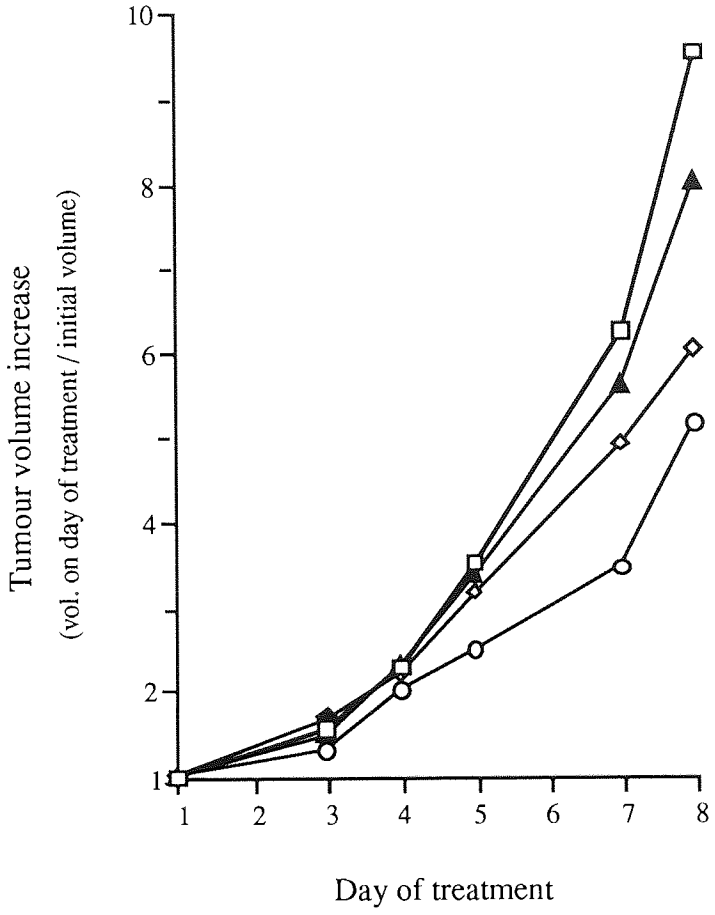


Fig. 3.12. Effect of temozolomide on MAC26 murine tumour volume increase. Mice possessing established MAC26 tumours were injected on a daily schedule (at days 1-5) *i.p* with doses of 40 (◇), 50 (▲), and 60 mg/Kg (○) temozolomide. The increase in tumour volume was monitored and compared to that for the tumours of solvent treated control mice (□). Values are the mean for at least 3 tumours. With the exception of control tumours on Day 8 (±SEM 28.1), standard errors did not exceed 20 %.

Table 3.5. Activity of temozolomide against established murine solid tumours.

		tumour volume increase (%T/C ± SEM)				
		(daily dose temozolomide mg/Kg)				
Tumour type	Day	0	40	50	60	80
M5076	6	100 ± 11.1*	92.1 ± 8.1*	-	-	95.5 ± 7.5*
MAC13	6	100 ± 16.1	69.3 ± 5.8*	62.9 ± 4.8	64.3 ± 6.8	70.2 ± 5.1
	8	100 ± 6.4	51.7 ± 6.9	-	-	54.3 ± 7.4
MAC26	6	100 ± 18.4	82.1 ± 10.4	90.3 ± 3.9*	70.8 ± 10.3*	-
	8	100 ± 28.1	63.0 ± 10.9	70.3 ± 13.6*	53.4 ± 5.0*	-

Values are the mean ± SEM for at least 3 tumours in an experiment or *mean of 2 experiments.

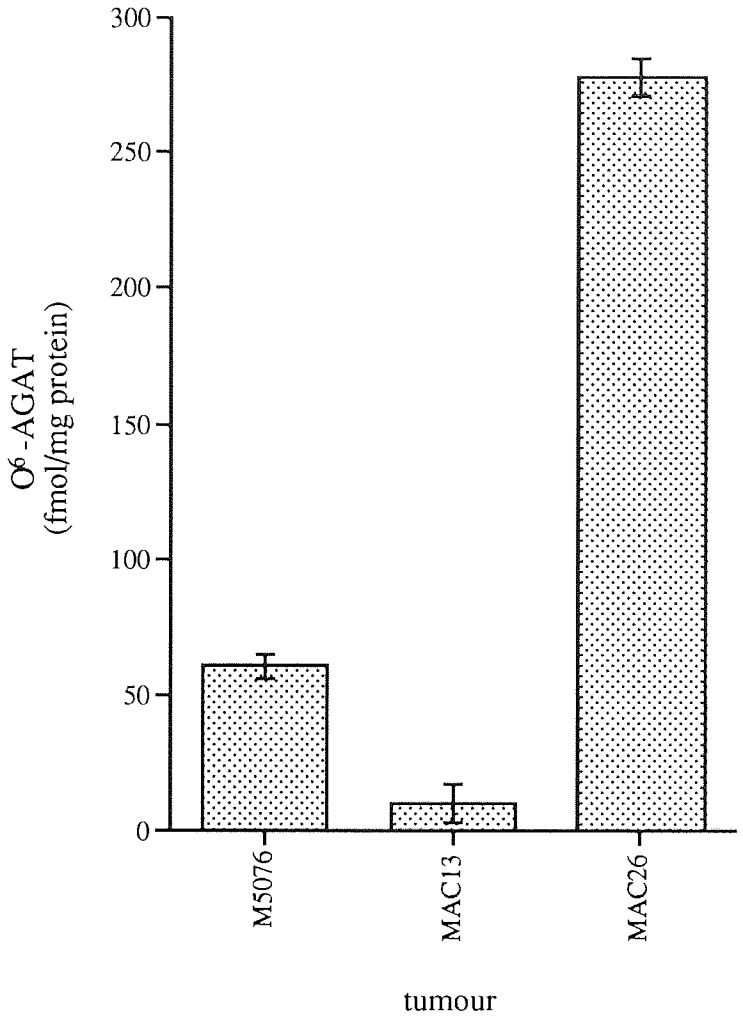


Fig. 3.13. Murine tumour O^6-AGAT levels.
Values are the mean ± SEM for at least 3 separate solid tumours.

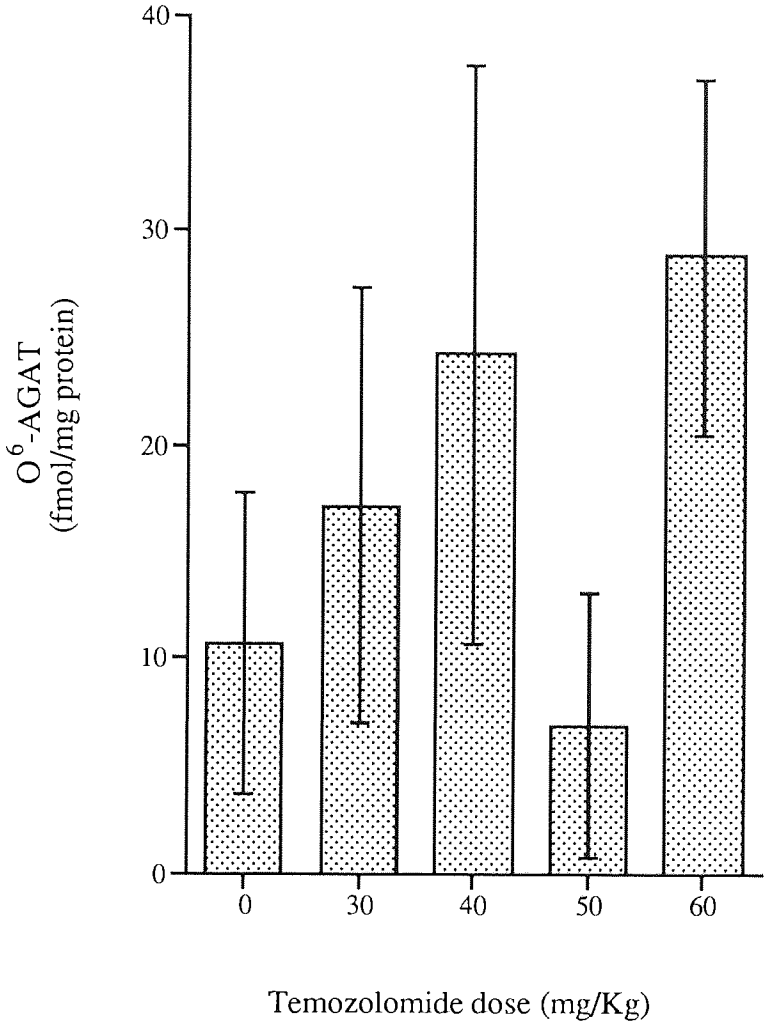


Fig. 3.14. Effect of temozolomide on MAC13 tumour O^6-AGAT levels. Mice with established MAC13 solid tumours were treated on a daily schedule with 5 doses of temozolomide (0-60 mg/Kg). 24 Hr after the final drug dose (Day 6) tumours were excised and the tumour O^6-AGAT levels determined. Values are the mean ± SEM for 5 tumours.

3.4 Discussion.

The imidazotetrazinones are a novel group of antitumour agents that have proved active against a range of murine tumours and human tumour xenografts, and more importantly within the clinic (Stevens *et al.*, 1987, O'Reilly *et al.*, 1993). They act as prodrugs for acyclic triazenes, formed under aqueous conditions, and it is these unstable triazenes which are thought to actively alkylate DNA bases. Considerable experimental data has implicated alkylation at the O^6-position of guanine in the cytotoxicity of imidazotetrazinones, nitrosoureas and triazenes: however, a mechanism by which the lesion might lead to cytotoxicity remains unproven and some authors have suggested that defects other than lack of O^6-AGAT are responsible for the sensitivity of Mer- cell lines to killing by alkylating agents (Karran and Williams, 1985). The aim of this project is to further elucidate the mode of action of the imidazotetrazinones.

The sensitivity to a range of agents has been determined for two cell lines differing in their ability to repair the O^6-position of guanine: The GM892A cell line has been shown to be deficient in O^6-AGAT, and the Raji cell line has been shown to be proficient, possessing approximately 20-fold higher repair capability (Table 3.3). The results are in agreement with previous studies (Hickman *et al.*,1985, Tisdale,1987), which have observed strict structural requirements for the 3-alkyl group of imidazotetrazinone compounds in order to exert activity. Active substituents are limited to a chloroethyl group or a methyl group, the 3-ethyl substituent losing selective cytotoxicity. In the present study, a 3-trimethylsilylmethyl-substituted imidazotetrazinone (**4**) appeared to possess differential activity, as did the pyrazole analogue of temozolomide, although surprisingly, in contrast with temozolomide (Stevens, 1987),this agent has been reported as inactive in *in vivo* screening against the NCI P388 and L1210 leukaemia (Cheng *et al.*,1986) despite being regarded a prodrug for the pyrazole analogue for MTIC. In addition, other 3-substituted imidazotetrazinones have been tested and shown not to display selective toxicity towards the alkyltransferase deficient cell line, although the DNA reactivity of these agents has not been determined.

In this study, repair of the O^6-position appears important to the cytotoxicity of both temozolomide and mitozolomide, and also the nitrosoureas MNU and MNNG and

the triazene product of temozolomide ring-opening MTIC, since, as found by previous studies (Tisdale, 1987, Gibson *et al.*, 1986, Scuderio *et al.*, 1984a & b), these agents also displayed selective toxicity towards an alkyltransferase deficient cell line,

In contrast with these alkylating agents methylmethanesulfonate (MMS) showed only minor selectivity towards the alkyltransferase deficient line. MMS produces primarily N-alkylated bases, with O^6-methylguanine as only a minor product (0.25% of total DNA alkylation products), further implicating O^6-methylguanine residues as deleterious DNA lesions (Table 1.4, Beranek *et al.*, 1980).

By comparison, the difference in cell line ID_{50} concentrations for ethazolastone was less marked, suggesting that alkyltransferase repair is not important to the cytotoxicity of this agent. This is corroborated by the shapes of growth inhibition curves where the role of O^6-AGAT in the cytotoxic effects of temozolomide and mitozolomide, but not ethazolastone, is reflected by the shoulders on curves which are considered to imply the necessity for depletion of a repair enzyme before maximal toxicity can be elicited.

It should be noted, however, that in these studies the toxicity displayed by DTIC was unexpected, since the triazene DTIC requires metabolism to the active alkylating form MTIC, and therefore may be expected to show little differential activity in cell lines (Tsang *et al.*,1991). In addition, the differential activity of MMS appears higher than might be anticipated for an agent that produces such a minor amount of O^6-methylguanine. These observations suggest that in addition to lack of the O^6-AGAT repair enzyme other factors may contribute, albeit to a minor extent, towards the sensitivity of the GM892A cell line. Uptake of the imidazotetrazinones in these lymphoid lines has been shown to be rapid and consistent with passive diffusion, and is insufficient to explain the observed differential toxicities of each drug (Bull, 1989), although it may contribute to the effects of other agents. Minor discrepancies must, however, be expected for non-isogenic cell lines and do not detract from the correlation between these agents, alkylation at the O^6-position and alkyltransferase repair capability.

Further support for the importance of O^6-alkylation of guanine in the cytotoxicity of these agents has come from depletion of O^6-AGAT using the free base O^6MeG. Previous studies have shown a marked increase in the sensitivity of Mer+ cell lines to cell

killing by CNU after pretreatment with O^6MeG, which is probably a weak substrate for the enzyme (Dolan *et al.*, 1985, Yarosh *et al.*, 1986). However, treatment of one Mer+ cell line, Raji, did not sensitise to MNNG or CNU, which led to the suggestion that adducts at the O^6-atom of guanine in DNA are not potentially cytotoxic lesions (Karran and Williams, 1985). Tisdale (1987) has shown increased sensitisation of a range of Mer+ cell lines to the cytotoxic effects of temozolomide and mitozolomide but not ethazolastone, although again the Raji cell line showed insignificant sensitisation to these agents, which it was suggested was due to induction of O^6-AGAT following exposure to the alkylating agents. In the present study, O^6MeG pretreated Raji cells were sensitised to the growth inhibitory effects of temozolomide, and increased cytotoxicity correlated with a decrease in measured cellular methyltransferase levels, despite the cell line having been previously reported as atypical in response. GM892A (Mer-) cells, however, remained unaffected by such O^6MeG pretreatment, whilst in addition sensitisation of Raji cells to MMS toxicity was only minor (and not significant $p>0.05$). This sensitisation to temozolomide and mitozolomide, but not ethazolastone or MMS, would appear to implicate the O^6-position of guanine as a target for the cytotoxic action of the 3-methyl and 3-chloroethyl imidazotetrazinone, but question the role of this site in the cytotoxicity of the 3-ethyl analogue. Some studies have implied nucleotide excision repair (NER) to contribute to the protection of human cells from the cytotoxic effects of N-ethyl-N-nitrosourea (ENU).Transfection of plasmid expressing O^6-AGAT into Mer- CHO cells and NER deficient CHO UV41 cells did not alter sensitivity of the cell lines to ENU, whilst UV41 cells were 2-to 3-fold more sensitive than wild-type CHO cells, suggesting that ethylation at the O^6-position has no role in cytotoxicity (Wu *et al.*, 1992). In other studies, a xeroderma pigmentosum (group A) cell line with a deficiency in nucleotide excision repair but normal expression of O^6-AGAT, a cell line with normal excision repair capacity but lacking in O^6-AGAT, and a cell line efficient in both types of repair responded differently to ENU indicating the lack of either repair mechanism to impair the ability of cells to withstand ethylation damage. Furthermore, the inhibition of O^6-AGAT, using O^6-benzylguanine, increased ENU toxicity in the NER+Mer+ cell line but not the xeroderma pigmentosum cells, suggesting that NER and O^6-AGAT co-operate in the

removal of DNA ethyl adducts (Bronstein *et al.*, 1992a & b). It has, however, been argued that the correlation between sensitivity and a deficiency in NER is weak, and that other consequences of the lack of NER may explain the poor ability to repair O^6-ethylguanine demonstrated (Goldmacher, 1992).

In consideration of the suggestion that defects other than lack of O^6-AGAT are responsible for the sensitivity of Mer- cell lines, methylphosphotriesters and O^4-methylthymine appear to be excluded from consideration as targets for lethality as protein fractions containing O^6-AGAT activity have little capacity to repair these lesions and so it is unlikely that O^6-AGAT depletion by O^6MeG would alter repair of these sites (Yarosh, 1985). Little evidence has been put forward to suggest a lethal role for the major DNA alkylation product 7-alkylguanine and other than this and the O^6-lesion the only product produced in significant quantities by temozolomide is 3-methyladenine (Bull, 1988). It can be more clearly understood why 3-methyladenine might be a potentially cytotoxic lesion, as blocks to DNA synthesis, introduced into DNA by MNNG, and used as an *in vitro* template for primed synthesis by the polymerase 1 Klenow fragment, occurred most frequently at the position of adenine residues, showing no particular tendency for O^6-methylguanine to cause chain termination (Larson 1985).

In view of the suggestion (Matejasevic et al.,1991) that glioma cell lines deficient in O^6-AGAT were also deficient in 3-methyladenine-DNA-glycosylase (3MAG) the level of 3MAG has been determined in a range of cell lines previously characterised for O^6-AGAT concentrations, and which were shown to correlate with the cytotoxicity of temozolomide (Tisdale, 1987). The results confirm cytotoxicity to be correlated to repair of the O^6-methylguanine lesion, whilst indicating 7-methylguanine to be negligibly repaired. The 3MAG activities did not vary between the cell lines and the levels do not correlate with the level of O^6-AGAT or with sensitivity towards temozolomide, despite a 40-fold variation in the latter. This suggests that although 3-methyladenine is a major product of temozolomide alkylation of DNA, repair enzyme activity is sufficient in most cells to prevent it from being an important cytotoxic lesion.

Recently, experiments have been performed in which clones of the N-methylpurine-DNA-glycosylase repair gene have been introduced into cells in attempts to

clarify further the role of this enzyme. Transfection of a clone of the human gene was shown to instigate up to 16-fold enhancement in levels of enzyme activity, however, cell lines did not develop increased resistance to the cytotoxic effects MMS or DMS, and at high doses of these agents cells over-expressing the enzyme even showed increased sensitivity (Ibeanu *et al.*, 1992). Thus, it would appear that the rate-limiting factor in the multi-step repair pathway of 3-methyladenine in CHO cells is not N-methylpurine-DNA-glycosylase activity but repair of apurinic sites, which appear cytotoxic. However, these results cannot be extrapolated to all mammalian cells, since expression of a rat gene encoding 3MAG in *Irs1* cells (mutants of the V79-4 Chinese hamster cell line) induced a 3-fold increase in resistance to MMS (Habraken and Laval, 1993). It seems that the initial levels of 3MAG in the *irs1* mutant are too low to remove all the lethal lesions from the cellular DNA, a feature that may prove salient in other cells and may offer a minor role for the lesion in cytotoxicity. However, 3MAG-negative cell lines have yet to be reported, suggesting any potential role to be only minor by comparison with O^6-AGAT repair.

In view of the success shown by temozolomide in the clinical treatment of gliomas, alkyltransferase studies have been repeated for 3 cell lines isolated from a single glioma tumour. The comparison of such cell lines is of particular interest since previous studies were for a variety of cell types of both murine and human origin, for which care must be taken when interpreting results. In agreement with the former studies the glioma cell lines displayed a strong correlation between O^6-AGAT levels and the cytotoxicity of temozolomide, as well as mitozolomide and the nitrosoureas MNNG and BCNU, while alkyltransferase depletion, using O^6-methylguanine, also potentiated the cytotoxicity of temozolomide. By contrast, the differential activity between these cell lines was less dramatic than that found between GM892A and Raji cell lines despite a similar difference in O^6-AGAT levels. This may further imply other factors to contribute to the sensitivity of the GM892A cell line, although the glioma cell line with the lowest O^6-AGAT would appear to possess higher levels of alkyltransferase than the GM892A cell line since this cell line was slightly sensitised by O^6MeG pretreatment. Despite these variations in potency, a good overall correlation between alkyltransferase and glioma cell line sensitivity has been demonstrated.

The maximum plasma concentration attained in patients receiving temozolomide

on a repeated dose schedule is approximately 50 μM (Newlands *et al.*, 1992), which is similar to the ID_{50} value shown for the glioma cell line with low levels of alkyltransferase. Studies in mice have shown that temozolomide, like mitozolomide, has good tissue distribution including penetration into tumour tissue and across the blood-brain barrier (Brindley *et al.*, 1986, O'Reilly 1993) , and in view of temozolomides clinical activity it is of particular interest that Mineura *et al.* (1991) have shown that tumours from 6 out of 17 glioma patients had low levels of O^6-AGAT (<100 fmol/mg protein with 4/17 <60 fmol/mg protein) whilst non-glial tumours appeared significantly higher. Unfortunately, in addition this study may illustrate limitations for the success of temozolomide treatment in that if tumours show similar heterogeneous cell populations, resistant tumours may re-establish following destruction of the alkyltransferase deficient cell population.

Having established a strong *in vitro* correlation between alkyltransferase and the toxicity of temozolomide, further studies have been performed in murine tumours in an attempt to corroborate these results *in vivo*. The murine reticulum sarcoma tumour M5076 and murine colon adenocarcinoma tumours (MAC tumours) were selected as solid tumour models. The M5076 sarcoma is a chemosensitive tumour with a long doubling time, a property that is a characteristic of most human solid tumours and which may be considered desirable within a tumour model (Langdon *et al.*, 1984). The tumour has also been shown to respond well to temozolomide when treated from the day of implantation of tumour cells. Mice treated with 17 daily doses of 10mg/Kg temozolomide develop tumours only 8% of the volume of controls with no measurable tumours forming at higher concentrations (Stevens *et al.*, 1987). Whilst, the series of murine adenocarcinomas of the colon (MAC tumours) have been shown to be similar in sensitivity to solid cancers in man where responses to standard agents are normally only seen close to the maximum tolerated dose (Double and Ball, 1975).

In contrast with previous murine tumour studies (Stevens *et al.*, 1987), solid tumours were allowed to establish before commencement of temozolomide treatment, in order to more closely parallel the situation on clinical presentation. Despite possessing only a low alkyltransferase repair capability, established M5076 tumours responded only poorly to temozolomide, whilst the MAC13 tumour which had even lower alkyltransferase levels showed an improved response, although treatment of the tumour

failed to reflect the tumour regression observed in patients. In these experiments tumour volume inhibition has been used as a measure of tumour cell kill, however, far from being a measure of viable tumour cell number, dying and necrotic tissue awaiting removal may contribute to the final volume of the tumour. Consequently, the effects of drug treatment may take some time to be manifested as tumour volume reduction and may partly explain a lack of dramatic response in these rapidly growing tumour models. Comparison of the inhibition of MAC13 tumour volume with that for a repair proficient MAC26 tumour, displaying a similar tumour volume doubling rate, revealed a correlation between alkyltransferase levels and response to temozolomide, although the difference in sensitivity was less than anticipated. Consequently, the proposal that induction of the alkyltransferase enzyme following DNA damage may result in improved resistance of tumour cells has been investigated in MAC13 tumours. Tumours were found to display up to a 2.7-fold increase in O^6-AGAT levels 24 hr after the final temozolomide treatment, however, overall results were far from conclusive. Previous reports of alkyltransferase induction have been mainly confined to rat hepatoma cells (Frosina and Laval, 1987)(Laval, 1991), although a human hepatoma and human glioblastoma cell line have shown 2-to 3-fold enhancement following DNA damage (Lefebvre *et al.*, 1993), as more dramatically have Chinese hamster V79 lung fibrobast cells surviving selection with low doses of mitozolomide and chlorambucil (Morten and Margison, 1988).

If genuine, the observed increases in O^6-AGAT may contribute to resistance, although it is uncertain whether such nominal increases in repair enzyme level would be of great significance. However, an actual elevation in O^6-AGAT expression may be greater than the increases detected since depletion of cellular O^6-AGAT levels due to repair of temozolomide alkylated DNA must be anticipated (Tisdale, 1987). Alternatively, the observed induction may be biased by the survival and selection for cells with higher alkyltransferase levels after temozolomide treatment. Consequently, these results remain difficult to interpret, and the lack of a major differential response between the MAC13 and MAC26 tumours, along with the lack of response of the M5076 tumour, may imply alkyltransferase not to be the sole determinant of *in vivo* response.

Discrepancies with *in vitro* studies are often attributable to the role of pharmacokinetic behaviour and bioavailability of the drug in determining tumour responses *in vivo*. Temozolomide, however, has good tissue distribution and penetration

into tumours (O'Reilly *et al.*, 1993), although poor tumour vascularisation may account for the low sensitivity of tumours, since M5076 tumours respond well if treated from implantation (Stevens *et al.*, 1987). In addition, a good differential response to nitrosoureas has been shown for both MAC13 and MAC26 tumours treated 2 days after implantation (Lunn *et al.*, 1989)(Bibby *et al.*, 1988). In these studies, the low resistance of the MAC26 tumour has been attributed to its high vascularisation.

Temozolomide has exhibited marked schedule dependency in both *in vivo* murine studies (Stevens *et al.*, 1987), as well as clinical studies where the drug has shown little activity when given as a single dose (Newlands *et al.*, 1992). In investigation of a possible basis for this feature of the agents activity, the GM892A and Raji cell lines were treated with temozolomide in divided doses and growth inhibition compared with that for a single treatment. No improvement was found on a single dose although activity was maintained in the alkyltransferase deficient GM892A cell line, by contrast with the loss of toxicity towards the alkyltransferase proficient cell line on division of the dose over a 12 hr schedule. This implies that multiple dosing may be beneficial in terms of maximising tumour selectivity through reduced toxic effects to normal alkyltransferase proficient cells. Such an effect could be attributed to a necessity for prior alkyltransferase depletion to elicit maximum toxicity of temozolomide, as suggested by the recent results of Baer *et al.* (1993). In these studies, whilst multiple doses of temozolomide, by itself, were less toxic than a single dose, after treatment of MCF-7 breast carcinoma and MAWI colorectal cell lines with O^6-benzylguanine, an inhibitor of O^6-AGAT (Dolan *et al.*, 1991), a 24 hr repeat dosing schedule showed dramatic potentiation of temozolomide toxicity when compared with single doses (Baer *et al.*1993).

Thus, it appears that O^6-benzylguanine alkyltransferase-depletion permits a build up of DNA damage, and significantly, such depletion has been shown to enhance the anti-tumour effects of BCNU in a human glioma tumour xenograft (Dolan *et al.*, 1990), a human colon cancer xenograft (Gerson *et al.*, 1993), and Dunning R3327G rat prostate tumours (Dolan *et al.*, 1993). Similarly, alkyltransferase inhibitors may have the potential to increase the anti-tumour activity of temozolomide, although potentiation of cytotoxicity may also occur in normal cells, and high toxicity has been reported for the combination of O^6-methylguanine and BCNU in L1210/BCNU bearing mice (D'Incalci *et al.*, 1991).

The results presented in this chapter strongly suggest that the primary cytotoxic lesion produced by both temozolomide and mitozolomide is alkylation at the O^6-position of guanine. The ineffectiveness of ethazolastone could be explained by one or more factors other than the lack of toxicity of the O^6-ethylguanine lesion: (a) the ethyl-analogue has a comparatively low overall reaction with DNA (Bull, 1988); (b) the production of a different spectrum of DNA lesions to temozolomide and mitozolomide, most notably, the production of ethylphosphotriesters as the major DNA alkylation product; or (c) the involvement of repair enzymes other than alkyltransferase in removing ethyl-lesions. With mitozolomide, once a crosslink has formed after initial alkylation at the O^6-position, it would appear no longer susceptible to alkyltransferase repair, but possibly subject to nucleotide excision repair (Wu *et al.*, 1992). This would account for the fact that whilst mitozolomide is more potent the difference in toxicity between GM892A (Mer-) and Raji (Mer+) cells is less with mitozolomide than temozolomide.

Chapter Four

Imidazotetrazinone DNA Binding and Sequence Selectivity

4.1 Introduction.

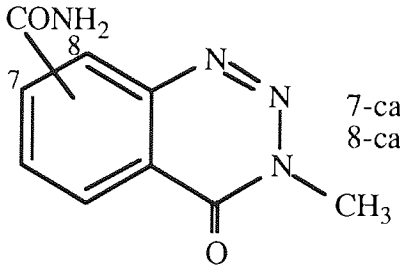
It is clear that many antitumour agents interact with DNA and that the damage caused is an important initial step in cancer chemotherapy. However, while there has been much investigation of the lesions formed by various agents, the importance of the location of DNA damage to this process is unknown.

Most alkylation reactions show a degree of sequence selectivity, and subtle changes in alkylation patterns in DNA between, and within, the common groups of alkylating agents has indicated an important role for the non-alkylating or 'carrier' moiety of the agent (Beranek *et al.*, 1980, Bull, 1988). Consequently, it has been suggested that this moiety is involved, in principle, in an initial recognition step by which noncovalent binding to specific preferred sequences present in the DNA structure may confer sequence specificity to the covalent modification of DNA.

The imidazotetrazinone antitumour agents posses a carboxamide substituent at the C^8-atom, and structure-activity studies of mitozolomide derivatives with N-monosubstituted carboxamides at this position have shown several to retain good antitumour activity. Aromatic and bulky substituents were in general adverse to activity, while an N,N-dimethylcarboxamide derivative required metabolic demethylation to become active, and a variety of esters were inactive (Lunt *et al.*, 1987, Clark, 1991). Since chemical hydrolysis studies have shown that changes in the rate of ring-opening to the active triazene species under aqueous conditions could not account for the 8-substituents affect on cytotoxicity (Clark, 1991), it appears that the presence of a hydrogen-bond donor at this position may aid sequence recognition crucial to antitumour activity. This postulated role for the 8-carbamoyl group in sequence recognition has been supported by molecular modelling studies (Clark *et al.*, 1990, Lowe *et al.*, 1992). However, corroboration with experimental data has proved difficult to achieve due to the inherent instability of the tetrazinone ring system towards nucleophiles.

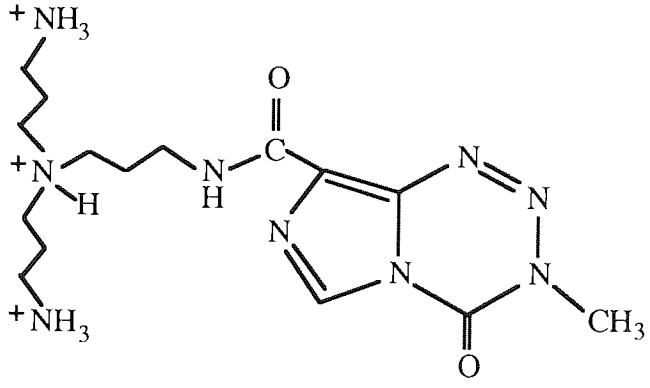
In the present study, various methods have been utilised to probe the interaction between imidazotetrazinones and DNA. DNA footprinting techniques have been used in attempts to investigate whether the imidazotetrazinones have preferred DNA binding sites, while the DNA nucleotide sequence specificity of covalent modification by imidazotetrazinones has been examined using both sequencing and polymerase stop

assays. In addition, it was considered that the synthesis of 3-substituted-1,2,3-benzotriazin-4(3H)-ones (**12,13**) and pyrazolo[4,3-d]-1,2,3-triazin-4(3H)-ones (**14**)(Fig.4.1)(Clark, 1991) would aid these studies by giving compounds which could mimic the noncovalent binding to DNA of tetrazinones, whilst due to their lack of the bridgehead N atom it was anticipated that they would not undergo ring-opening or alkylate DNA.

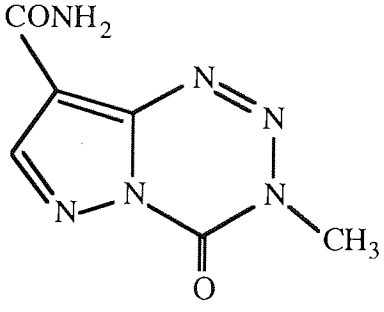


7-carbomyl benzotriazinone (**12**)
8-carbomyl benzotriazinone (**13**)

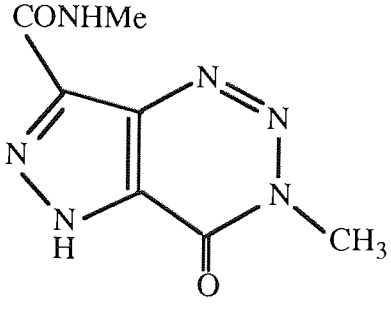
Benzotriazinones



Spermidine - temozolomide conjugate (**15**)



3-Methylpyrazolotetrazinone (**10**)



Pyrazolotriazinone (**14**)

Fig. 4.1. Structure of compounds used in DNA binding and specificity studies.

4.2 Methods.

4.2.1 Benzotriazinone and temozolomide binding competition studies.

The ID_{50}'s for the series of benzotriazinones (**12,13**) and a pyrazolotriazinone (**14**) were determined in Raji and GM892A cell lines from growth inhibition studies as described in section 3.2.1.

In investigation of an ability for these compounds to inhibit the biological activity of temozolomide, GM892A cells were seeded at 0.8 x 10^5 cells/ml in T25 flasks and treated with a range of concentrations of a benzotriazinone or pyrazolotriazinone. After incubation for 2 hours at 37°C the cells were centrifuged, washed with PBS, resuspended in media and then aliquoted into Nunc 24 well plates before being challenged with temozolomide. Cells were incubated for 3-4 days at 37°C. The ability of temozolomide to inhibit growth of cells pretreated with the inactive benzotriazinones or pyrazolotriazinone was compared with the inhibition elicited by the drug alone in solvent treated controls.

4.2.2 DNA footprinting.

4.2.2.1 *Tyr*T DNA fragment isolation and labelling.

The plasmid pKMΔ-98, a derivative of plasmid pBR322, containing the tyrosine tRNA promoter, its flanking sequences and the ampicillin resistance gene, ß-lactamase (Lamond and Travers, 1983), was isolated from a culture of ampicillin selected *E.coli* . Colonies were grown in 500 ml L.B containing 50 μg/ml ampicillin to O.D. 0.6 before the addition of 170 μg/ml chloramphenicol. The culture was incubated for a further 12 hr and the cells were harvested by centrifugation for 10min at 4,000 rpm. The bacteria were washed with STE buffer (0.1M NaCl-10mM Tris/HCl-1mM EDTA, pH 8.0), re-centrifuged and then re-supended by vortexing in 50 mM glucose-10mM EDTA-25mM Tris/HCl (pH 8.0). The cells were lysed by the addition of lysozyme (2mg/ml) to the suspension which was then vortexed and stored at room temperature for 5 min before the addition of 2 vol of fresh 0.2N NaOH-1% SDS. The tube was inverted 3 times and chilled on ice for 10 min and 0.25 vol of sol I (5M potassium acetate/glacial acetic acid/H_2O - 120:23:57) was added and after vortexing and storage on ice for 10 min the cell debris was removed by centrifugation for 10 min at 12,000 rpm. The supernatant

was decanted through glass-wool and then vortexed with phenol/chloroform/*iso*amylalcohol (25:24:1) and the aqueous layer was re-extracted with chloroform/*iso*amylalcohol (24:1). Plasmid DNA was precipitated by the addition of 0.6 vol *iso*propanol and was washed with 70% ethanol. Following air drying, the DNA was dissolved in TE (10mM Tris-Cl-1mM EDTA, pH 8.0) /10μg/ml pancreatic RNAase (DNAase free).

The 150 base pair *tyr*T DNA fragment was labelled following cutting of plasmid DNA by *Eco*RI and AvaI restriction enzymes by incubation with α-[^{32}P]-dATP (3000 Ci/mmole), dTTP and reverse transcriptase. The radioactive fragment, singly end-labelled at the EcoRI site, was isolated following ethanol precipitation by electrophoresis on a 6% non-denaturing polyacrylamide gel.

4.2.2.2 DNAase 1 footprinting.

2-3 μl of the labelled DNA (20-30 CPM), in 10mM EDTA-10mM Tris buffer, (pH 8.0) were incubated with various concentrations of drug (dissolved in Tris-sodium buffer-DMSO<1%), and 0.3 units/ml DNAaseI enzyme in a final volume of 10μl. The incubation was carried out at 4°C or 37°C and stopped after either 1 or 5 min by the addition of 3μl of DNAaseI stop buffer and freezing on dry ice. The fragment products of digests were visualised following electrophoresis and autoradiography (section 4.2.2.5).

4.2.2.3 DMS footprinting (Maxam-Gilbert method).

Dimethylsulphate (1μl) was added to 10μl of *tyr*T DNA in 200 μl Tris-sodium buffer in the presence of various concentrations of drug. The reaction was stopped by the addition of 50μl of G-stop buffer (1.5M sodium acetate-1.0M mercaptoethanol, pH7.0) after incubation on ice for 4 minutes. Drug was omitted from the reaction in order to produce a guanine marker track. The DNA was then ethanol-precipitated, dried and 50μl of 10% piperidine was added. Following boiling at 95°C for 15 min to produce breaks specifically at sites of N^7-guanine alkylation, the products of fragment cleavage were lyophilised and dissolved in DNAaseI stop buffer prior to gel electrophoresis.

4.2.2.4 Diethyl pyrocarbonate (DEPC) footprinting.

It was hoped the ethylating agent DEPC might offer improved sensitivity on the DMS methylation protection technique in the detection of small molecules, given the bulkier substituents of this agent. As a variation on the technique, footprinting was performed replacing DMS in method 4.2.2.3 with 5µl DEPC.

4.2.2.5 Gel electrophoresis.

The products of *tyrT* cleavage or digestion were heated at 100°C for 4 minutes prior to analysis on 0.3mm 8% (w/v) polyacrylamide gels containing 7M urea and Tris-borate EDTA buffer (pH 8.3). After 2 hr at 1500V the gel was soaked in 10% acetic acid for 10 minutes, transferred to Whatman 3MM paper, dried under vacuum at 80°C and subjected to autoradiography at -70°C with an intensifying screen. . Bands in the pattern of radioactive fragments thus visualised were assigned by using guanine markers from a dimethylsulphate-piperidine digest.

4.2.3 DNA Sequence Selectivity.

4.2.3.1 Determination of Guanine-N^7 Alkylation Sites in Defined DNA Sequences.

The DNA sequence specificities of guanine-N^7 alkylation produced by temozolomide, its pyrazolotetrazinone analogue, ethazolastone and a spermidine-temozolomide conjugate were examined using a modification of the Maxam and Gilbert chemical cleavage technique for DNA sequencing. The basic method was as described by Hartley *et al.*(1986).

4.2.3.1a DNA fragment isolation and labelling.

pBR322 plasmid DNA (20µg) was digested by incubation with BamHI restriction enzyme for 1 hr at 37°C. Following ethanol precipitation, centrifugation (10 min., 13,500 rpm) and lyophilisation, the 5′ ends of the linearised plasmid were dephosphorylated by incubation (1hr, 65°C) with bovine alkaline phosphatase. Protein was extracted from the DNA by brief vortexing of the incubation mixture with phenol / chloroform / isoamylalcohol (25:24:1) and any phenol contaminating the aqueous

fraction was removed using chloroform / isoamylalcohol (24:1). The DNA was again ethanol precipitated and lyophilised and then ^{32}P end-labelled using T4-polynucleotide kinase and [γ-^{32}P] ATP (5000 Ci/mmol). After incubation for 40 min at 37°C, the reaction was stopped by the addition of 0.1 vol ammonium acetate (7.5 M) and 3 vol ethanol and the labelled DNA was precipitated on dry ice. The supernatant was removed and the DNA precipitate washed by the addition of 20 µl of sodium acetate (0.3 M) and 60 µl ethanol followed by re-centrifugation after 10 min on dry ice, and then lyophilisation. Labelled linear pBR322 DNA was cut with a second restriction enzyme, SalI, releasing a 276 base-pair fragment and following precipitation and drying of the DNA, the restriction products were re-suspended in loading buffer and separated by electrophoresis on 0.8% agarose gel (70 V, 2 hr). Labelled fragments were located on the gel by autoradiography (30 min), excised from the agar and then electroeluted. The 276 base-pair Bam HI-SalI fragment of pBR322 5′-labelled at the BamHI site was ethanol precipitated, lyophilised, re-dissolved in 200 µl of water, phenol / chloroform extracted and finally re-suspended in TEOA buffer.

A 346 base pair HindIII-BamHI fragment of pBR322 DNA 5′-labelled at the HindII restriction site was prepared by the same method following initial cutting by HindIII.

4.2.3.1b Fragment alkylation and piperidine cleavage.

The DNA fragment (5000 cpm per sample) was alkylated in TEOA buffer at 37°C for 2 hr using a range of concentrations of drug in a final reaction volume of 50 µl (drugs were dissolved in DMSO to give a concentration of 2% DMSO in the final reaction mixture). The reaction was stopped by the addition of an equal vol of ice cold stop buffer (0.6 M sodium acetate, 100mM tRNA, 20 mM EDTA) followed by 300 µl ethanol. DNA was precipitated on dry ice (10 min), centrifuged and lyophilised, and then washed by re-precipitation. The alkylated DNA was treated with 100 µl of a fresh solution of 10% piperidine at 90°C for 15 min to produce strand-breaks specifically at the sites of N^7-guanine alkylation. Samples were lyophilised, re-dissolved in water, frozen and re-lyophilised and this step repeated once. The DNA fragments were then dissolved in 3 µl of formamide dye solution, heated at 90°C for 2 min and separated

using electrophoresis on 0.4 mm x 90 cm x 20 cm 6% acrylamide 7M urea (section 2.4.3) denaturing polyacrylamide gels with a tris-boric acid-EDTA (TBE) buffer system. Gels were pre-run at 3000 V for 30 min, samples run in to the gel at 1000 V for 5 min, and then run for 3 hrs at approximately 3000 V, maintaining a constant temperature of 55-65°C. Following gel drying, bands were visualised by autoradiography and relative band intensities determined by microdensitometry.

4.2.3.2 Measurement of the Sequence Specificity using *Taq* DNA Polymerase stop assay.

The sequence specificity of covalent DNA modification by temozolomide and ethazolastone has been examined using a polymerase stop assay as originally described by Ponti *et al.* 1991.

pBR322 DNA was double digested with Bam HI and SalI restriction enzymes and purified by phenol/chloroform extraction and ethanol precipitation. DNA (0.5 μg per sample) was alkylated with drug in TEOA buffer at 37°C for 1 hr in a final volume of 50 μl and ethanol precipitated twice before lyophilisation. The 276 base-pair BamHI-SalI fragment was then used as a template for extension of a 5′[α-^{32}P]-end labelled 20 base oligonucleotide primer. The polymerase chain reaction was carried out in a total volume of 100 μl containing 0.5 μg of alkylated or control DNA, 0.25 ng labelled primer, 10 μl 10 x buffer (670mM Tris pH 8.4, 20mM $MgCl_2$), 250 μM of dNTP and 1U *Taq* polymerase. The samples were mixed and overlaid with 2 drops of mineral oil and then incubated in a thermal cycler. A linear amplification procedure, permissible due to the thermostability of *Thermus aquaticus* DNA polymerase, was carried out for 30 cycles, each consisting of 1 min denaturation at 95°C, 2 min annealing at 60°C and 2 min chain elongation at 72°C. Following completion of the cycles, samples were cooled on ice, extracted with chloroform/*iso*amylalcohol (24:1), and precipitated with ethanol and dried. DNA fragments were separated by denaturing polyacrylamide gel electrophoresis and visualised by autoradiography.

4.3 Results.

4.3.1 Benzotriazinone and temozolomide binding competition studies.

The 1,2,3-triazinones were synthesised (Clark, 1991) in the hope that they would display similar non-covalent bonding properties to the tetrazinones yet have increased stability, due to the lack of a bridgehead N atom, and thereby be unable to act as alkylating agents. Indeed, *in vitro* cytotoxicity assays for the 7- and 8-carbamoyl 3-methyl-substituted benzotriazinones (**12,13**) and the 3-methyl-substituted pyrazolotriazinone (**14**) against the GM892A (Mer-) and Raji (Mer+) cell lines clearly indicated a lack of any antitumour activity for these compounds in stark contrast to the differential toxicity displayed by temozolomide (Table 4.1).

Table 4.1: Comparison of the *in vitro* cytotoxicities of 3-methyl-1,2,3-benzotriazinones a 3-methyl-pyrazolo-1,2,3-triazinone and the 3-methyl-imidazotetrazinone (temozolomide).

Compound	ID_{50} (μM) (± SEM) GM892A	Raji
Temozolomide	7.1 ± 2.1	160 ± 3.9
7-Carbamoyl benzotriazinone (**12**)	a	541 ± 52
8-Carbamoyl benzotriazinone (**13**)	980 ± 59	562 ± 32
Pyrazolotriazinone (**14**)	b	c

a,b,c.Due to the insolubility of these compounds insufficient concentratrations for ID_{50} determination were attained, however at 500μM (a) 35% ; (b) 31.1% and ; (c) 37.5% inhibition of growth was seen. Values are the mean ± SEM for 3 determinations.

Competition assays were performed to assess the ability of these stable compounds to inhibit the cytotoxic action of temozolomide, the results for which are presented in Fig 4.2. Pre-incubation of the GM892A cell line with the 7- and 8-substituted benzotriazinones (**12,13**) over a range of concentrations did not appear to

inhibit the cytotoxic properties of temozolomide suggesting that these compounds do not compete with temozolomide for binding sites in DNA. However, in a preliminary experiment cells exposed to 3-methylpyrazolotriazinone (**14**) (500μM) appeared less susceptible than untreated cells to the growth inhibitory effects of temozolomide, with the triazinone treated GM892A cell line showing 93% control growth compared with 70% for untreated cells at a 5μM concentration of temozolomide, while 10μM temozolomide reduced growth to 42% in the absence and but only 64.5% in the presence of the pyrazolotriazinone (**14**). Unfortunately, insufficient 3-methylpyrazolotriazinone was available for further studies and as a single experiment this result must be viewed with caution, especially when the vagaries of tissue culture are considered. However, if accurate based on this evidence there may well exist an imidazotetrazinone specific binding region which clearly warrants further study.

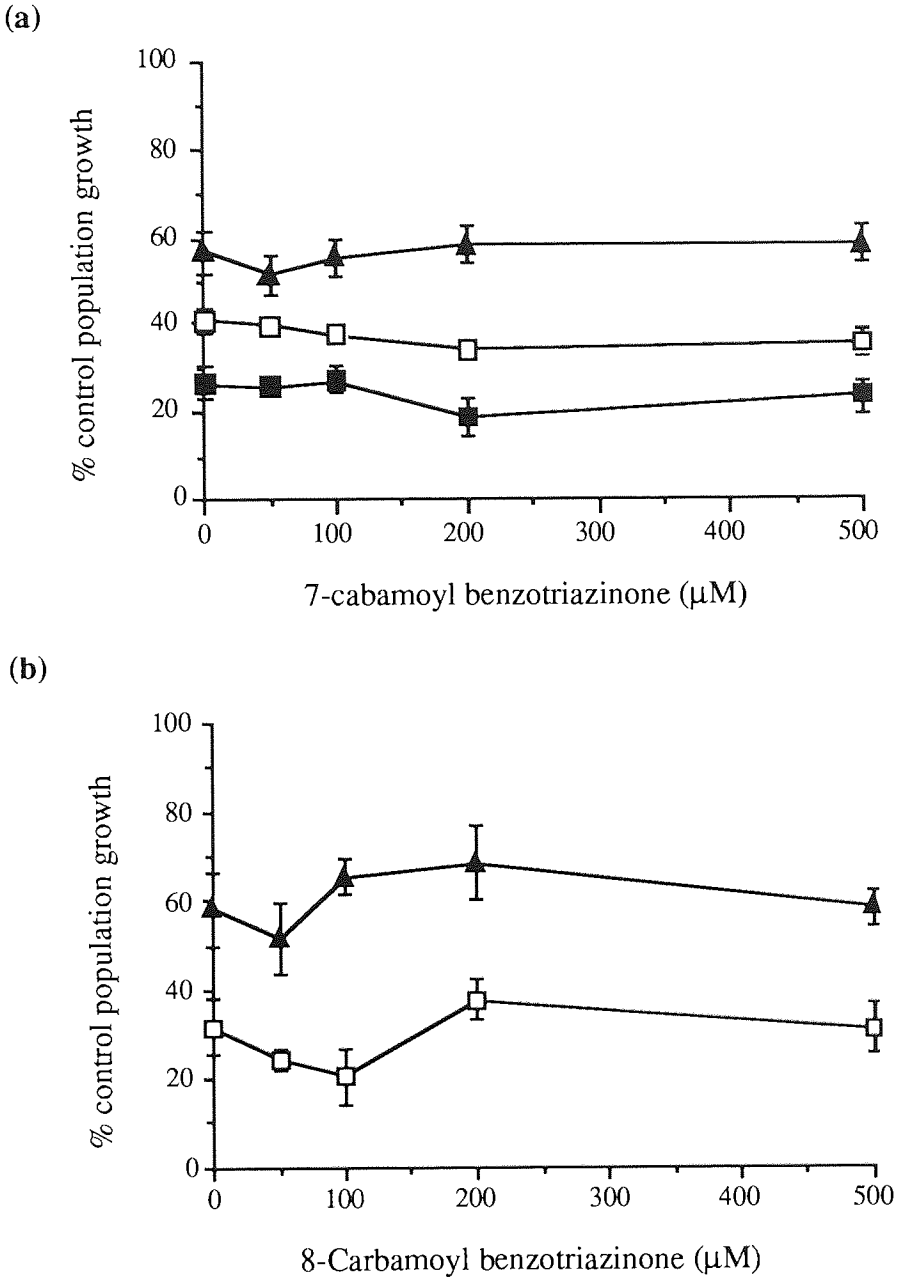


Fig. 4.2. Effect of pretreatment of GM892A cells with benzotriazinones on temozolomide cytotoxicity. GM892A cells were exposed for 2 hr to a range of concentrations of (**a**) 7-carbomyl benzotriazinone (**12**) and (**b**) 8-carbomyl benzotriazinone (**13**). Following resuspension in fresh medium, cells were treated with 5μM(▲), 10 μM(□) or 20 μM(■) temozolomide and the effect on cell population growth was examined relative to appropriate untreated control populations. Values are the mean ± SEM for 3 determinations.

4.3.2 DNA Footprinting Studies.

Various DNA footprinting techniques have been used in attempts to determine potential binding sites for temozolomide. A homogeneous sample of double-stranded labelled DNA was digested by DNAaseI in the presence and absence of drugs, and the fragments were visualised by autoradiography following electrophoresis (Fig.4.3). DNA-binding sites for the drug echinomycin were detected as distinct gaps near positions 35, 55, 75 and 100 corresponding to sections where the DNA remained uncut by DNAase1 due to protection by the drug (Appendix A). However, there were no observable differences from solvent controls for DNA digested in the presence of temozolomide or MTIC at 37°C or at the lower temperature of 4°C, which it had been considered may promote more stable DNA binding (Fig. 4.4).

Further footprinting studies have been performed using a modification of the Maxam and Gilbert sequencing technique to examine the ability of drugs to protect DNA from alkylation. Temozolomide did not appear to protect guanine residues from DMS methylation nor MTIC, DTIC or temozolomide from DEPC ethylation (Fig.4.5), however, these methods of footprinting may be complicated by the alkylating activity of the agents under study themselves. Indeed, by omitting DMS from the incubation the preferential sites of guanine alkylation have been investigated in section 4.3.3.

It was hoped that the benzotriazinones compounds (**12,13**) might show more stable DNA binding, however, these agents too showed no impedance of DNAaseI mediated DNA cleavage and it would seem that if an imidazotetrazinone specific receptor site exists these methods appear unable to aid clarification of such interactions.

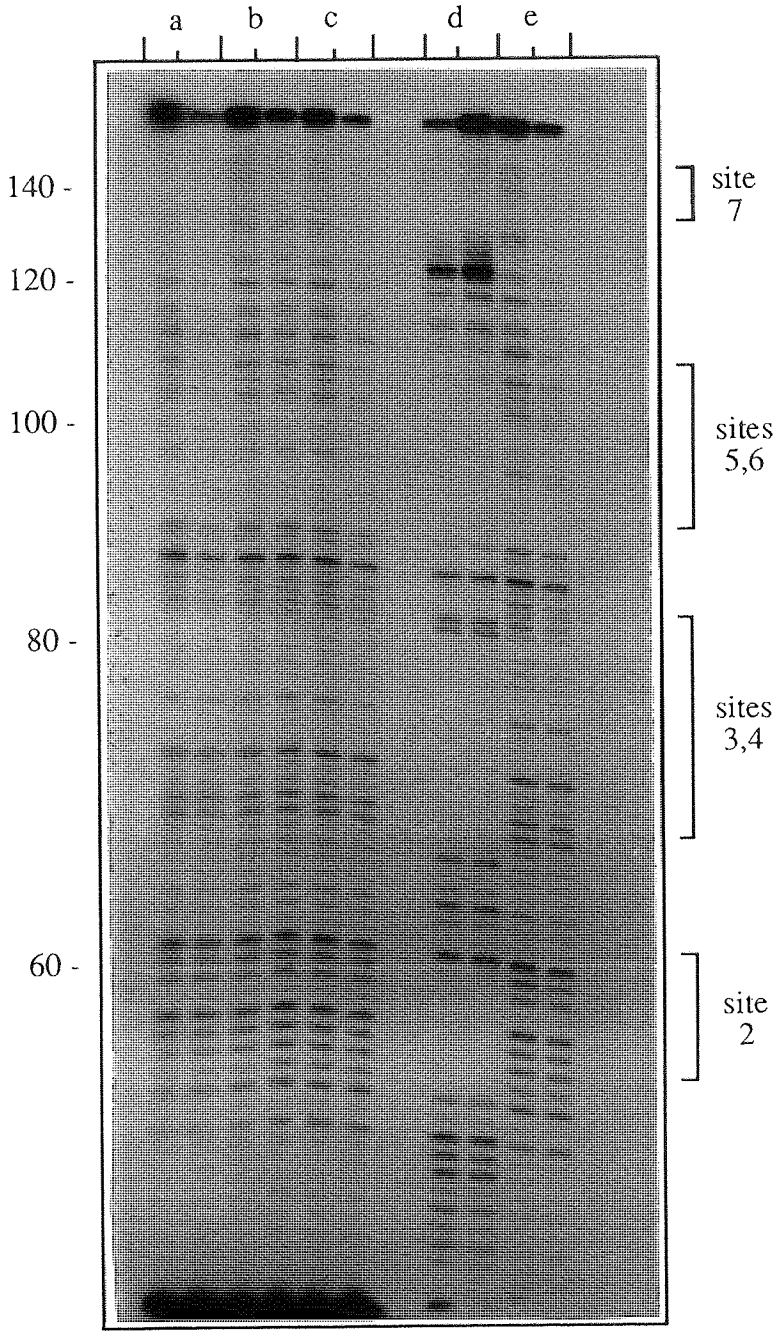


Fig.4.3. DNAase I footprinting with temozolomide.
A homogenous sample of [^{32}P]- 3' single end-labelled tyr T DNA was partially digested by DNAase I at 37°C in the presence of (a) 1mM, (b) 200 μM and (c) 20 μM temozolomide, (d) a positive control of echinomycin (100μM) and (e) a negative control of the drug solvent (1% DMSO). Digestion was stopped after 1 min (first lane) or 5 min (second lane) at each drug concentration and the products were visualised by electrophoresis and autoradiography. Numbers on the left refer to the base numbering scheme (Appendix A), while sites of protection from DNAase I digestion by echinomycin are identified on the right.

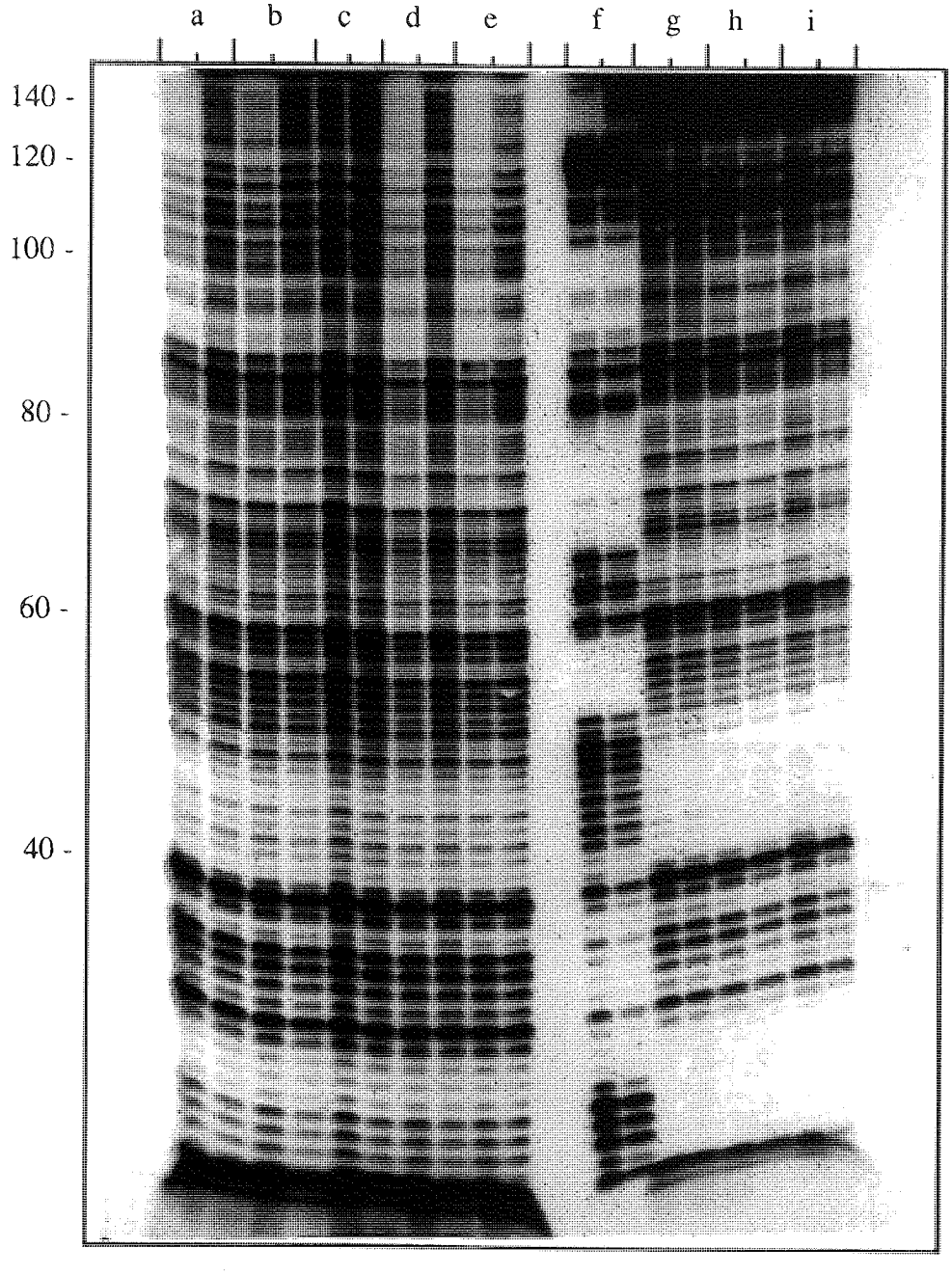


Fig. 4.4. DNAase I footprinting with MTIC.
TyrT DNA was digested by DNAase I at 37 °C (a-e) and at 4 °C (f-i), for 1 min (first lane) or 5 min (second lane) in the presence of (a, g) 1 mM, (b) 200μM, (c) 20μM, and (d, h) 1 μM MTIC, with controls of (e, i) DMSO (1%) and (f) echinomycin (100μM). Labelled fragments were visualised by electrophoresis and autoradiography.

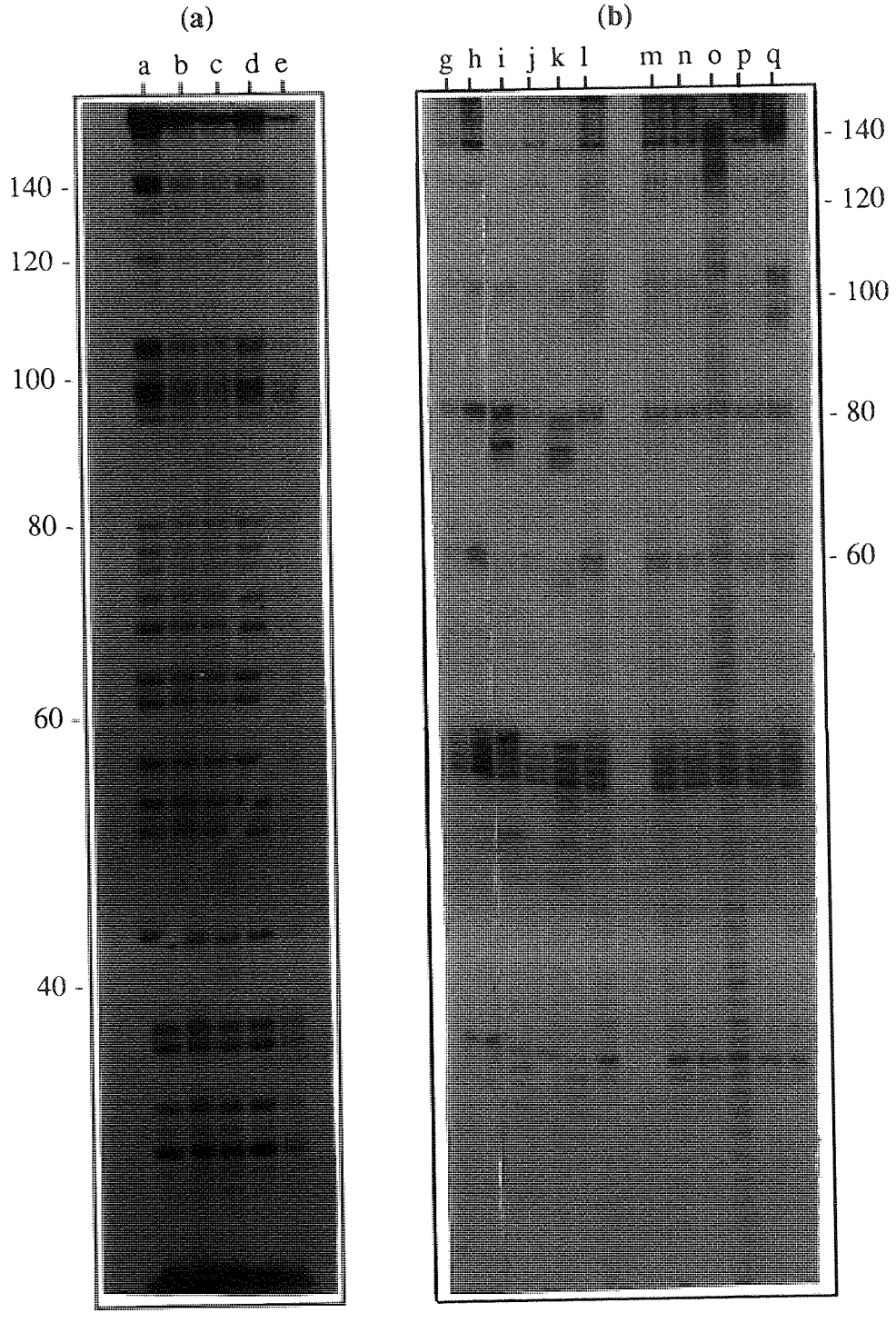


Fig. 4.5. (a) DMS footprint with temozolomide and (b) DEPC footprints with temozolomide, MTIC and DTIC. *Tyr*T DNA was digested by piperidine-cleavage of DNA alkylated by **(a)** DMS in the presence of a) 1 mM, b) 200μM and c) 20 μM temozolomide; d) solvent control; and e) guanine marker track, or by **(b)** DEPC with g) 20μM and h) 200 μM DTIC; i) 20 μM and k) 40 μM nogalamycin; l) 20μM , m) 200μM and n) 1mM temozolomide; o) 20μM, p) 200μM and q) 1mM MTIC; j) solvent control.

4.3.3 Sequence specificity of covalent modification of DNA.

In order to examine the DNA sequence selectivities of temozolomide, its inactive 3-ethyl analogue, a pyrazole analogue (**10**) and a spermidine-temozolomide conjugate (**15**), a modification of the standard Maxam and Gilbert sequencing technique was used to examine guanine-N7 alkylation. 5′ End-labelled BamHI-SalI or HindIII-BamHI DNA fragments were incubated with drug at 37°C for 2 hr, and the DNA was cleaved with piperidine to produce breaks at sites of guanine-N^7 alkylation. The pattern of fragments obtained are shown in figures 4.6, 4.7a and 4.7b.

Bands predominated at sites corresponding to runs of 3 or more contiguous guanine bases indicating temozolomide, the pyrazole analogue (**10**), and the spermidine conjugate (**15**) to preferentially alkylate these sites, with the intensity of reaction increasing with the number of adjacent guanines. Ethazolastone and the 3-benzyl pyrazolotetrazinone (**11**) appeared marginally less discerning. In addition, qualitative examination of the level of fragment cleavage following alkylation indicates ethazolastone to have low reactivity by comparison with temozolomide, a factor which undoubtedly contributes to its lack of antineoplastic activity.

It was anticipated that conjugation with spermidine would provide temozolomide with improved DNA-directed activity, and indeed a significant increase in DNA reactivity was detected, although whether the improved reactivity of the conjugate is translated into increased cytotoxicity remains to be determined. Initial studies indicate that antitumour activity may be limited, since at 500μM the % control population growth in GM892A and Raji cells was 52.5% and 85% respectively while toxicity was not improved by depletion of cellular polyamine levels, using DFMO (0.5mM,72hr), prior to exposure of these cell lines to the conjugate. Hence, cellular delivery of this agent would appear to be hampered for most cells by the necessity for a spermidine uptake mechanism, although this in turn may allow the targeting of tumour cells possessing such an uptake system (Holley *et al.*, 1992).

The piperidine cleavage assay is confined to the detection of guanine-N^7 alkylation, and so sequence selectivity has also been examined using a primer extension procedure utilising multiple cycles of polymerisation with the thermostable DNA

polymerase from *Thermus aquaticus*. Following annealing of a 5′-end-labelled primer to template DNA, extension with *Taq* DNA polymerase produced a full length fragment of 263 bp in a solvent control, while the ability of temozolomide alkylations to block the progress of the polymerase, causing premature termination of chain-elongation is presented in Fig.4.8. Unmodified DNA showed very few sites of early termination, while with temozolomide alkylated DNA termination occurred at the sites of guanines, again indicating a preference for temozolomide alkylation at clusters of guanines over isolated guanines.

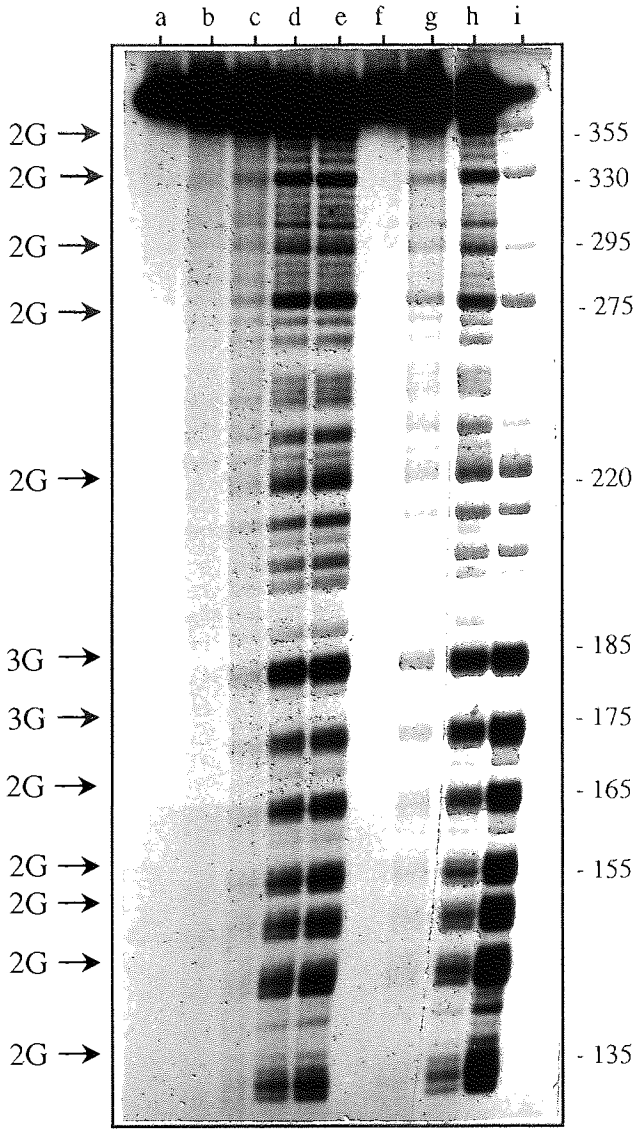


Fig. 4.6. Sequence specificity of guanine-N^7 alkylation of HindIII-BamHI fragment. Sites of guanine N^7-alkylation produced in the 346 bp HindIII-BamHI fragment of pBR322, 5' labelled at the HindIII site. Lane (a) 10 μM, (b) 100 μM, and (c) 1 mM ethazolastone; (d) 10 μM and (e) 100 μM temozolomide-spermidine conjugate (**15**); f) solvent control; (g) 10 μM, (h) 100μM, and (i) 1 mM temozolomide. Figures refer to the base sequence (appendix B) for which the sites of 2 or more contiguous guanine residues are marked.

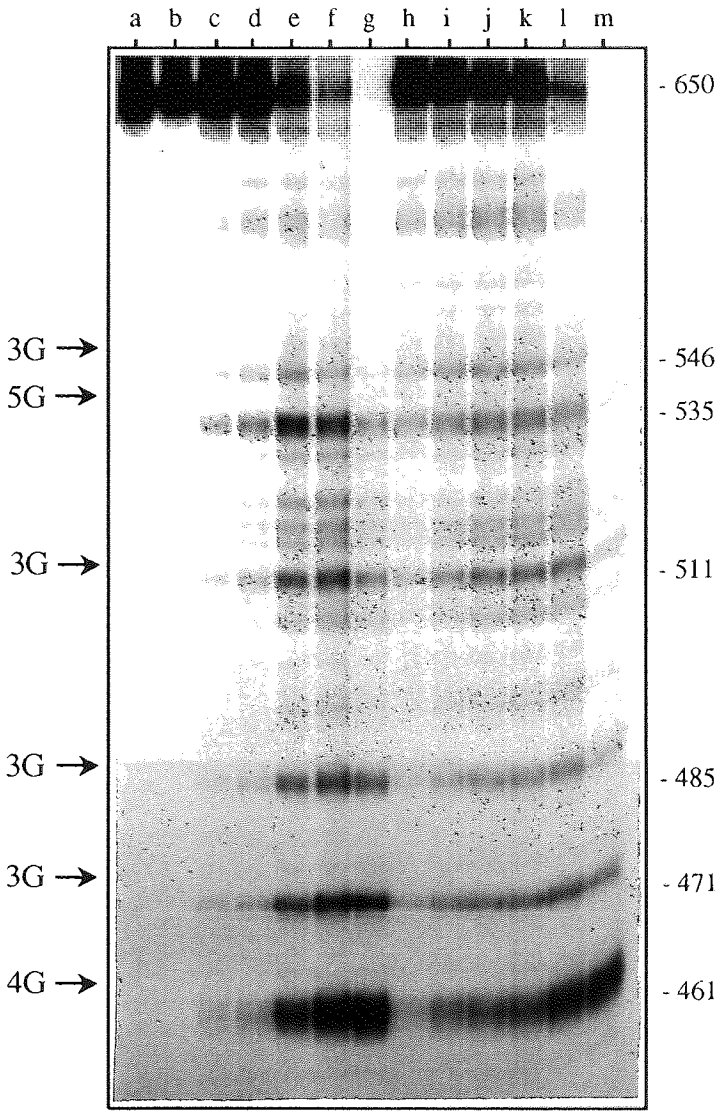


Fig. 4.7a. Sequence specificity of guanine-N^7 alkylation of BamHI-SalI fragment. Sites of guanine N^7-alkylation produced in the 276 bp BamHI-SalI fragment of pBR322, 5' labelled at the BamHI site. Lane (a) 2.5 mM, (b) 5mM ethazolastone; (c) 50 μM, (d) 100 μM, (e) 250 μM, (f) 500μM μM and (g) 1mM temozolomide; (h) 2 μM, (i) 10 μM, (j) 50 μM, (k) 100 μM, (l) 500 μM and (m) 1 mM temozolomide-spermidine conjugate (**15**). Figures refer to the base sequence (appendix B) for which the sites of 3 or more contiguous guanine residues are marked.

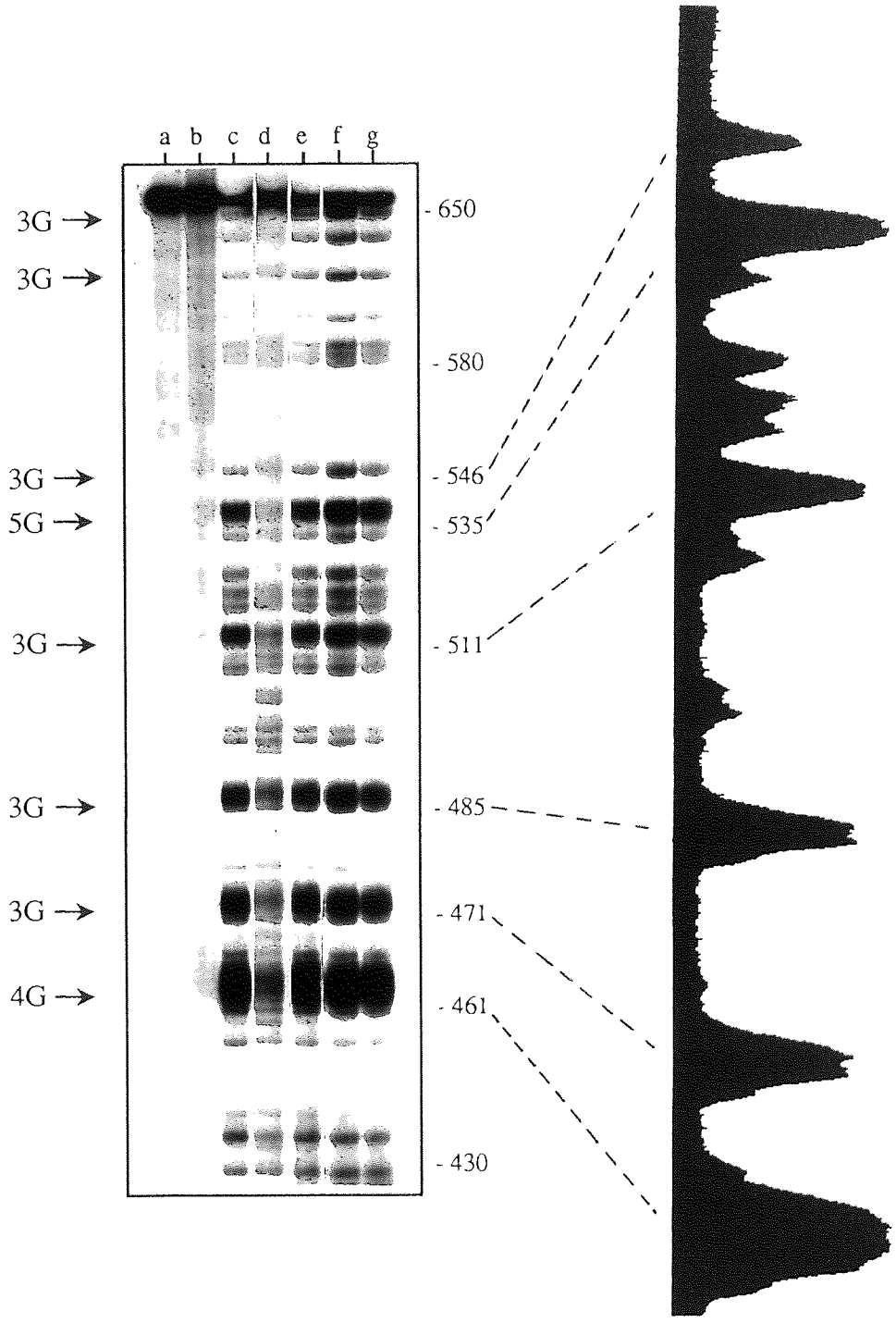


Fig. 4.7b. Sequence specificity of guanine-N^7 alkylation of BamHI-SalI fragment. Sites of guanine N^7-alkylation produced in the 276 bp BamHI-SalI fragment of pBR322, 5' labelled at the BamHI site. Lane (a) solvent control; (b) formic acid purine marker lane; (c) 250 μM 3-methyl pyrazolotetrazinone (**10**); (d) 2.5mM 3-benzyl pyrazolotetrazinone (**11**); and (e, f and g) 250μM temozolomide. Figures refer to the base sequence (appendix B) for which the sites of 3 or more contiguous guanine residues are marked. The preferential akylation of runs of guanines by temozolomide is highlighted by an optical densitometric scan of lane (e).

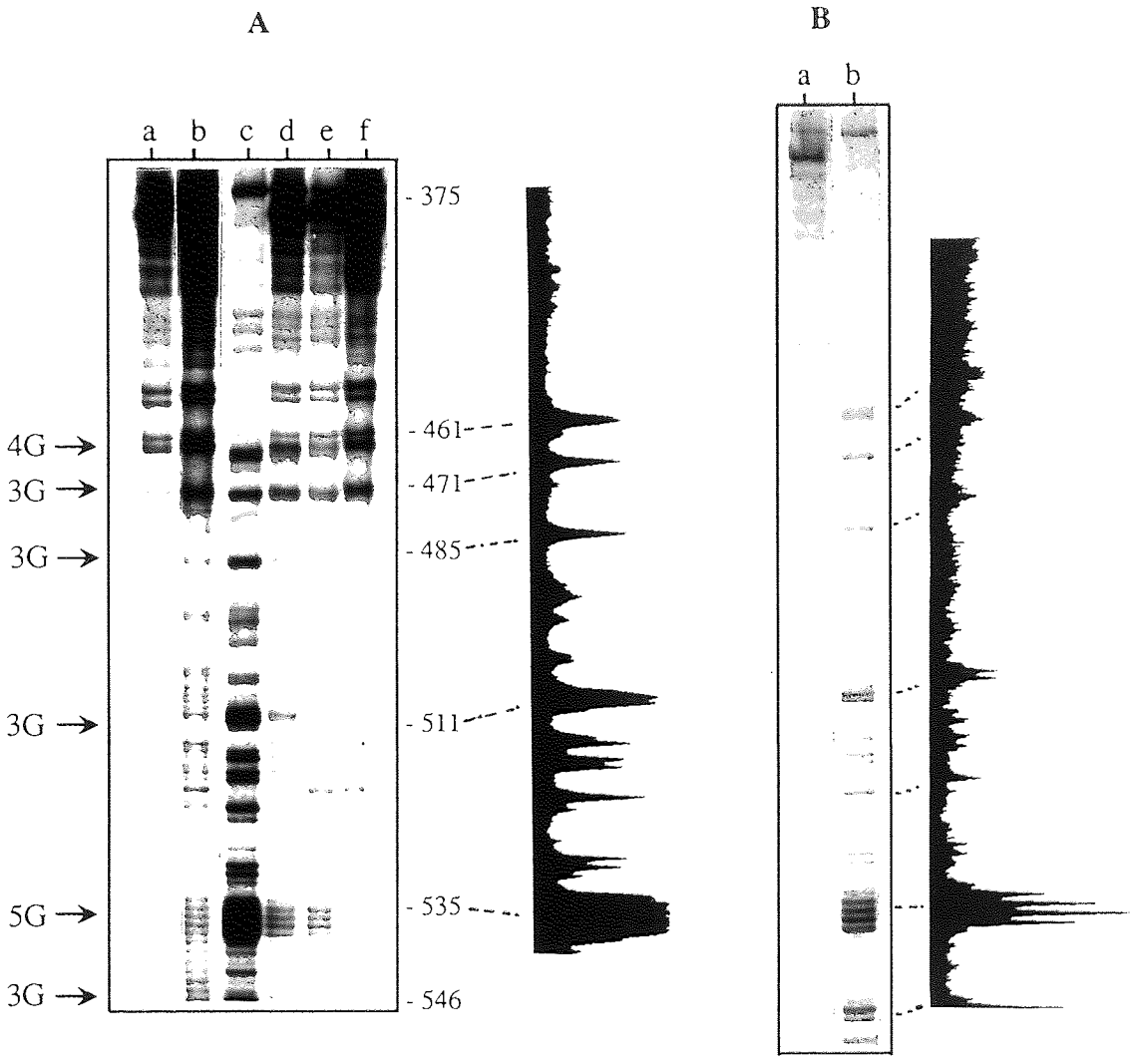


Fig. 4.8. Autoradiogram showing the blocks to *Taq* DNA polymerase produced by alkylation. Bands indicate the sites of termination for extension of a 5'-end labelled primer, complimentary to bases 621-640 of the BamHI-SalI fragment of pBR322 (bases 375-650) . **A)** Lane (a) is control, unmodified fragment; (b) 10μM and (c) 1mM temozolomide; (d) 10μM and (e) 100μM temozolomide- spermidine conjugate (**15**); and (f) 2.5 mM ethazolastone. **B)** Lanes a) and c) following reduced autoradiogram exposure time. Figures refer to the base sequence (appendix B), for which the sites of 3 or more contiguous guanine residues are marked. The densitometric scans shown are of lane (e).

4.4 Discussion.

To date, much research into covalent DNA modification has concentrated on the types of lesions produced by agents and the relationship between DNA alkylations and cell death. Thus, the ability of the imidazotetrazinones to produce O^6-alkylguanine has been correlated with cytotoxicity. However, if a knowledge of the mechanism of action of these agents is to aid design of improved drugs a better understanding of the molecular nature of their interaction with DNA is required.

The products of DNA alkylation are largely determined by the nucleophilicity and steric situation of the atoms of DNA bases, but in addition, an important role for the non-alkylating or 'carrier' moiety is indicated by the variation in the types and amounts of alkylations produced by different agents, even within the same class.

Examination of guanine-N^7 alkylation by temozolomide has indicated a sequence preference for runs of guanines, a specificity common among alkylating agents. The selectivity of nitrogen mustards for the middle guanine in a run of three has been correlated with the negative molecular electrostatic potential shown by the DNA sequences and has been rationalised by the consideration that a positively charged aziridinium intermediate is attracted to these regions (Kohn *et al.*, 1987). A similar theory has been proposed for the preference for these sequences displayed by chloroethylnitrosoureas via the generation of a partial chloronium ion (Hartley *et al.*, 1986). However, factors other than charge attraction have been suggested to be responsible for promoting the selective alkylation of guanine-rich regions exhibited by imidazotetrazinones (Lowe *et al.*, 1992), although recent studies have identified a positively charged methyldiazonium species as a product of aqueous decomposition of temozolomide (Wheelhouse and Stevens, 1993).

On the basis of crystallographic and molecular modelling studies a model has been proposed in which the imidazotetrazinone prodrugs are preferentially ring-opened to their active forms by water 'activated' in the nucleophilic microenvironment of guanine-rich DNA sequences (Clark *et al.*, 1990, Lowe *et al.*, 1992). The importance of a free NH group in the C^8-substituent for a series of mitozolomide derivatives has indicated that an ability to participate in hydrogen bonding interactions may be crucial for maintaining

the antitumour activity of imidazotetrazinones (Lunt *et al.*, 1987, Clark, 1991). Thus, it is suggested, sequence specificity may be conferred by a step preceding covalent modification of DNA involving non-covalent attraction of the non-alkylating moiety to DNA.

The theory for a DNA receptor site for the imidazotetrazinones relies heavily on these molecular modelling studies of mitozolomide binding in the major groove of DNA, while there remains a paucity of corroborating experimental evidence. Studies of the carcinogenic epoxide of the mycotoxin aflotoxin B_1 have demonstrated the epoxide to exhibit sequence-specific guanine-N^7 alkylation, while an inhibition of alkylation by nonreactive analogues has suggested this alkylating compound to possess clearly defined binding sites in DNA (Muench *et al.*, 1983). Analogously, it was envisaged that the inactive 3-methyl-1,2,3-benzotriazinones (**12,13**) and a 3-methyl-pyrazolo-1,2,3-triazinone (**14**) might provide firm experimental evidence for the existence of an imidazotetrazinone receptor site. However, *in vitro* experimental results have indicated the presence of the benzotriazinones to have no observable effect on temozolomide mediated growth inhibition, although, initial results with the pyrazolotriazinone have proved more encouraging.

Further attempts to analyse potential binding sites for temozolomide in DNA using footprinting techniques have proved similarly discouraging. It was hoped that such studies may discern targeting differences between temozolomide and its ring-opened product. However, while dramatic changes were seen for DNA digested in the presence of echinomycin, there were no detectable variations with temozolomide or MTIC. This may be explained by the small size of these compounds, a lack of strong interactions with DNA, and their high reactivity, although attempts at footprinting the stable benzotriazinones provided no further enlightenment of the subject. Indeed, reports of successful footprinting appear confined to compounds of larger molecular weight and possessing multiple potential hydrogen-bonding sites (Fox, 1987), while as proposed major groove binders, the minor groove cutting nature of the DNAase1 endonuclease may prevent this technique from identifying DNA binding sites for imidazotetrazinones.

The fact that many agents show similar sequence preferences yet differ significantly in structure, may argue against a role for the non-alkylating residues in influencing selectivity, especially given the low potential for non-covalent DNA binding

of small compounds other than an apparent charge attraction. Consequently, Warpehoski and Hurley (1988) have suggested that sequence selectivity may result from the covalent bonding step due to stabilisation of an incipient positive charge in the transition state of the electrophilic substrate by specific nucleophilic nucleotide sequences. According to the model proposed by Clark *et al.* (1990) the imidazotetrazinones should have an enhanced potential for sequence-selective alkylation of DNA compared with MTIC. However, Hartley *et al.* (1988) have demonstrated that while DMS and monoethyltriazenes exhibit little specificity, the monomethyltriazene MTIC, although slightly less striking than mitozolomide, does show a preference for the middle guanine in clusters. In addition, studies of the rate of mitozolomide hydrolysis in the presence of synthetic G-C rich double-stranded DNA have failed to show any catalytic effect and this theory remains unsubstantiated by experimental data (Clark, 1991). As such, the relative contributions of an initial noncovalent DNA binding step and the covalent modification step to DNA sequence selectivity of the imidazotetrazinones have as yet to be established.

The sequence selectivity of guanine-N^7 alkylation by temozolomide has been determined using the piperidine cleavage technique, and comparable results obtained using a polymerase stop assay, which is not confined to detection of guanine-N^7 alkylations. However, these techniques cannot elucidate the sequence specificity of O^6-guanine alkylation, the lesion correlated with cytotoxicity. The evidence that N^7-alkylation by temozolomide is dependent on the base sequence surrounding the site of alkylation may prove applicable to the O^6-lesion. Indeed, the model for DNA binding proposed by Clark *et al.*(1990) allows for ring-opening leading to alkylation of the N^7- or O^6-position of guanine, while the formation of O^6-methylguanine in an MNU alkylated oligonucleotide has been shown 5-6-fold higher in the middle and end guanine of a run of 3 in the oligonucleotide in its double- but not single-stranded form (Richardson *et al.*, 1989). On the other hand, a recent study of the distribution of O^6-methylguanine in the rat H-ras gene sequence modified with MNU, has identified the central guanine of GGPy (Py, pyrimidine) as preferentially alkylated (Mironov *et al.*, 1993). Interestingly, O^6-methylguanines adjacent to other guanine residues have been shown to be poorly repaired when compared with isolated O^6-methylguanines (Dolan *et al.*, 1988), and so it appears

that these sequences represent sites of preferential alkylation and poor repair. However, while this non-random alkylation and repair appears to be a factor in mutagenesis (Richardson *et al.*, 1989, Bishop *et al.*, 1993) and hence possibly related to carcinogenesis (Georgiadis *et al.*, 1991), its importance in cytotoxicity can only be speculated on. It may be of significance that certain oncogene promoter regions are high in content of triplets of GGG (Mattes *et al.*, 1988) and could thus constitute regions predisposed to the alkylating action of temozolomide. Whether preferential reaction with such sites may partially explain chemotherapeutic activity remains an intriguing possibility.

As regards the relevance of sequence selectivity to antitumour activity, there are no clear examples of agents that act by virtue of their ability to discriminate between different sequences. However, while much research has examined DNA alkylation products at the genomic level and the relationship between overall cellular capacity for repair of these DNA lesions and cytotoxicity, the possibility that chromatin and chromosome structure may influence the susceptibility of DNA sequences to alkylation as well as to repair, be it sequence-specific or even gene-specific, should not be overlooked. Thus, the suggestion that alkylation is directed by the 'carrier' moiety to guanine rich regions at sites in DNA governed by DNA structure is not inconceivable and may in theory help to explain the tumour specificity shown by temozolomide.

Attempts have been made to improve the activity of imidazotetrazinones by the incorporation of additional hydrogen bonding acceptor and donator functionalities, including derivatives with amino acids and peptides that may be implicated in promoting DNA binding, but to date these have proved disappointing (Clark, 1991). An alternative approach has been the attachment of the cationic DNA affinity binder spermidine to cytotoxics, which it was envisaged would improve DNA directed activity, while the complexities of cellular delivery of such agents might allow selective targeting of tumour cells with polyamine uptake systems. Conjugation of spermidine to chlorambucil results in significantly increased DNA reactivity and *in vitro* cytotoxicity when cellular uptake of the agent is facilitated by active transport (Holley *et al.*, 1992). Similarly, in the present sequence specificity studies, a temozolomide-spermidine conjugate has shown enhanced DNA alkylation although it did not display any significant change in sequence specificity. However, despite the anticipated selective accumulation of these agents, in initial *in vivo*

murine studies of the chlorambucil conjugate in a tumour with an active uptake mechanism it was found that despite having increased potency the therapeutic index was not increased. This may also prove the case for the temozolomide conjugate and whether the improved DNA reactivity observed for this agent is reflected by increased cytotoxic activity and, moreover, if the polyamine targeting of cells is of clinical use, remains to be seen.

In conclusion, while the extent that binding and covalent bonding steps contribute to the sequence preferences of imidazotetrazinones remain undetermined, the specificity for alkylation of certain sites observed *in vitro* may implicate some sequences or genes as targets *in vivo*. It remains to be seen whether targeting of DNA damage to a certain gene or sequence will have a specific chemotherapeutic result, yet the answer to this question and a greater understanding of the mechanisms contributing to the sequence selectivities of agents offer the most exciting prospects in terms of the rational design of drugs with enhanced sequence preferences and the ultimate goal of tumour selective agents.

Chapter Five

Imidazotetrazinones and Gene Expression

5.1 Introduction

The cytotoxicity of many alkylating agents has been attributed historically to an ability to crosslink DNA and thus inhibit strand separation and DNA replication. However, while this remains possible for mitozolomide, temozolomide is chemically incapable of such crosslinking and consequently a mechanism by which O^6-methylation may exert lethality is less clear. Intriguingly, while methylation at this position alters the coding specificity of the base (Singer *et al.*, 1989), there has been little evidence to suggest that this lesion constitutes a major block to DNA polymerase (Larson *et al.*, 1985, Dosanjh *et al.*, 1991).

If we consider other protein-DNA interactions, temozolomide (but not ethazolastone) has been shown to induce differentiation in K562 human erythroleukaemia cells suggesting an ability for the methylating agent to alter gene expression (Tisdale, 1985a). This differentiation is accompanied by a decrease in the total 5-methylcytosine content of the DNA (Tisdale, 1986), a change that appears to result from inhibition of DNA 5-methyltransferase by O^6-alkylated guanine residues adjacent to cytosines (Tisdale, 1989, Hepburn *et al.*, 1991). Methylation of these cytosines is associated with regulation of proto-oncogene expression (Bird, 1984), however hypomethylation is more normally related to increased oncogene expression and tumourigenesis (Diala *et al.*, 1983, Gama-Sosa *et al.*, 1983). Again this may account for the carcinogenic activity of the O^6-lesion, but in addition interference with gene expression could disrupt cellular functioning with potential lethality.

This effect on the interaction of a protein with DNA is not an isolated example. The presence of O^6-methylguanine in DNA has been demonstrated to inhibit recognition and / or cleavage of DNA by some restriction enzymes (Voigt and Topal, 1990), and also to affect binding of transcription factors to regulatory sequences and thereby possibly disrupt regulation of gene expression (Bonfanti *et al.*, 1991). This is of particular interest given the marked preference that mitozolomide (Hartley *et al.*, 1986) and temozolomide, in common with many other alkylating agents, have shown for alkylation of runs of three or more contiguous guanine residues, sequences which are common to the promoter regions of oncogenes (Mattes *et al.*, 1988). These studies have only revealed the

sequence specificity of guanine-N^7 alkylation while information concerning alkylation at the O^6-position is limited, although similar sequence dependent preferences for MNU alkylation of DNA have been described (Richardson *et al.*, 1989, Mirinov *et al.*, 1993). Furthermore, these O^6-alkylated sequences appear poorly repaired by comparison with single O^6-methylguanines and thus may represent sites resilient to repair and of significant cytotoxic importance.

The relevance of these phenomena to cytotoxicity and a role for the involvement of inhibition of gene expression in eliciting cell death has yet to be realised, although the observation that transcriptionally active nucleosomal gene sequences appear more prone to MNU alkylation than inactive genes (Palombo *et al.*, 1992, Boffa *et al.*, 1992) may imply that the cytotoxicity of temozolomide could be further targeted towards specific genes. This provides the exciting possibility that the cytotoxic activity of temozolomide could involve not only inhibition of gene transcription, but that chemotherapeutic activity is perhaps as a result of inhibition of aberrant oncogene expression.

This hypothesis has prompted experiments examining the effect of temozolomide alkylation on gene expression using a transient expression assay. A plasmid expressing a reporter gene under the control of the GC rich promoter region of the human H-ras gene has been alkylated with temozolomide and introduced into cells, and the effect on expression determined. In order to relate the findings to alkylation at the O^6-position of guanine, and hence to cytotoxicity, expression has been compared in cell lines deficient and proficient in O^6-AGAT, and also following depletion of alkyltransferase using the free base O^6-methylguanine. In addition, expression has been determined following alkylation with ethazolastone and DMS, agents for which alkylation at the O^6-position appears not to be important to cytotoxicity.

5.2 Methods

5.2.1 Experiments to examine the effect of alkylation on gene expression.

The effect of alkylation on gene expression has been assessed within CHO (mer-) and MCF7 (mer+) cell lines using a transient expression system employing the plasmid pHrasSVOCAT consisting of a 551 base-pair Ha-ras-I gene promoter region and a reporter gene, chloramphenicol acetyl transferase (Ishii *et al.*, 1985).

5.2.1.1 Determination of cell line O^6-AGAT levels.

The O^6-AGAT levels of CHO and MCF7 cells was determined as described in section 3.2.3.

5.2.1.2 Effect of direct drug treatment on cell population growth.

The levels of temozolomide, ethazolastone and DMS required to cause a 50 % inhibition of cell population growth were determined by incubation of 1 x 10^5 CHO cells or MCF7 cells, in Nunc 6 well plates, with a range of concentrations of drug. Cells were incubated for 72 hr before counting, and the population growth inhibition was determined by comparison with solvent controls.

Similarly, the effect of O^6-AGAT depletion on the sensitivity of MCF7 cells to temozolomide was determined following pretreatment of cells with 0.5 mM O^6-methylguanine for 16 hr.

5.2.1.3 Effect of plasmid alkylation on CAT reporter gene activity.

5.2.1.3a Plasmid purification.

Plasmid DNA was introduced into competent *E.Coli* as described by Sambrook *et al.* (1989) and transfected colonies selected for by resistance to ampicillin. Colonies were grown in 500 ml L.B containing 50 μg/ml ampicillin to O.D. 0.6 before the addition of 170 μg/ml chloramphenicol and plasmid DNA was isolated following the basic method as described in section 4.2.2.1. Cells were lysed by an alkaline SDS solution at low temperature, and the solution then made acidic by the addition of

potassium acetate (SolI), causing precipitation of denatured genomic DNA and cellular proteins. Residual proteins were removed by phenol extraction, RNA was digested with RNAase and plasmid DNA was recovered by ethanol precipitation.

5.2.1.3b Plasmid alkylation

Plasmid DNA was incubated for 2 hr at 37°C with temozolomide, ethazolastone or DMS over a range of concentrations, or with solvent controls (final concentration of DMSO = 2.5%) in a reaction mixture containing 10 µg DNA in TEOA buffer (1mM EDTA-25mM triethanolamine/HCl, pH7.2) in a final volume of 50 µl. The DNA was precipitated by the addition of an equal vol of 0.6M sodium acetate followed by 3 vol ice-cold ethanol and storage on dry-ice for 10 min. The DNA was isolated by centrifugation in an Eppendorf microcentrifuge and was washed with 2% Sodium acetate-70% ethanol and re-centrifuged, and finally rinsed with ethanol before being air dried.

Following alkylation plasmids were analysed by electrophoresis on 0.8% agar gels to ensure that drug treatment had not altered the integrity or topology of the plasmid. For comparison of the effects on transcription elicited by temozolomide treatment with those by ethazolastone the extent of alkylation was determined by carrying out the above procedure using equivalent concentrations of [^{14}C]temozolomide (26.3 mCi/mmol) or [^{14}C]ethazolastone (16 mCi/mmol) (of purity 93% and 78% respectively, as determined in section 2.4.4) followed by extensive re-precipitation of the DNA to constant specific activity.

5.2.1.3c Transfection of plasmid DNA into cells

Plasmid DNA was introduced into cells by calcium phosphate transfection methods performed essentially as described by Sambrook *et al.* (1989). 24 hr prior to transfection three T25 flasks for each drug concentration used were seeded at 2 x 10^5 cells/flask and refed 1 hr before transfection. 10 µg of DNA in 10% TE was combined with an equal volume of 0.5 M $CaCl_2$ and, whilst being mixed by a stream of air bubbles, was slowly added to an equal volume of 2 x HEPES buffered saline. The precipitate was allowed to stand for 20 min and then added directly to the cell monolayer, and the cell medium replaced avoiding disturbance of calcium

phosphate/DNA coprecipitate. The cells were incubated for 5 hr before removal of the precipitate, following which the cells were glycerol shocked by incubation with 15% glycerol (in medium) for 2 min. The monolayer was then washed with PBS and refed with medium.

5.2.1.3d CAT assay

Cellular levels of chloramphenicol-acetyltransferase were assayed by the basic procedure of Gorman *et al.* (1982). At 48 hr after transfection, the monolayers were washed with PBS and the cells were detatched from the flask by scraping into PBS. An aliquot of each cell sample was counted to confirm that the introduction of alkylated DNA into the cells had no effect on cell population growth. Cells were harvested by low speed centrifugation and the pellet was re-suspended in 1ml ice-cold PBS and centrifuged at 12,000 rpm for 10 sec. Following re-suspension of the cells in 0.25M Tris/HCl (pH 7.8), a cell extract was prepared by freeze/thawing x3 the suspension in dry-ice/ethanol and centrifuging at 12,000 rpm for 5 min at 4°C. Cell extracts were normalised to equal protein concentration using BioRad Bradford assay and were then heated at 65°C for 10 min. CAT activity was assayed by incubating 50 µl of enzyme extract with 50 µl of 1M Tris/HCl (pH 7.8), 20 µl acetyl CoA (3.5 mg/ml, freshly prepared) and 0.25 µCi [^{14}C]chloramphenicol (50.1 mCi/mmol). After incubation for 1hr at 37°C, the reaction was stopped by extraction with 1 ml ice-cold ethyl acetate, which after vortexing and centrifugation (12,000 rpm, 5 min) was evaporated to dryness in a speed vac and dissolved in 25 µl of ethyl acetate. Equal aliquots were spotted on silica gel thin layer chromatography plates, which were developed in 95% chloroform/5% methanol. The labelled products were visualised by autoradiography and for quantitation, the acetylated forms of [^{14}C]chloramphenicol were cut from the TLC plates and liquid scintillation counted in HisafeIII scintillant. The enzyme activity determined by this assay was found to be time and protein dependent.

5.2.1.4 Effect of O^6-AGAT depletion in MCF7 cells on CAT activity

Plasmid DNA was alkylated with 1mM temozolomide as described above and transfected into MCF7 cells which had been incubated with 0.5 mM O^6-methylguanine for 16 hr prior to transfection. After 48 hr, CAT assays were performed on alkylated-plasmid transfected cells and on untreated plasmid controls, and the level of inhibition caused by alkylation was determined and compared with that observed in non O^6-methylguanine pretreated control cells.

5.2.1.5 Effect of alkylation on plasmid uptake.

To determine whether alkylation has any effect on transfection, tritiated plasmid was alkylated and its uptake into cells determined. Tritiated plasmid (3000 dpm/μg) was isolated from *E.Coli* cultures grown in the presence of 0.5 μCi/ml [^{3}H]thymidine and treated with drugs as in 5.2.1.3. Following transfection of untreated plasmid or alkylated plasmid into cells and incubation overnight, cells were washed extensively before being lysed with 1ml lysis buffer and tritium counted. Controls were carried out omitting HEPES buffered saline and Plasmid DNA and plasmid uptake was calculated by the subtraction of non-specifically bound radioactivity from that for transfected cells.

5.3 Results

5.3.1 Experiments to examine the effect of alkylation on gene expression.

The alkyltransferase repair capabilities of the CHO and MCF7 cell lines, and the ID_{50} concentrations for temozolomide, ethazolastone and DMS in these cell lines are shown in table 5.1. Temozolomide showed differential toxicity towards these cell lines, consistent with alkylation of the O^6-position of guanine being important to cytotoxicity, although the differential activity of temozolomide was less marked than might be expected considering the 180-fold differential level of repair. Ethazolastone and DMS, as might be anticipated, showed similar activity towards both cell lines.

The effect of alkylation in the transient expression system is illustrated in Fig 5.1. This shows an autoradiograph of the products of the CAT assay following introduction of temozolomide alkylated plasmid into the CHO (Mer-) cell line, for which the radioactive counts have been quantitated in Fig 5.2a, while that for the MCF7 cell line is shown in Fig 5.2b. Control populations that were not transfected with the

Table 5.1: O^6-AGAT repair capabilities and drug sensitivities of CHO and MCF7 cell lines.

Cell Line	O^6-AGAT fmol/mg prot.(±sem)	ID_{50} (μM)±sem Temozolomide	Ethazolastone	DMS
CHO	4.72 ± 12.4	94 ± 23.5	>750[a]	72.1 ± 8.1
MCF7	886 ± 150	485 ± 18.5	>1000[b]	63.3 ± 6.5
(MCF7 +O^6MeG)	-	382 ± 21	-	-

a. at 750μM % growth inhibition = 37.5 ± 6.3
b. at 750μM % growth inhibition = 20.0 ± 5.1; at 1mM= 31.5 ±10.6

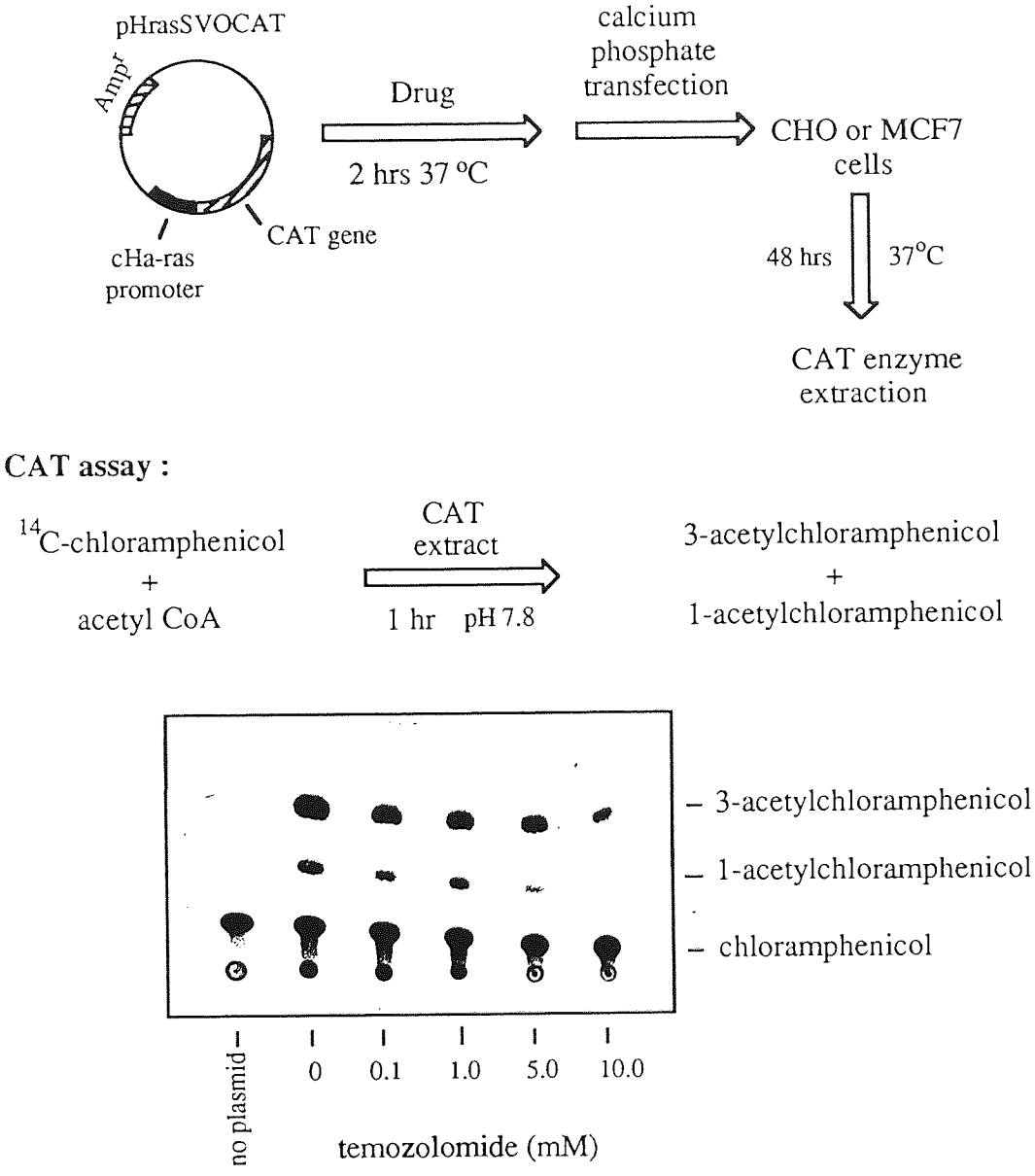
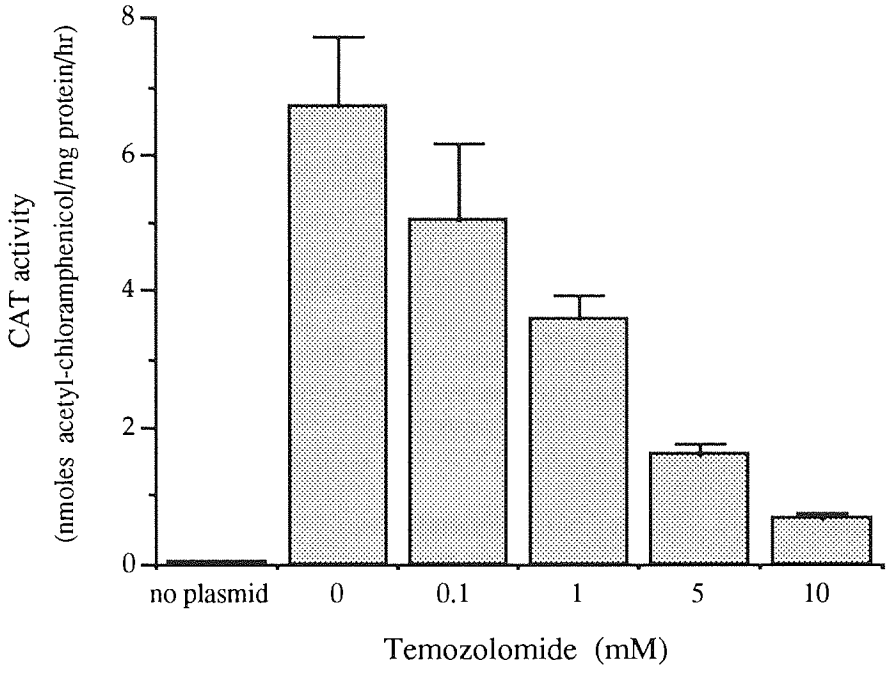


Fig.5.1. Determination of the effect of plasmid alkyation on expression of a reporter gene. pHrasSVOCAT plasmid, containing the gene for chloramphenicol acetyl transferase (CAT) under the control of the guanine-rich promoter of the Ha-ras proto-oncogene, was exposed to various concentrations of drug and introduced into CHO (Mer-) or MCF7 (Mer+) cells. After 48 hr a cell extract was prepared and assayed for CAT activity. The labelled products of the assay were separated by TLC and visualised by autoradiography. An autoradiograph for the products of the assay for CAT activity in CHO cells transfected with temozolomide alkylated plasmid DNA is shown. Spots corresponding to 1- and 3-acetylchloramphenicol were then excised and the radioactivity counted. Activity was determined as moles acetylchloramphenicol / mg protein assayed / hr.

a. CHO cell line.



b. MCF7 cell line.

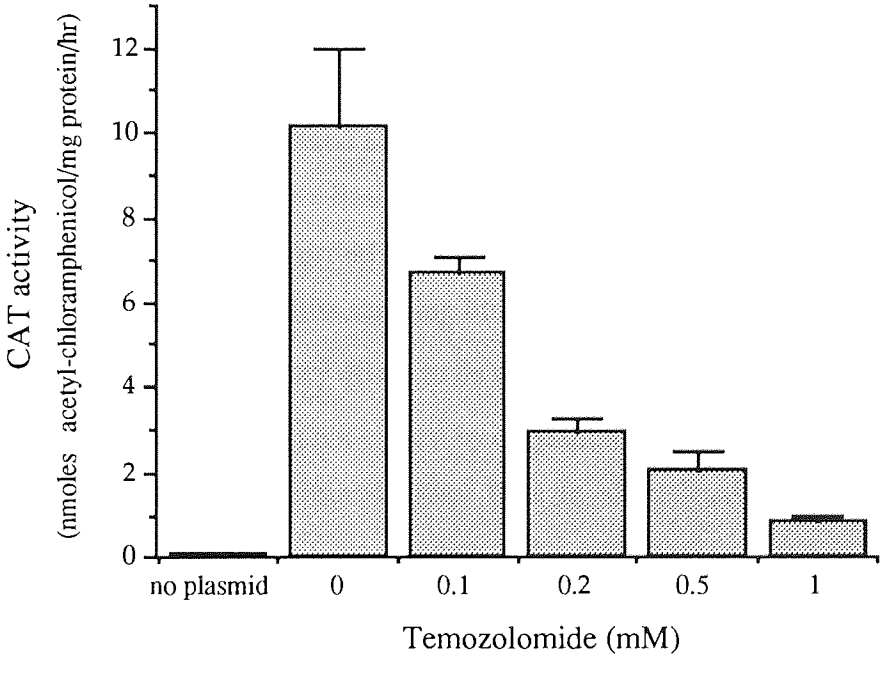


Fig. 5.2. Effect of temozolomide alkylation on plasmid CAT expression: CAT activity was determined in (**a**) CHO (Mer-) and (**b**) MCF7 (Mer+) cells in the absence of plasmid DNA (no plasmid) and following transfection of cells with untreated plasmid DNA, or plasmid DNA that had been exposed to a range of concentrations of temozolomide. Values are the mean ± SEM determined from a single experiment consisting of triplicate cell cultures.

plasmid had insignificant endogenous expression of CAT in either cell line, while temozolomide alkylation of the plasmid dramatically inhibited CAT activity in both cell lines. Comparison of the inhibition elicited in each cell line over several experiments is shown in Fig 5.3 in which the activity for alkylated plasmids has been expressed as a percentage of the CAT activity determined for cells transfected with non-alkylated plasmids . Comparison of the inhibition surprisingly indicated the MCF7 cell line to be considerably more susceptible to this effect, inversely to cytotoxicity, while studies with ethazolastone showed this agent both equitoxic in these cell lines and similar in inhibition of CAT activity (Fig. 5.4). It was considered that, this might suggest the inhibitory effect of temozolomide on CAT activity to be related to repair of alkylation at the O^6-position, a proposal which was investigated by the depletion of alkyltransferase in the MCF7 cell line using O^6-methylguanine. This was shown to sensitise the cell line to the cytotoxicity of temozolomide by 21% (Table 5.1), while expression of plasmid DNA treated with 0.1mM temozolomide in pretreated MCF7 cells resulted in a 50% decrease in CAT activity while a control treated population transfected with the same DNA showed a 34% inhibition (Fig. 5.5). However, while the inhibition of CAT activity caused by temozolomide (0.1 mM) alkylation was significant ($P<0.01$) the variation between untreated and alkyltransferase depleted cells was not significant (2 way ANOVA - $F_{(1,11)}= 0.01$ $P>0.05$).

DMS alkylation of plasmid DNA resulted in a similar differential effect to that of temozolomide (Fig. 5.6) whilst showing little differential cytotoxicity towards the cell lines suggesting the inhibitory effect to be due to methylation of bases other than O^6-methylguanine, and an effect not caused by ethylation. Differences in repair capabilities between the cell lines examined, other than in alkyltransferase capacity, remain undetermined.

To relate the observed effects to the DNA reactivity of the imidazotetrazinones, the extent of plasmid alkylation has been determined for temozolomide and ethazolastone (Fig. 5.7). The results are expressed as the number of alkylations per plasmid for a given concentration of imidazotetrazinone (estimated in Appendix C). While providing little information regarding the different effects observed for these agents, since these figures represent approximate levels of alkylation before exposure to the repair mechanisms of the cells, the results indicate ethazolastone to be 2.3-fold less

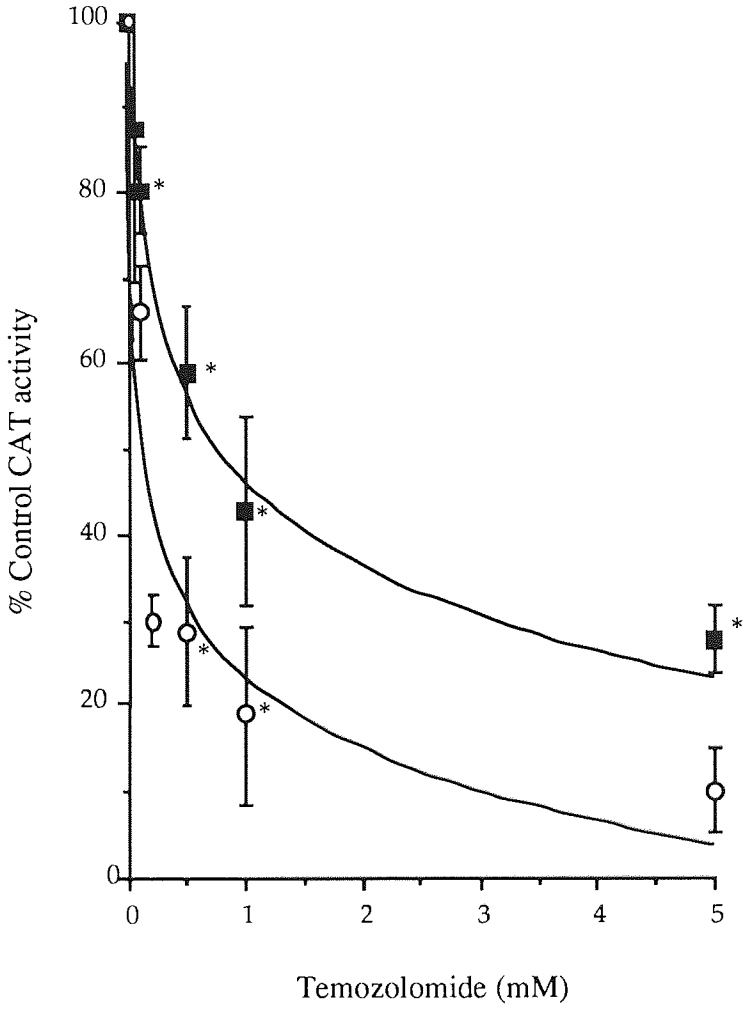


Fig. 5.3. Effect of plasmid alkylation by temozolomide on CAT expression. as a % of control activity. Temozolomide treated plasmid DNA was transfected into CHO (■) and MCF7 (O) cells and the activity of the plasmid reporter gene, chloramphenicol acetyl transferase (CAT), was determined after 48 hr. Values are the mean ± SEM for triplicate cell cultures.* Mean ± SEM of two separate experiments. Curves show a logarithmic correlation; CHO correlation coefficients r^2= 0.98; MCF7 = 0.74.

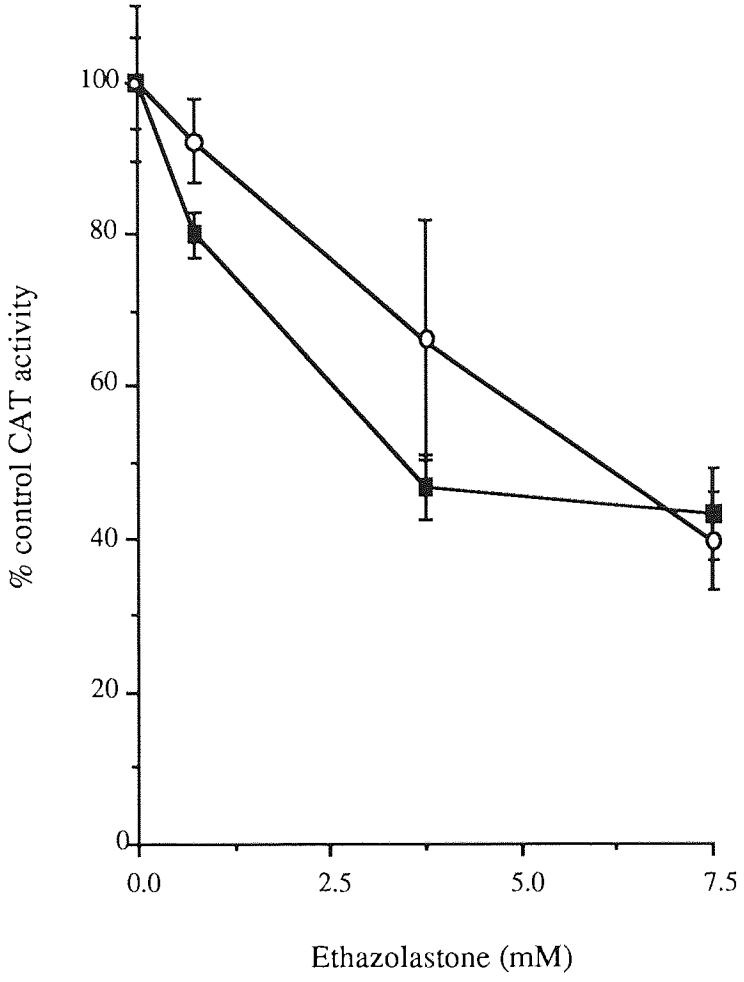


Fig 5.4. Effect of alkylation by ethazolastone on plasmid CAT expression.
Values are the mean ± SEM for triplicate cultures of CHO (■) and MCF7 (o) cells.

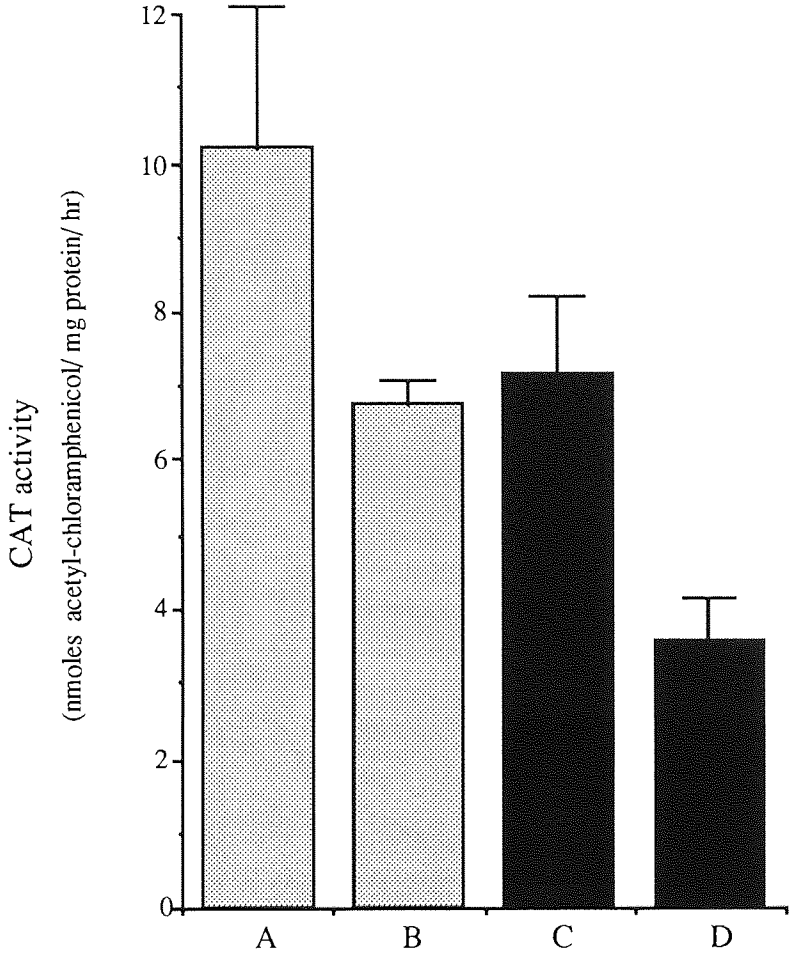


Fig. 5.5. Effect of alkyltransferase depletion on CAT activity.
The CAT activity for controls (A, C) and plasmid DNA alkylated with temozolomide (0.1 mM) (B, D) was determined following transfection into MCF7 cells (▨) and compared with that for MCF7 cells with depleted alkyltransferase levels (■), following pretreatment with O^6-methylguanine (0.5 mM, 16 hr). CAT activity for B = 66.1 % of A; D = 49.7 % B. Values are the mean ± SEM for triplicate cell cultures.

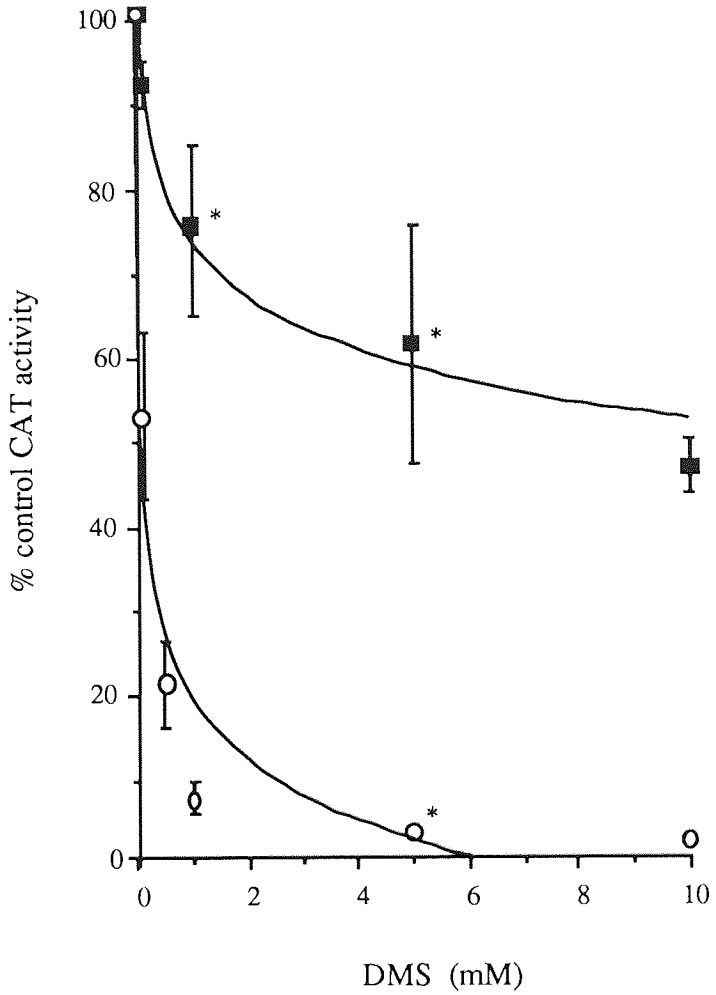


Fig. 5.6. Effect of alkylation by DMS on plasmid CAT expression.
Values are the mean ± SEM for triplicate cultures of CHO (■) and MCF7 (O) cells. Logarithmic correlation coefficient $r^2 = 0.76$ for the CHO cell line; MCF7 = 0.83. * Mean ± SEM for two separate experiments.

reactive towards plasmid DNA than temozolomide at concentrations of 0.5mM. In addition, from these figures it is possible to estimate the number of O^6-methylguanine lesions, as 5.3% of the total alkylations (accepting that this percentage as determined by Bull (1988) for temozolomide alkylation of calf thymus DNA remains applicable to this plasmid DNA). As such, 1mM temozolomide, a concentration that causes approximately 55% and 80% inhibition of CAT expression in CHO andMCF7 cells respectively, produces approximately 4 O^6-methylguanines per plasmid.

In order to confirm the validity of the observed results, various other potential factors have been investigated. While alkylation did not appear to radically disrupt plasmid topology or have an antiproliferative effect on the cells, to ensure that the observed effects were not due to alkylation of the plasmid altering the ability to transfect the DNA, the uptake of alkylated and non-alkylated [^{3}H]-labelled plasmid DNA was determined for both cell lines. The uptake of alkylated plasmid DNA by CHO and MCF7 cells relative to the uptake for untreated plasmid DNA (0.7 and 0.5 $\mu g/10^6$ cells transfected respectively) is shown in Fig 5.8. Only minor differences in uptake were observed after treatment with high concentrations of temozolomide (10mM), ethazolastone (7.5 mM) or DMS (10mM). Such discrepancies in uptake are insufficient to explain the inhibitory effect on CAT activity or the differential effects between CHO and MCF7 cells.

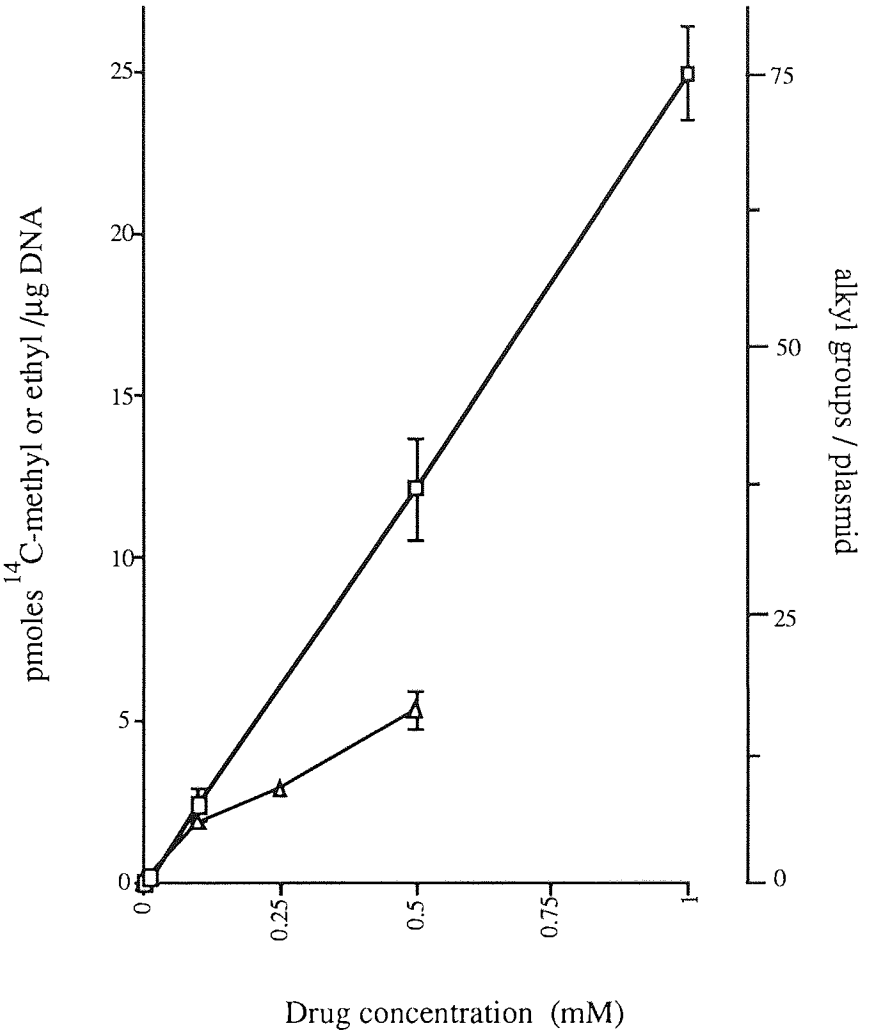


Fig. 5.7. Extent of reaction of imidazotetrazinones with plasmid DNA: Plasmid DNA was treated for 2 hr with various concentrations of temozolomide, [3-methyl- ^{14}C] (□), and ethazolastone,-[3-ethyl-^{14}C] (Δ), and labelled DNA precipitated to constant activity. Values are the mean ± SEM for 3 samples.

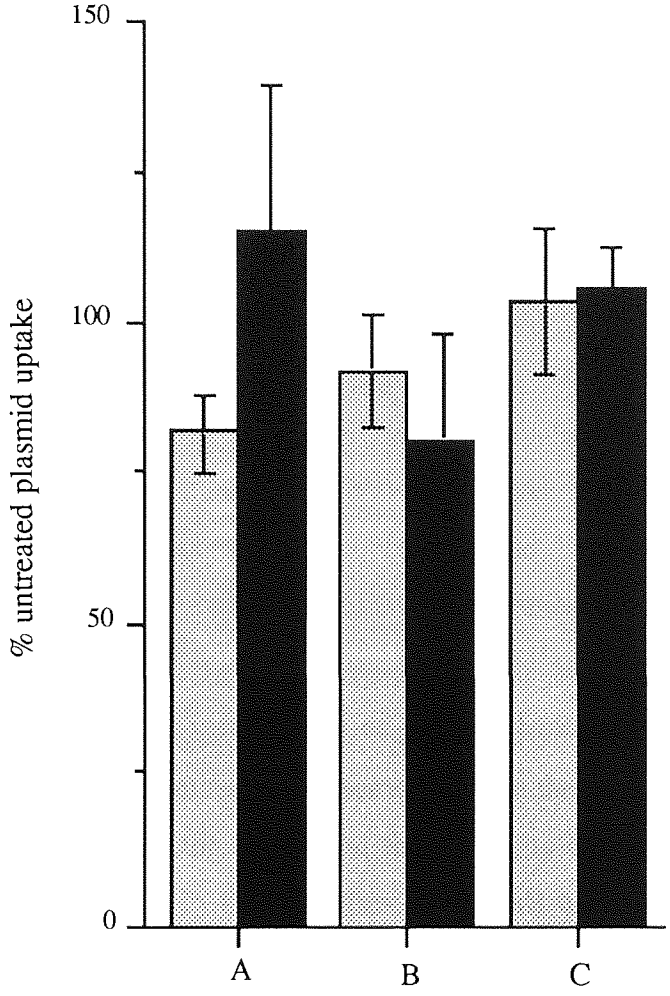


Fig. 5.8. Uptake of [^{3}H] plasmid DNA into transfected cell lines.
[^{3}H]-Thymidine labelled plasmid DNA was transfected into CHO (▨) and MCF7 (■) cells and cellular uptake determined 24 hr after transfection, for (A) plasmid DNA alkylated with temozolomide (5 mM); (B) with ethazolastone (7.5 mM); and (C) DMS (10mM). Values are expressed as a % of the uptake for untreated plasmid (0.7± 0.06 μg and 0.5± 0.02 μg DNA / 10^6 cells transfected for CHO and MCF7 cells respectively), and are the mean ± s.d. for triplicate cell cultures.

5.4 Discussion

The general disruption of gene expression seems an unlikely mechanism of action for temozolomide since Bull (1988) has examined the effect of temozolomide treatment on the incorporation of ^{3}H-leucine into protein in GM892A and Raji cells, and the ability of genomic DNA extracted from cells treated with temozolomide to support *E.Coli* RNA polymerase activity, and found little effect. This would suggest that if the toxicity of temozolomide is mediated by inhibition of gene expression this action is specific to a small number of genes.

One possible mechanism by which this could occur is through disruption of the regulation of gene expression. The regulation of transcription is facilitated by the formation of stable complexes between DNA-binding proteins and specific DNA sequences in the promoter and enhancer regions of genes. In theory, interference with the binding of cellular factors to these sequences could disrupt expression, while specific genes may be affected since some regulatory sequences are specific for a given gene or group of genes. Such an ability to impair the recognition of binding sequences has been demonstrated following methylation of guanine bases at the O^{6}-position within the binding sites of the serum response factor (SRF) in the c-fos promoter, and in the sites for the transcription factor NFxB and those for the cellular factor sp1 in HIV LTR (Bonfanti *et al.*, 1991). In these studies, the NFxB and sp1 binding sites that were methylated contained runs of 3 or 4 adjacent guanines while the SRF binding site contains 2 pairs of guanines that when methylated inhibited factor binding.

In view of the apparent preference of temozolomide for alkylation of such sequences the effect of alkylation on gene expression has been determined for a plasmid containing the chloramphenicol acetyl transferase reporter gene under the control of the H-ras promoter (positions 116-666). This region contains six GC box elements (GGGCGGG) which are sequences common to many viral and animal oncogenes that have been shown to bind the sp1 transcriptional factor (Ishii *et al.*, 1986).

Expression was examined in the CHO cell line, which was clearly illustrated to be alkyltransferase deficient, while the MCF7 cell line was highly proficient. The repair capacities showed some correlation with cell line sensitivity to temozolomide, but not to ethazolastone or DMS, although the differential response to temozolomide was less

emphatic than might be anticipated from the cellular levels of O^6-AGAT. While temozolomide alkylation of plasmid DNA caused inhibition of the CAT activity extracted from the transfected cell lines and, unlike ethazolastone, had a differential effect on activity, this effect correlated inversely with the cytotoxicity of temozolomide towards the cell lines. It was hoped that alkyltransferase depletion in the MCF7 cell line may provide further evidence for such a relationship, however, the MCF7 cell line appeared only poorly sensitised by O^6-methylguanine, and the effects on expression were not conclusive.

From examination of the extents of plasmid alkylation by the imidazotetrazinones, it is apparent that the poor DNA reactivity of ethazolastone may contribute to the low CAT inhibition by this agent, although clearly this cannot explain the lack of effect in the MCF7 cell line by comparison with the methylating agent. Anomalously, DMS plasmid alkylation caused an almost identical inhibition to temozolomide in both the CHO and MCF7 cell lines, however, while it may be anticipated to be more reactive than temozolomide (DMS-DNA reactivity was not determined) it produces less than 0.3% O^6-methylguanine (Lawley and Warren, 1976, and cited Ibeanu *et al.*, 1992) and therefore it remains unlikely that alkylation or repair of the O^6-lesion is responsible for the observed effects.

The results could not be accounted for by alkylation affecting plasmid topology, the growth of transfected cells, or the uptake of the plasmid into the cell lines, although the maintenance of intact plasmid once in the cells has not been investigated. Thus, it would appear likely that an alternative methylated lesion produced by both DMS and temozolomide either directly, or following partial repair, causes disruption of transcription. N^7-Methylguanine is a lesion that has also been demonstrated to alter the DNA binding of transcription factors (Staudt *et al.*, 1986) and DNA-cytosine methylase (Drahovsky and Morris, 1972), while being a lesion produced far in excess of O^6-methylguanine by both DMS and temozolomide. Since DMS alkylations differentially inhibited CAT activity in CHO and MCF7 cells it appears that these cell lines may possess major differences other than in O^6-AGAT levels, however, it is unlikely that differences in repair for N^7-methylguanine account for the effect, since this lesion has been reported

as only poorly repaired (Medcalf and Lawley, 1981). Consequently, the experimental results have proved difficult to interpret, and it appears that the cell lines used in the study form a poor model for examining this effect. Despite being Mer- the CHO cell line is surprisingly resistant to temozolomide, while the MCF7 line is only poorly sensitised by alkyltransferase depletion, possibly due to its high level of constitutive expression of the repair enzyme. Although an alternative Mer+ cell line, the A549 cell line, was examined, insignificant quantities of reporter enzyme were detected following transfection, undoubtedly due to the failure of the calcium phosphate precipitation technique with this cell line. The repetition of studies in a Mer+ cell line using the more potent O^6-AGAT inhibitor O^6-benzylguanine, and including a non alkylated plasmid expressing a reporter gene such as β-galactosidase which would allow correction for variation in transfection success and minimisation of standard errors, could provide a more definitive answer to the points in question.

While the results obtained for DMS alkylation indicate the involvement of lesions other than O^6-methylguanine, the involvement of G-rich sequences and the importance of inhibition of factor binding to the decreases in CAT activity seen have not been established. Since DMS lacks the sequence preference for runs of guanines (Hartley *et al.*, 1988) shown by temozolomide, this might question the necessity for alkylation at such sites, a situation that could be determined by examination of the expression of the reporter gene in the same plasmid vector with alternative promoters. Location of alkylation may prove of little importance to the observed inhibition, and alkyl groups or perhaps apurinic sites from glycosylase repair of N-methylpurines may simply interfere with RNA polymerase progression. In either scenario, since the differential cytotoxicity of DMS towards the cell lines remains small, such inhibition of gene expression would seem of little importance to cytotoxicity.

The possibility remains that O^6-alkylation of specific sequences in other genes has a role in lethality. Given a possible preference for O^6-alkylation of GGPy sequences (Mironov *et al.*, 1993), regions which are contained in the serum response element of the c-fos gene, O^6-methylation of which has been shown to inhibit the binding of serum response factor (Bonfanti *et al.*, 1991), it may prove interesting to examine such promoters in similar expression systems. In addition, it appears that the presence of

O^6-methylguanine may not necessarily in itself alter protein-DNA interactions. Whilst single residues of O^6MeG in binding site I of the SV40 origin of replication inhibited binding of T-antigen, the presence of three O^6MeG bases in the region facilitated the helicase activity of the T antigen (Bignami *et al.*, 1991). Hence, the effects may be related more to its presence in regions of important secondary structure or its ability to introduce structural distortions in particular sequence contexts. This considered, the inhibition of DNA replication by O^6-methylated guanines in particular sequences may yet prove the main route to cytotoxicity. It is apparent that O^6-methylguanine will cause a delay to DNA polymerase (Dosanjh *et al.*, 1993), however, present reports of whether it constitutes a significant block to replication that could effect cytotoxicity remain ambiguous (Larson *et al.*, 1985, Voigt and Topal, 1993, Pfeiffer *et al.*, 1993). The possibility that distortion of DNA structure in specific sequences, perhaps even at runs of O^6-alkylations, could be sufficient to terminate chain elongation may be worthy of investigation. Alternatively, the apparent correlation between the inhibition of T-antigen binding and DNA replication found in the study by Bignami *et al.* (1991) may imply a role for O^6-methylation and interference with other proteins involved in DNA replication as a possible cytotoxic mechanism.

In summary, while it appears that for the cytotoxicity of temozolomide to be mediated by disruption of gene expression this action would have to be specific to certain genes, the present experiments have found no evidence to suggest that alkylation at the O^6-position and inhibition of binding of cellular factors to the H-ras promoter is important to the cytotoxicity of this agent. This may not rule out the importance to cytotoxicity of alkylation at other specific DNA-protein binding sites. However, the observations of Drahovsky and Morris (1972) and Staudt *et al.* (1986) that N^7-methylated guanines also inhibit protein-DNA interactions may suggest this to be a general effect of alkylation in cells that while possibly contributing to toxicity, is probably not responsible for the chemotherapeutic activity of temozolomide.

Chapter Six

Temozolomide-Induced DNA Damage and Potential Mechanisms of Cell Death

6.1 Introduction.

The cytotoxicity of temozolomide may not be as a result of the presence of an alkyl group in DNA itself, but perhaps manifested by alternative forms of DNA damage. While, unlike mitozolomide, cross-linking of DNA is impossible for this methylating agent, it has been postulated that single-stranded DNA breaks arising from methylation may ultimately be responsible for cell lethality (Section 1.5).

Most breaks occurring following alkylation are as a result of metabolic participation by the cell, and not as a direct consequence of drug action. Thus, N-methylated purines are converted into apurinic sites in DNA as an initial step in repair of these lesions. Introduction and high expression of a gene for N-methylpurine glycosylase repair in cells has been shown to sensitise to MMS and DMS (Ibeanu *et al.*, 1992), suggesting that strand-breaks resulting from repair of these sites are potentially cytotoxic. This enhanced cytotoxicity may be reconciled by the observation that single-strand breaks have been shown to cause transient inhibition of replication (Painter and Young, 1976). However, a biochemical mechanism by which O^6-methylguanines could form strand breaks, and whether such breaks would be of cytotoxic significance, remains to be established.

Studies by Hepburn and Tisdale (1991) have suggested that the cytotoxicity caused by DNA alkylation may not be due to the direct disruption of DNA function. Transfection of cells with DNA extracted from GM892A or Raji cells that had been treated with temozolomide, had a dose-dependent growth inhibitory effect that appeared to correlate with the alkyltransferase repair capabilities of GM892A and Raji cells. Thus, it appears that alkylated DNA may be capable of exerting other actions in the cell and that sites beyond DNA may be responsible for cytotoxicity.

The enzyme poly (ADP-ribose) transferase (ADPRT) is an enzyme that is dependent on DNA strand breaks for activity. It catalyses the conversion of NAD+ to chains of poly ADP-ribose, which are used for covalent modification of numerous nuclear proteins, thereby modifying their activity (review Boulikas, 1993). The role of this modification has remained controversial. Inhibition of ADPRT activity using nicotinamide analogues, such as 3-aminobenzamide, has been shown to potentiate the cell killing by several methylating agents (Lunn and Harris, 1988), which has led to the idea

that the enzyme is involved in DNA repair. Initially, it was postulated that ADPRT may regulate DNA ligase II, since strand breaks have been shown to accumulate following methylation in the presence of ADPRT inhibitors. However, a decrease in ligation activity has not been substantiated (Lunn and Harris, 1988). Treatment of L1210 murine leukaemia cells with MNNG has been shown to cause consumption of large quantitities of NAD+ and cause drastic alterations in carbohydrate metabolism, suggesting that extensive DNA damage may activate ADPRT to a sufficient degree to cause lethal depletion of NAD+, and hence ATP, pools (Berger *et al.*, 1986).

In this study, possible relationships between O^6-methylguanine formation and repair, single-strand DNA breaks, and the cytotoxicity of temozolomide have been investigated. DNA strand breakage has been assessed by the alkaline elution method for normal and alkyltransferase depleted Raji cells treated with temozolomide, and the potential role for strand-breaks and the enzyme poly(ADP-ribose) polymerase in cell lethality has been investigated in cell lines with differing sensitivities to temozolomide. A potent inhibitor of ADPRT, 3-aminobenzamide has been used to examine the potentiation of the toxicity of temozolomide in these cell lines, and cellular spermine levels have been depleted using DFMO in attempts to investigate the possible importance of chromatin structural changes to this effect. In addition, post-replication repair has been investigated as a further possible source of cellular resistance to temozolomide, and as a potential mechanism by which methylation at the O^6-position of guanine could result in strand-breakage.

6.1 Methods

6.2.1 Determination of single-strand DNA breaks by the alkaline elution method.

The ability of temozolomide to cause single-strand DNA breaks has been examined using the alkaline elution method of Kohn *et al.* (1981), a technique that discriminates single-strand sizes by utilising nitrocellulose filters to impede the passage of long DNA strands.

6.2.1.1 Cell DNA labelling.

Exponentially growing cells were incubated overnight with 0.05 μCi/ml [^{3}H] thymidine. After labelling the cells were washed with PBS twice and incubated for a further hour in medium, in order to chase the label into high mw DNA.

6.2.1.1 Drug treatment and DNA elution.

Raji cells were incubated with temozolomide or solvent controls for 1 hr and then washed with PBS x3 following low speed centrifugation. Avoiding exposure to ambient light, 0.6-1.0 x 10^6 cells were isolated on nitrocellulose filters and then lysed by the addition of 5mls of sarkosyl lysis solution. The lysates were collected for future counting, and the filters were treated with a further 2ml sarkosyl lysis solution containing 500 μg/ml proteinase K. After incubation for 30-60 min in the dark, 40 ml of alkaline elution buffer (pH 12.1) was pumped through each filter at 2ml/hr and 5x 6ml fractions collected over a period of 18 hr. The excess alkali remaining in the funnel reservoir was discarded, the filter was removed and the dead volume of buffer in the line tubing and filter holder was collected ("the line"). The funnel and tubing were washed with 10 ml of 0.4M NaOH to give a further fraction ("the wash") and the filter was incubated with 400 μl 1M HCl at 65°C for 1hr followed by the addition of 2.5 ml 0.4 M NaOH. Samples from each elution fraction collected, "the wash" , "the line" and filter samples were counted following the addition of 10 ml Aquasol containing 0.7% glacial acetic acid and from these counts the fraction of DNA retained on the filter over the time course of elution was determined.

6.2.2 Experiments to investigate the effect of transfection of cells with DNA extracted from temozolomide treated cells.

6.2.2.1 Raji and GM892A drug treatment and DNA isolation.

Raji and GM892A cells (Approx. 2 x 10^7 cells) were exposed to a range of concentrations of temozolomide for 6hr, or alternatively exposed to the ID_{50} concentration (10 μM with GM892A, 185 μM with Raji cells) or a solvent control (0.5% DMSO) for a period of 6 hr.

DNA was isolated essentially as described by Warren *et al*. (1984). Cells were isolated from the culture medium by low speed centrifugation (1,500 rpm -Heraeus bench top centrifuge), washed with 0.9% NaCl, and re-suspended in 2 ml 6% p-amino salicylic acid (sodium salt). After vortexing for 5 min the cells were lysed by incubation for 5 min at 37 °C with 0.1 vol of 10% SDS. The lysate was vortexed with an equal volume of phenol/chloroform/*iso*amylalcohol (25:24:1) in polypropylene tubes and then centrifuged at 3000 rpm for 10 min.The aqueous phase was carefully removed and any residual phenol extracted with an equal volume of chloroform/*iso*amylalcohol. DNA was precipitated by the addition of 1.5 vol 2-ethoxyethanol, was collected using a glass rod and was washed repeatedly with 70% ethanol/ 2% sodium acetate(w/v) and then with absolute ethanol, before being air dried.

Measurement of the O.D 260/280nm ratios of DNA solutions proved the DNA preparations to be essentially protein free (ratio 1.9 - 2.0), and agarose gel electrophoresis indicated the DNA to be mainly of high molecular weight DNA (Mw > 25 Kb).

6.2.2.2 Transfer of DNA into eukaryotic cells by calcium phosphate transfection.

The day prior to transfection, MAC13 or CHO cells were seeded at 5 x 10^4 cells/flask in 9 ml of fresh medium in T25 flasks, and were allowed to adhere overnight. DNA for transfection was sterilised by precipitation, dried and re-dissolved in sterile water. The concentration of DNA was found using an ethidium bromide-fluorometric assay (section 2.4.2), and 30 μg of DNA was co-precipitated with calcium phosphate. The precipitate was prepared by diluting the DNA to 450 μl with sterile water and adding 50 μl of 2.5 M $CaCl_2$. With continual mixing by aeration, the

solution was slowly added to 500µl of sterile 2 x HeBS and, after vortexing for 5 sec, a calcium phosphate/ DNA precipitate was allowed to form over 20 min at room temperature. The precipitate was added directly to the monolayer and was distributed evenly over the the surface of the cells, before the medium was gently returned to the flask. The cells were then incubated for 5 hr, after which the medium was removed and the cells washed twice with 5 ml of 1 x phosphate buffered saline. The cells were then fed with 10 ml of complete medium, and incubated in an atmosphere of 5% CO_2 in air for 3 or 4 days. Cells were harvested by trypsin treatment and the cell populations were counted.

6.2.2.3 Experiment to confirm the uptake of DNA into CHO and MAC13 cells.

6.2.2.3a Preparation of [^{3}H] labelled DNA.

Exponentially growing GM892A and Raji cells were seeded at 4 x 10^5 cells/ml in 50 ml of media and 0.5µCi/ml [^{3}H]thymidine was added. After incubation for 24 hr DNA was extracted from the cells as in section 6.2.2.1. [^{3}H]DNA samples were mixed with optiphase HiSafeIII scintillant and counted on Packard Tri-Carb 2000CA liquid scintillation counter and DNA concentrations were determined by ethidium-bromide assay. The DNA was re-precipitated by ethanol precipitation until constant activities were obtained. (Raji: 18,000 cpm/µg, GM892A 8000 cpm/µg).

6.2.2.3b Transfection of [^{3}H]DNA and determination of uptake.

MAC13 and CHO cells were seeded at 0.5x10^5 cells/ml in T25 flasks and following incubation overnight, were transfected (section 6.2.2.2) with [^{3}H] GM892A or Raji DNA (0-30µg). 18 hr after removal of the calcium phosphate/DNA precipitate, the cells were trypsinised, pelleted and then washed extensively with PBS. The cells were then lysed with 0.5ml of cell lysing buffer and the amount of radioactivity associated with the cells was determined by liquid scintillation counting. In order to account for non-specific binding of labelled DNA to the cell membrane controls were carried out in which [^{3}H]DNA was added to the cells in the absence of calcium

phosphate.

6.2.2.4 Transfection of DNA into cells by liposome transfection.

As an alternative to the calcium phosphate transfection technique, DNA was introduced into cells using liposomes formed from the cationic lipid N-[1-(2,3-Dioleoyloxy) propyl] -N, N, N-trimethylammoniummethylsulphate (DOTAP). This interacts spontaneously with DNA to form stable complexes which adhere to the cell surface, fuse with the cell membrane and release the DNA into the cytoplasm.

Transfection mixtures were prepared by incubating 3 μg of DNA, which had been prepared from temozolomide treated cells as described in section 6.2.2.1, with 5 μg DOTAP liposomes in 40μl of HBS (20 mM Hepes, 150 mM NaCl, pH 7.4), for 10 min at room temperature in polystyrene reaction vials. The transfection-reagent-DNA mixture was added directly to CHO, MAC13 (0.2×10^5 cells) or GM892A cell cultures (0.8×10^5 cells) seeded in 1 ml of medium in 24 well Nunc plates. After incubation for 24 hr, the medium was aspirated from the monolayer cells and replaced by fresh medium, and the cells were incubated for a further 48 hr before counting.

6.2.3 Experiments to investigate a role for poly(ADP-ribose) synthetase in the cytotoxic mechanism of temozolomide.

6.2.3.1 Determination of cellular ATP levels following temozolomide treatment.

6.2.3.1a Drug treatment and perchloric acid extraction.

Raji and GM892A cells (2×10^5 cells/ml in exponential growth) were treated with the concentrations of temozolomide or MNNG as indacted in Fig 6.5. At the times indicated cell samples were removed, cell number counted and viability determined by trypan blue exclusion. The cells were centrifuged, resuspended in five volumes 0.6N perchloric acid, and the suspensions sonicated and centrifuged (5000g, 5min). Dipotassium hydrogen phosphate (20μl of 0.1M per 100μl supernatant) was added, the pH adjusted to 7.2-7.4 with 3 N KOH and the supernatant stored at -70°C.

6.2.3.1b Determination of adenine nucleotide levels following temozolomide treatment.

Perchloric acid extracts were prepared from cells treated with various concentrations of temozolomide or MNNG, and were analysed by HPLC, performed as essentially described by Crescentini and Stocchi (1984) using a 5 μm C-18 LichoCART column (0.5x20 cm) protected by a C-18 guard column with absorbance monitored at 254 nm. Gradient elution was used at a flow rate of 1ml/min with a first eluent of 0.1M potassium dihydrogen phosphate buffer (pH 6.0) followed by elution with 0.1M potassium dihydrogen phosphate buffer (pH 6.0) containing 10% methanol. The chromatographic conditions consisted of elution for 4 min at 100% of the first buffer and then a linear gradient up to 100% of the second buffer over a 9 min period. The gradient was then returned to 100% of the first buffer in 1 min and the initial conditions allowed to regenerate over 6 min. Under these conditions, retention times for ATP, ADP, AMP, NADP+ and NAD+ markers were 4.3, 5.2, 10.1, 11.9 and 14.7 min respectively.

ATP, ADP, AMP, NAD^+, and $NADP^+$ standards were prepared as 5mM stock solutions in 0.1 M potassium dihydrogen phosphate buffer solution (pH 6.0) (stored at -20°C) and were diluted and analysed to give standard concentration curves for determination of the nucleotide levels in perchloric acid extracts, which were expressed per 10^6 viable cells.

6.2.3.2 Effects of inhibition of ADPRT on the sensitivity of cell lines to temozolomide.

GM892A (mer-) and Raji (mer+) cells were seeded at 0.8×10^5 cells/ml with or without 5mM 3-aminobenzamide, a potent inhibitor of ADPRT. The cells were treated with a range of concentrations of temozolomide or solvent controls and, after incubation for 72 hr, the cells were counted. The level of inhibition of cell population growth caused by temozolomide was determined and compared in the presence or absence of 3-aminobenzamide.

6.2.3.3 Effect of cellular spermine depletion on cell line sensitivity to temozolomide.

Raji and GM892A cells were incubated for 72 hr with or without 0.5 mM DFMO. The cells were then seeded in fresh medium and the growth inhibition elicited by temozolomide was determined as in section 3.2.1.

6.2.4 Investigation of the involvement of post-replication repair in resistance to temozolomide.

6.2.4.1 The effect of caffeine on cell line sensitivity to temozolomide.

Human and rodent cell lines were seeded at 0.2-0.8 x 10^5 cells/ml in the presence or absence of caffeine (0.75 mM), an inhibitor of post-replication repair, and treated with a range of concentrations of temozolomide. The effects on cell population growth were determined following incubation for 72 hr.

6.2.4.2 The sensitivity of cell lines to 6-thioguanine.

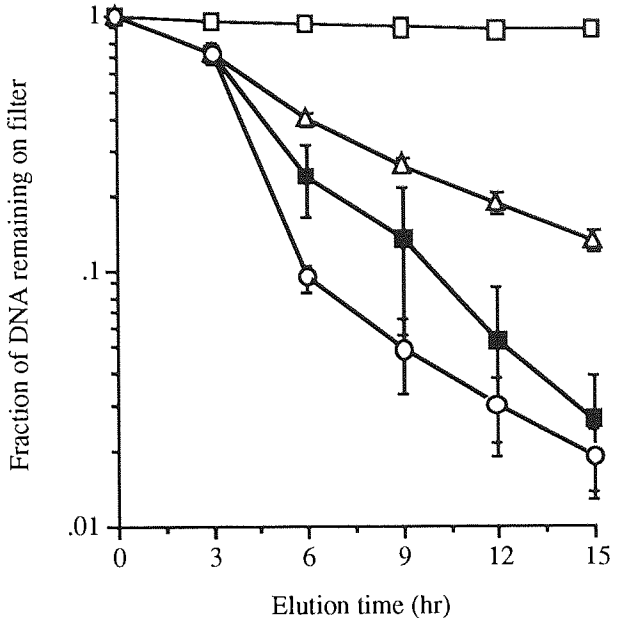
Population growth inhibition studies were performed over a range of concentrations of 6-thioguanine against GM892A, Raji, CHO, MAC13 and MAC16 cell lines. In addition, the effect of 0.75 mM caffeine on the sensitivity of the rodent cell lines to this agent was examined. ID_{50} concentrations were determined as described in section 3.2.1.

6.3 Results

6.3.1 Determination of temozolomide-induced single-strand DNA breaks.

The frequency of DNA single strand breaks induced by alkylation damage with temozolomide was studied by the alkaline (filter) elution technique. The elution profiles for temozolomide treated Raji cells show dose dependent formation of strand breaks (Fig. 6.1a), generally accepted as a feature of excision-repair of DNA lesions, although some apurinic alkali labile sites, may be converted to strand breaks at the pH(12.1) employed in the alkaline elution procedure. To investigate whether alkylation at the O^6-guanine could contribute to strand-breakage, elution rates have been compared for O^6-methylguanine treated and untreated Raji cells following exposure to temozolomide (250μM) (Fig 6.1b). While controls showed little strand breakage both for untreated and alkyltransferase depleted cell populations, after exposure to temozolomide Raji cells with a reduced capacity for repair exhibited a marked increase in strand breakage over alkyltransferase proficient Raji cells. Alkyltransferase depletion using the free base O^6-methylguanine has been demonstrated to sensitise Raji cells to the cytotoxic effects of temozolomide (section 3.3.2). Thus, the formation of DNA strand-breaks appears correlated with methylation at the O^6-position of guanine, and in addition may be important to cytotoxicity.

a)



b)

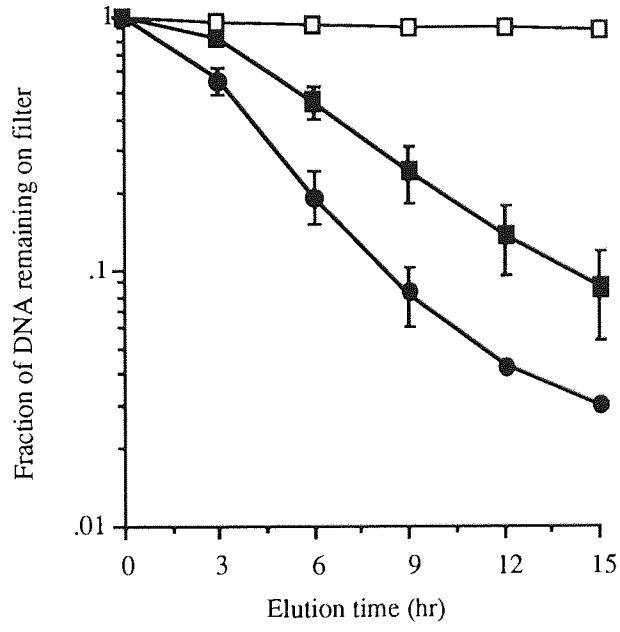


Fig.6.1. Single-strand breaks and alkali-labile sites in DNA after exposure of Raji cells to temozolomide. (**a**) Cells were treated with 100μM (△), 250 μM (■), and 500μM(○) temozolomide, and strand breakage was assessed by the alkaline-elution technique, and compared with that for untreated cells (□). The DNA strand-break frequency after temozolomide (250μM) treatment of control(■) and O^6-MeG (0.5 mM, 16 hr) treated, alkyltransferase depleted, Raji cells (●), is shown in (**b**), along with untreated controls for Raji and Raji + O^6-MeG (□), which had identical elution rates. Values are the mean for 2 experiments ± SD.

6.3.2 Experiments investigating the effect of transfection of cells with DNA extracted from temozolomide treated cells.

Attempts have been made to repeat the growth suppression by DNA from cells treated with imidazotetrazinones observed by Hepburn and Tisdale (1991). The effect on cell population growth of transfection into MAC13 cells of DNA extracted from GM892A and Raji cells 6hr after treatment with a range of concentrations of temozolomide is shown in Fig 6.2a. Little effect on cell population growth was observed at ID_{50} concentrations of temozolomide, compared with an approximate 75 % inhibition of growth reported by Hepburn and Tisdale (1991), or at concentrations far in excess of these values. Studies were repeated in an alternative cell line, CHO (Mer-) (Fig. 6.2b), however, these also proved disappointing. To ensure that differences in the efficiency of the calcium phosphate transfection procedure were not the cause of the lack of response, the uptake of [^{3}H]DNA into these cell lines has been examined (Fig.6.3). These confirmed uptake of calcium phospate-DNA precipitate by both cell lines, and although in these studies uptake by the MAC13 cell line appeared less efficient than in the studies reported by Hepburn and Tisdale (1990), discrepancies are insufficient to explain the results.

Further studies have been performed using liposome transfection as an alternative technique for introducing the temozolomide treated DNA into cells. This technique is less toxic than the calcium phosphate procedure and, in addition, can be used with non-adherent cell lines. However, although the level of cellular DNA uptake was not determined for this procedure, transfection of MAC13 and GM892A cells with DNA extracted from temozolomide treated cells using this technique again failed to produce an effect on cell population growth (Fig.6.4).

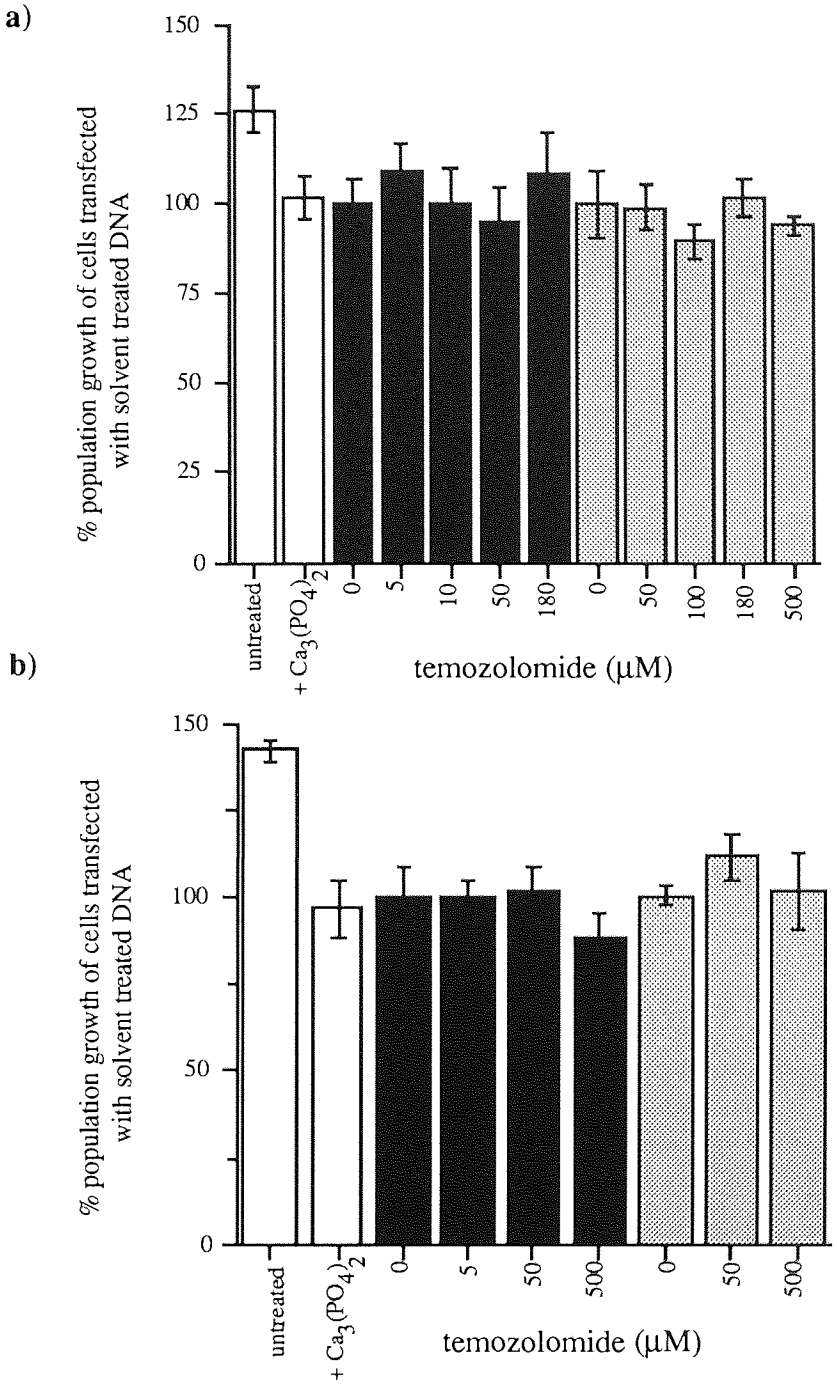


Fig.6.2. Effect on growth of (a) MAC13 and (b) CHO cells of $Ca_3(PO_4)_2$ transfection with DNA isolated from GM892A (■) and Raji (▨) cells. Cells were transfected with calcium phosphate co-precipitated DNA (30μg) isolated from solvent control cells or cells which had been exposed to a range of concentrations of temozolomide, including the ID_{50} concentrations, for 6 hr. The effect on the subsequent cellular proliferation was determined and compared with non-transfected cells (□). Values are the mean ±SEM for 3 seperate determinations.

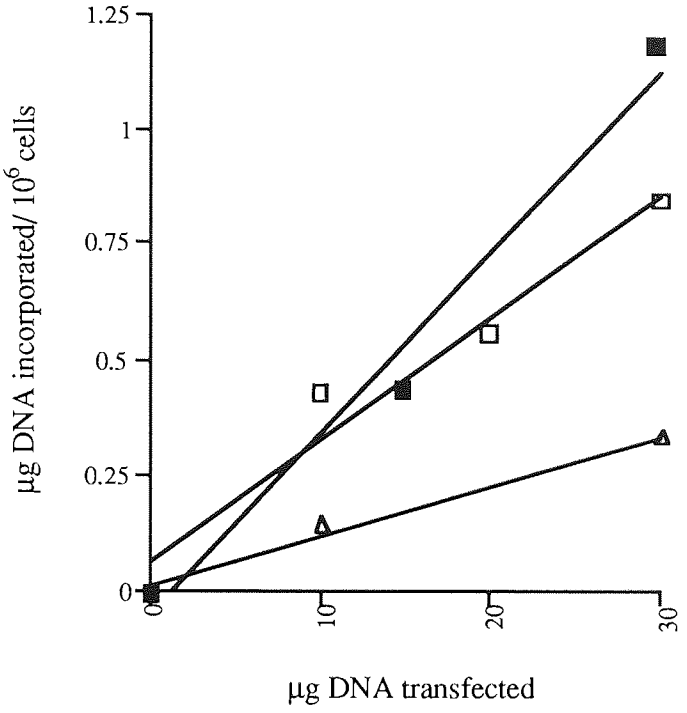
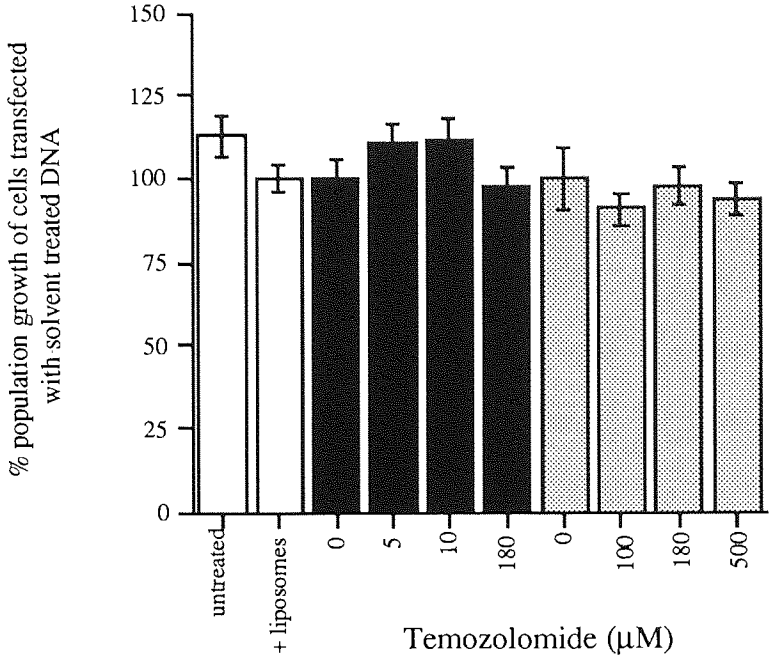


Fig.6.3. Level of [^{3}H] DNA incorporation into cell lines by calcium phosphate transfection. MAC13 (▲) and CHO (□, ■) cells were allowed to attach overnight and were treated with a calcium phosphate-[^{3}H] DNA co-precipitate prepared from labelled DNA that had been extracted from GM892A (closed symbols) or Raji cells (open symbols). Following incubation for 5hr the cells were extensively rinsed with PBS, and incubated in medium for 16 hr, afer which the cells were harvested and repeatedly washed. The amount of DNA was determined from radioactive counts following cell lysis. Non-specific binding of the labelled DNA to the cell membrane was excluded by comparison with controls in which cells were treated with [^{3}H]DNA in the absence of calcium phosphate. Values are the mean for 2 transfected cell populations.

a)



b)

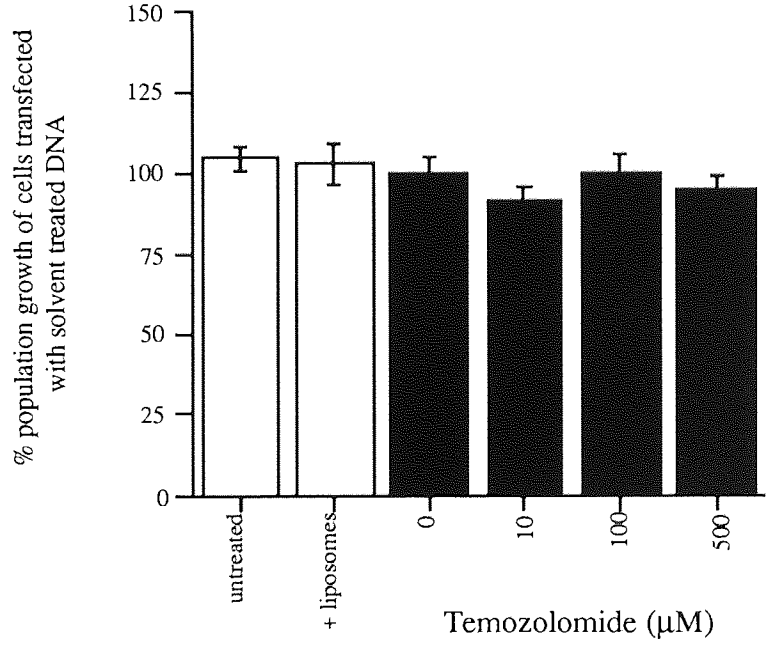


Fig.6.4. Effect on growth of (a) MAC13 and (b) GM892A cells of liposome transfection of DNA isolated from GM892A (■) and Raji (▨) cells which had been exposed to temozolomide. Cell population growth was compared with that for cells transfected with DNA extracted from solvent treatred populations, and also in the absence of DNA (□) and transfection reagents (untreated). Results are expressed as mean ± SEM, for at least 3 determinations.

6.3.3 Experiments investigating a role for poly(ADP-ribose) synthetase in the cytotoxic mechanism of temozolomide.

The activation of ADPRT, caused by the appearance of DNA strand-breaks, may result in the utilisation of NAD^+ for the synthesis of poly (ADP-ribose). A comparison has been made of the effects of temozolomide and MNNG on cellular levels of adenine nucleotides in two cell lines differing in alkyltransferase capacity. Treatment of Raji (Mer+) and to a lesser extent GM892A (Mer-) cells with MNNG (100μM) resulted in rapid and almost total depletion of NAD^+ and $NADP^+$ leading to a consequent reduction in ATP and hence accumulation of ADP and AMP (Fig. 6.5). While NAD^+, $NADP^+$ and ATP levels remained depleted at this concentration, ADP and AMP levels rapidly returned to approximately normal levels, an imbalance that probably reflects the leakage of nucleotides from the ATP depleted cells. However, this concentration of MNNG is associated with extreme growth inhibition and cell killing in both Raji and GM892A cell lines (Fig.6.7), and is far in excess of chemotherapeutic levels. At a lower concentration of MNNG (6μM), causing inhibition of viable cell population growth equivalent to 500μM temozolomide, the effect was much reduced. At this concentration only a slight decrease in NAD^+/ATP was evident and levels rapidly returned to normal. In addition, this depletion was greater in Raji than GM892A cells, illustrated most prominantly by the increases in ADP and AMP shown in Fig 6.5, suggesting that the effect was not related to repair of O^6-methylguanine. The most profound effect at this concentration of MNNG was a reduction in the $NADP^+$ pool size, although levels rose again after 3 hr. However, temozolomide (500μM) caused no depletion of $NADP^+$ in GM892A cells and only a slight decrease in the Raji cell line, while having little effect on NAD^+/ATP levels. Adenine nucleotide levels appeared unaffected upto 96 hr after treatment, with even a slight elevation in ATP levels (Fig.6.6), a feature that may be attributable to the cell cycle inhibitory effects of temozolomide.

These differences between Raji and GM892A cells, and MNNG and temozolomide, may be reflected by the level of cell necrosis (Fig.6.7). At 100μM

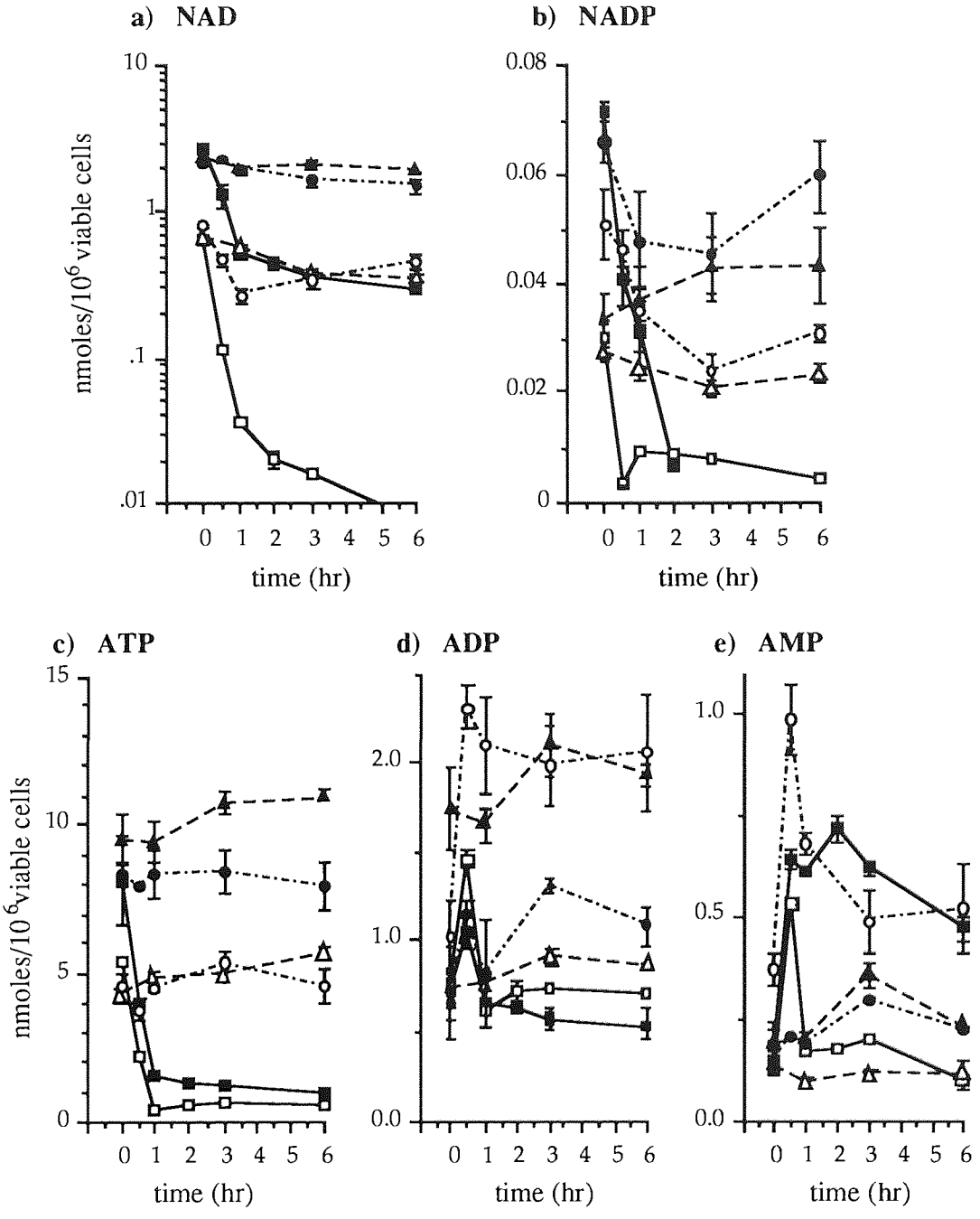


Fig. 6.5. Effect of alkylating agents on cellular adenine nucleotide levels .
GM892A (closed symbols) and Raji (open symbols) cells were treated with MNNG (6μM-●- /-○-, 100μM-■-,/-□-) or temozolomide (500μM-▲-/-Δ-) and cellular levels of (a) NAD, (b) NADP, (c) ATP, (d) ADP and (e) AMP were determined from HPLC analysis of perchloric acid extracts. Results are expressed as the concentration of nucleotide /10^6 viable cells, as determined by trypan-blue exclusion assay. Values are the mean ±SEM of extracts from 3 separately treated cell populations.

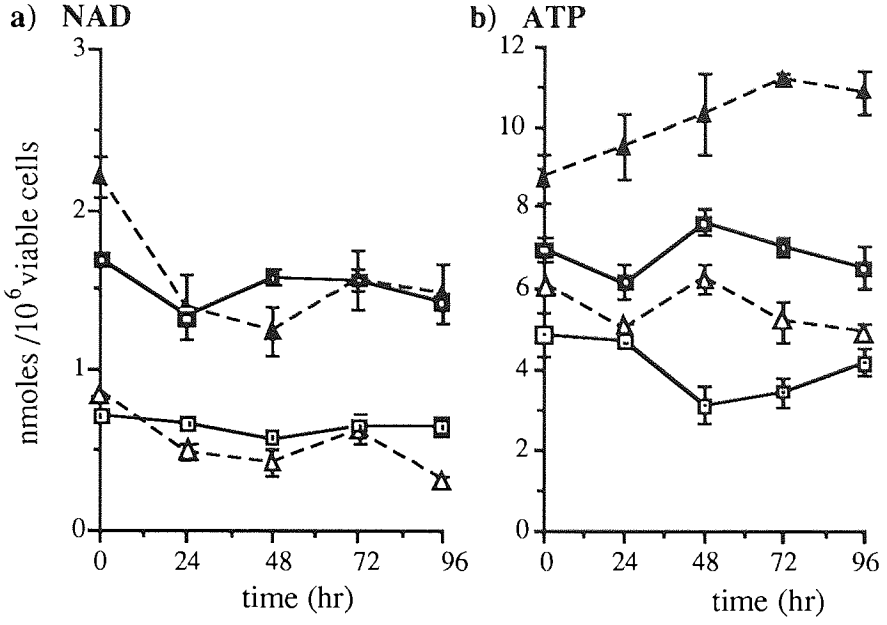


Fig.6.6. Cellular adenine nucleotide levels after treatment with temozolomide. (a) NAD and (b) ATP levels are shown for GM892A (closed symbols) and Raji cells (open symbols) for 96 hr following exposure to temozolomide (500μM, –▲– /–△–). Nucleotide levels are compared with those found for solvent treated control cell populations (–■– /–□–). Results are the mean of 3 determinations ±SEM.

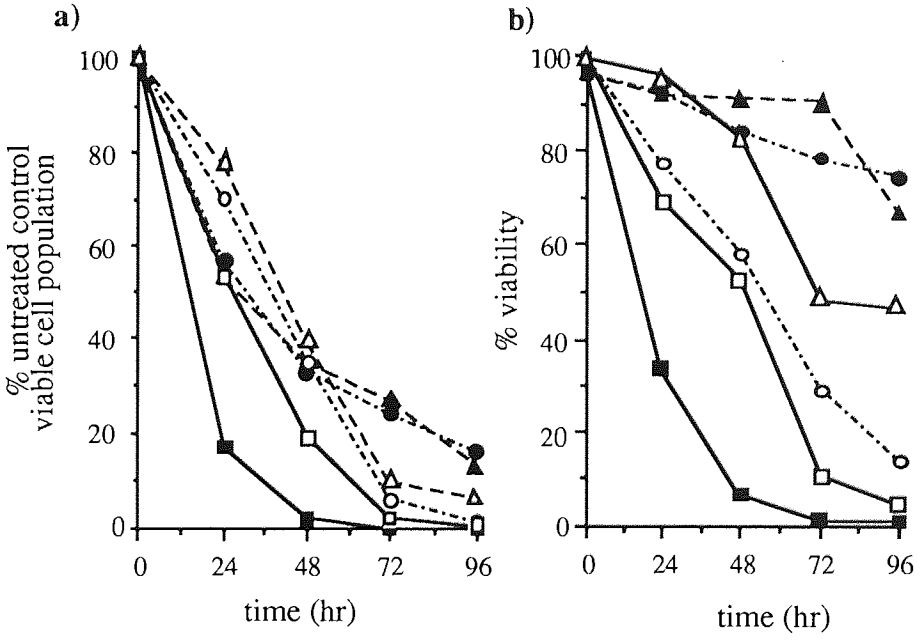


Fig.6.7. Effect of drug on (a) cell population growth and (b) viability. GM892A (closed symbols) and Raji cells (open symbols) were exposed to MNNG (6μM ··●·· /··○··, 100μM –■– /–□–) and temozolomide (500μM –▲– /–△–) and the viable cell population was determined by trypan-blue exclusion assay. Results are the mean of 3 treated cell populations. With the exception of 24 hr after exposure to 100 μM MNNG (± SEM= 19.5% and 12.8%, for GM892A and Raji cells respectively), standard errors did not exceed 10%.

MNNG cell viability, as determined by trypan-blue exclusion assay, decreased rapidly. However, necrotic cell death was less rapid than might be anticipated given the immediacy of the depletion of ATP. In addition, the decrease in cell viability was more rapid in GM892A than Raji cells, suggesting that other factors may influence loss of cellular integrity. Following exposure to 6μM MNNG and 500μM temozolomide the decrease in viability was less pronounced. While the number of viable cells was similar for both drugs (Fig.6.7a), unlike MNNG the % viability did not decrease significantly in temozolomide treated cell populations until 48-72 hr after temozolomide treatment (Fig.6.7b). Viable cell numbers remain equal since temozolomide treatment appears to inhibit cell division more effectively than MNNG at these concentrations, while not initiating cell death as rapidly.

These studies show that activation of ADPRT in response to DNA damage by high concentrations of MNNG can cause drastic alterations in metabolic pool sizes, which can result in rapid changes in cell viability. However, over the range of concentrations of temozolomide likely to be relevant *in vivo* it is unlikely that ADPRT activation could be responsible for cell death, and changes in cell viability are probably not manifested by indirect metabolic consequences of DNA damage.

The role of ADPRT in DNA repair has been investigated by inhibition of the enzyme by the nicotinamide analogue 3-aminobenzamide (3-AB). The effect of 3-aminobenzamide on the growth inhibition caused by temozolomide in GM892A and Raji cells is illustrated in Fig.6.8. Temozolomide cytotoxicity was greatly enhanced in Raji cells, with a reduction in the ID_{50} value from 170 ± 14μM to 79 ± 13μM. In contrast, there was no potentiating effect of 3-AB on the Mer- GM892A cells, and the ID_{50} value remained little changed (control = 14 ± 3μM,+3-AB = 10 ± 3μM).

To investigate the possible importance of modulation of chromatin structure by ADPRT to this effect, response to temozolomide has been determined in polyamine depleted cells (Fig.6.9). However, exposure to DFMO (0.5 mM) did not sensitise GM892A or Raji cells to the growth inhibitory effects of temozolomide.

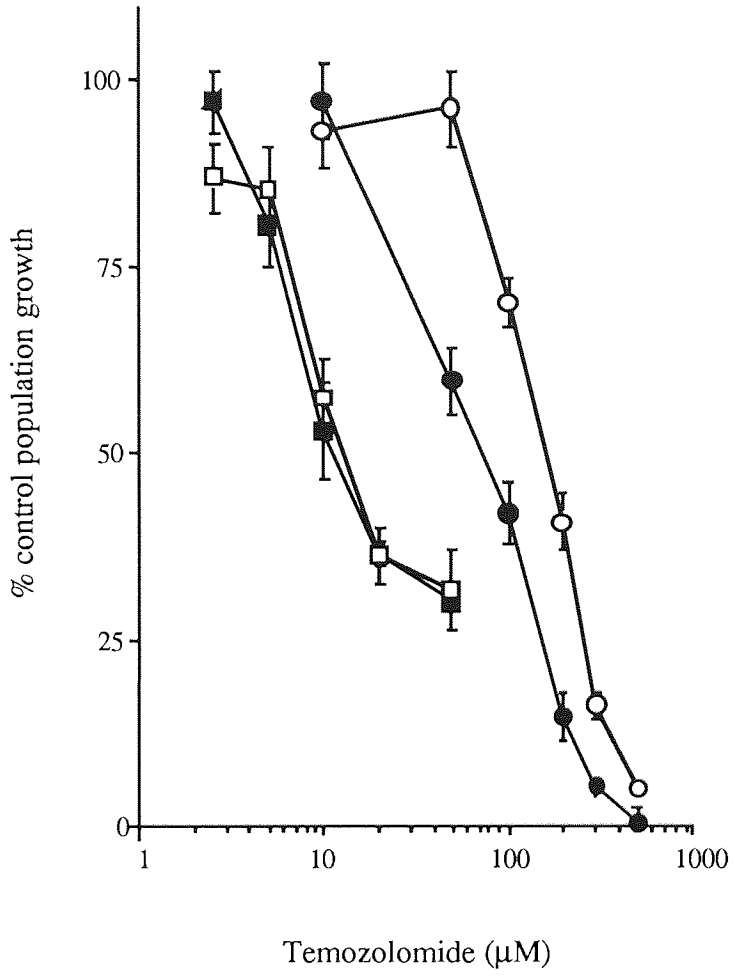


Fig. 6.8. Effect of 3-aminobenzamide (3-AB) on temozolomide cytotoxicity. Raji (Mer+) (○, ●) and GM892A (Mer-) cells (□, ■) were exposed to a range of concentrations of temozolomide in the presence (closed symbols) or absence (open symbols) of 3-AB (5mM). Population growth was determined relative to untreated control cell populations (± 3-AB). Values are the mean ± SEM for 3 experiments.

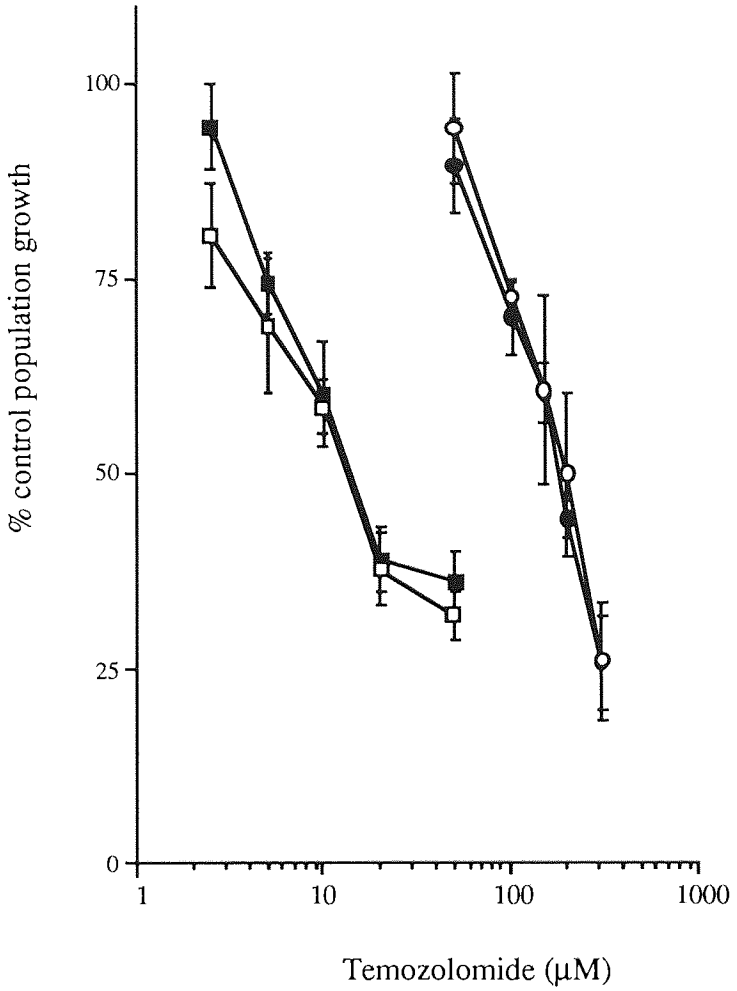


Fig. 6.9. Effect of DFMO on temozolomide cytotoxicity *in vitro*.
Raji (○ , ●) and GM892A cells (□ , ■) were exposed to a range of concentrations of temozolomide in the absence of DFMO (open symbols), or following pretreatment with, DFMO (0.5 mM, 72 hr) (closed symbols). Population growth was determined relative to untreated control populations (± DFMO). Values are the mean ± SEM for 3 experiments.

6.3.4 Experiments investigating a role for post-replication repair in resistance to temozolomide.

The contribution of post-replication repair to resistance to temozolomide has been investigated in a range of human and rodent cell lines. Growth inhibition studies were performed in cells treated with caffeine (0.75mM), a proposed inhibitor of post-replication repair, the results for which are shown in Fig.6.10 for the rodent cell lines. The ID_{50} values from these studies are compared in table 6.1, and show a marked potentiation of the cytotoxicity of temozolomide in murine MAC13 and MAC16 cell lines, and to a lesser extent in the the Chinese hamster CHO cell line. However, human glioma and lymphoid cell lines showed little variation from controls.

In addition, the toxicity of 6-thioguanine (6-TG) towards cell lines (6.11), and the effect of caffeine on 6-TG toxicity in the rodent cell lines has been determined, and the ID_{50} values compared with those for temozolomide (Table.6.1). Caffeine treatment resulted in a similar sensitisation to that found with temozolomide, although the potentiation of the MAC13 cell line was less pronounced. It was hoped that 6-TG toxicities might reflect the post-replication repair capacities of these cell lines, although the results are without doubt complicated by additional factors that determine sensitivity to this agent. It is however interesting that the CHO cell line appears more resistant than the GM892A to 6-TG, since temozolomide is similarly less active towards CHO cells despite both cell lines possessing minimal levels of O^6AGAT. Furthermore, the Raji cell line is more sensitive to temozolomide and 6-TG than the MAC16 cell line, while having similar alkyltransferase efficiency, features that could in part be explained by differences in the level of post-replication repair between these cell lines.

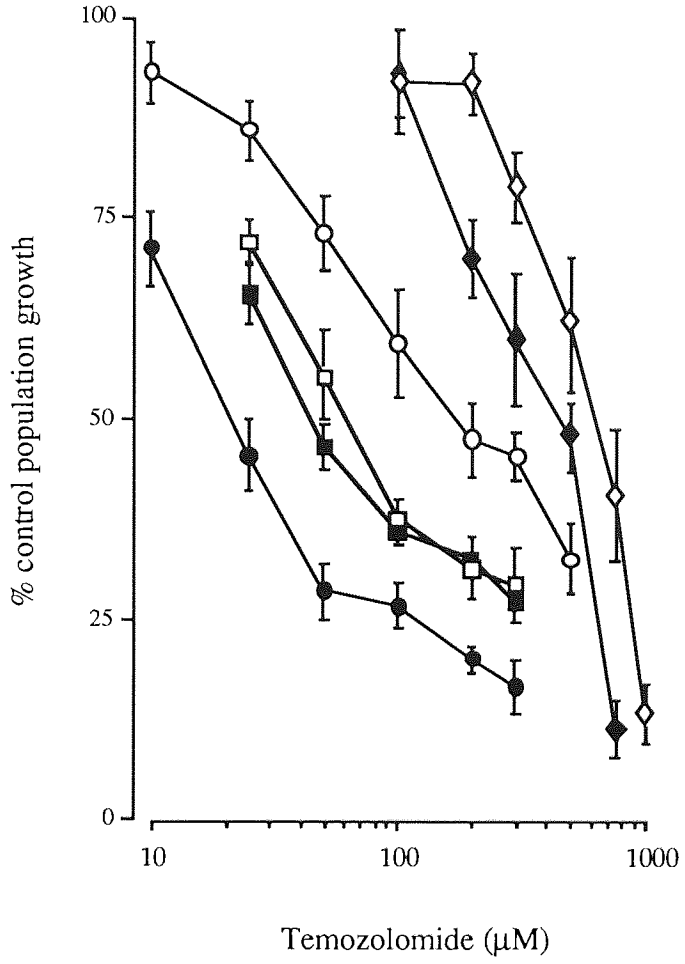


Fig. 6.10. Effect of caffeine on temozolomide cytotoxicity in rodent cell lines. MAC16 (◇, ◆), MAC13 (○, ●) and CHO (□, ■) cells were exposed to a range of concentrations of temozolomide in the presence (closed symbols) or absence (open symbols) of caffeine (0.75 mM). Population growth was determined relative to untreated control cells (± caffeine). Values are the mean ± SEM for three experiments.

Table 6.1. Effect of temozolomide and 6-thioguanine on population growth of (a) untreated cell lines and (b) cell lines exposed to caffeine (0.75 mM).

Cell line	O^6-AGAT * (fmol/mg protein)	ID_{50} (μM) ±SEM temozolomide			6-thioguanine		
		(a)	(b)	% b/a	(a)	(b)	% b/a
GM892A	22.8	6.8 ± 1.1	8.5 ± 1.0	125	0.42 ± 0.04	-	-
Raji	372	218 ± 24.5	225 ± 13.0	103	0.88 ± 0.08	-	-
GL 7	18.9	50.0 ± 13.1	58.5 ± 14.6	117	-	-	-
GL 18	127	150 ± 28	200 ± 38.5	133	-	-	-
GL 16	307	260 ± 37.5	285 ± 21.0	110	-	-	-
CHO	4.7	64.9 ± 12.8	45.8 ± 4.1	70.6	1.09 ± 0.20	0.84 ± 0.07	77.1
MAC 13	44	180 ± 65.2	22.7 ± 2.9	12.6	0.48 ± 0.06	0.34 ± 0.05	70.8
MAC 16	320	642 ± 55	466 ± 61	72.6	1.79 ± 0.28	0.63 ± 0.01	35.2

* Values from chapters 3 and 5.
Results are the mean of at least 3 determinations ± SEM.

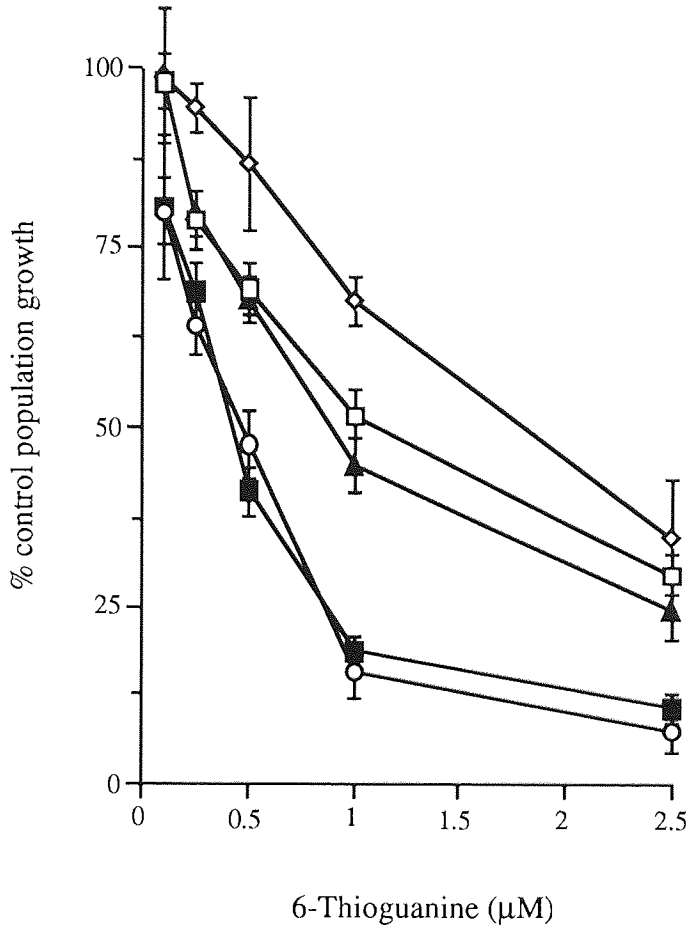


Fig. 6.11. Effect of 6-thioguanine (6-TG) on cell population growth.
CHO (□), MAC16 (◇), MAC13 (○), Raji (▲) and GM892A cells (■) were exposed to a range of concentrations of 6-TG and cell population growth determined relative to untreated controls. Values are the mean ± SEM for three experiments.

6.4 Discussion.

One consequence of the methylation of cellular DNA is the appearance of DNA single-strand breaks. If not rapidly filled in by DNA polymerase and re-ligated by DNA ligase such sites may be of cytotoxic significance. Glycosylase enzymic excision-repair activities and the slow spontaneous hydrolysis of N-alkylated purine bases are accepted as the major sources of strand-breaks. However, a mechanism by which alkylation of the O^6-position of guanine could result in strand breakage and whether such breaks constitute a cause of cytotoxicity remains unproven. An isogenic O^6-AGAT defective HeLa cell mutant has been found to exhibit elevated DNA strand breaks and cytotoxicity after exposure to MNNG but not DMS (Kalemegham *et al.*, 1988), while a similar occurrence of excess strand breaks has been observed in a Mer- cell line (A-172)(unpublished studies R.Kalemegham and R.Day). Consequently, it has been suggested that failure to remove O^6-methylguanine lesions results in strand breaks and that these are the primary cause of cytotoxicity. Further support for this proposal has come from the present study, in which DNA strand breakage has been determined following exposure to temozolomide of Raji (Mer+) cells depleted in cellular alkyltransferase levels by pretreatment with O^6-methylguanine. Alkyltransferase depletion caused a significant increase in the single-strand breaks elicited by temozolomide alkylation, an increase that corresponds with sensitisation to the cytotoxic effects of temozolomide (section 3.3.3). Similarly, Taverna *et al*. (1992) have shown alkyltransferase depletion of an L1210 BCNU resistant subline (L1210/BCNU Mer+) to potentiate the toxicity of temozolomide and result in increased DNA single-strand breaks, while L1210 (Mer-) cells remain little affected. In addition, alkaline elution studies in HT29 (Mer+) and BE (Mer-) cell lines have indicated increased breakage in the Mer- cell line 12 hr after exposure to equimolar doses of MTIC (Gibson *et al.*, 1986). However, while the Mer- cell line was considerably more sensitive to the cytotoxic effects of MTIC, at equitoxic doses the Mer+ cell line exhibited much greater DNA strand breakage. In these studies, the sensitivity of the alkaline elution reported by Gibson *et al*. (1986) appeared low, however the apparent discrepancies question the link between the observed strand breakage and cytotoxicity.

It has been suggested that an O^6-methylguanine excision-repair process may occur, and that this could account for single-strand breaks (review Day *et al.,* 1990). Alternatively, gaps may arise due to DNA mismatch repair activity opposite O^6-methylguanine, since Mer- human cells have been shown to perform more MNNG-induced DNA repair synthesis (Day *et al.*, 1980b, Scuderio *et al.*, 1984b). This DNA repair synthesis has recently been shown to be non-semiconservative and to depend on the presence of O^6-methylguanine in the substrate DNA in *in vitro* studies with human cell extracts (Karran *et al.*, 1993). It has been proposed that this repair synthesis may represent unsuccessful attempts to remove O^6-methylguanine from DNA due to incorrect targeting of mismatch excision repair to the poorly paired cytosine in the strand opposite O^6-methylguanine. The inability to find a complementary base for the methylated purine, would inevitably be associated with long-lived nicks in the DNA (Karran *et al.*, 1993). DNA replication *in vitro* is more frequently accompanied by the incorporation of thymine bases than cytosine bases opposite O^6-methylguanine (Singer *et al.*, 1989) and an incision activity at O^6-methylguanine:thymine mispairs in DNA has been identified in human cell extracts (Sibghat-Ullah and Day, 1992). Incisions both 5′ and 3′ to the T in a O^6MeG:T mismatch are thought to leave a one nucleotide gap which following endonuclease processing may be filled in by polymerase ß and religated. If the O^6-methylguanine remains unrepaired by O^6-AGAT dTMP may be preferentially incorporated across from O^6MeG regenerating the poorly matched O^6MeG:T substrate. This could account for the observation of elevated incorporation of [^{3}H] thymidine into the DNA of Mer- cells compared with that of Mer+ following MNNG treatment (Scuderio *et al.*,1984, Day, 1987). In addition, the pool sizes of dTTP and dCTP influence the frequency of formation of the O^6MeG mismatch in a cell free system (Abott *et al.*, 1979), and several studies have shown elevated dCTP levels to protect, and increased dTTP to sensitise cells to the cytotoxic effects of MNNG (Peterson and Peterson, 1982). Consequently, it appears that long-lived incisions could initiate biological effects specific to O^6-methylguanine including sister chromatid exchanges (Day *et al.*, 1980b) and more significantly in cell lethality.

Repair of O^6-methylguanine by an alternative mechanism to O^6-AGAT has been considered as a possible explanation for the hypersensitivity of some Mer+ cells (Scuderio *et al.*, 1984b) and the resistance of Mer- cells lines to MNNG (Ikenaga *et al.*, 1987). Following the isolation of a Mer- yet MNNG resistant TK6 cell line variant, one suggestion has been that cells posses a non-excision defence mechanism that allows tolerance of DNA damage (Goldmacher *et al.*, 1986). One model for tolerance has suggested that the loss of the mismatch repair system in tolerant cells may prevent its improper action, however, a definite explanation of the tolerant phenotype awaits clarification.

Proof that tolerance is specific for O^6-methylguanine has been provided by Green *et al.*(1989) who have demonstrated that transfection of parental MRC5 (Mer-) human fibroblasts with the *E.Coli ada* methyltransferase gene conferred resistance to MNU, while MRC5 (Mer-) cells previously selected for tolerance to MNU showed no increased resistance. A further property of the the tolerant MRC5 cells was their cross-resistance to 6-thioguanine (6-TG), a base analog that requires incorporation into DNA to elicit cytotoxicity (Green *et al.*, 1989). Aquilina *et al.*, (1990) have extended this observation for tolerance to both O^6-methylguanine and 6-TG in CHO clones, while resistance in CHO cell lines was due neither to a loss in hypoxanthine-guanine phosphoribosyltransferase (HPRT) activity nor to a decrease in incorporation of 6-TG into DNA. A mammalian gene has recently been cloned that increases resistance of a post replication repair defective and Mer- Indian muntjac mutant cell line (SVM) to UV, MNU, ENU and 6-TG, although not DMS (Godfrey *et al.*, 1992). This has suggested that the cytotoxicity of MNU is in part due to perturbation of DNA replication, and that replication blocks caused by UV, O^6-methylguanine and 6-TG lesions can be overcome by a common error prone post-replication repair mechanism. This repair involves a recombinational process that permits replication to be completed on a damaged template by leaving gaps where DNA polymerase is unable to read past a lesion in DNA. A section of the parent strand then fills in the gap created in the daughter strand. This process has been well characterised in *E.Coli*, while in mammalian cells the evidence is less convincing. However, the formation of transient gaps in DNA by this pathway could account for the apparent correlation between O^6-methylguanine and strand-breakage.

In order to examine the possible contribution of a tolerance mechanism to resistance to temozolomide, the cytotoxic effects of temozolomide have been compared with those of 6-TG in a range of cell lines. Since the level of incorporation of 6-TG into DNA has not been determined, little conclusive evidence can be drawn from these studies, although the higher resistance of the CHO (Mer-) cell line to both temozolomide and 6-TG in comparison with GM892A (Mer-) cells may reflect a mechanism of increased tolerance in this cell line. In addition, the ability to potentiate temozolomide and 6-TG toxicity of caffeine, a proposed inhibitor of post-replication repair, has been examined. Sensitisation to temozolomide was found in MAC13 and MAC16 murine cell lines and to a lesser extent in the Chinese hamster ovary cell line, although not in the human cell lines examined. These results appear to reflect species specific differences in response to caffeine since similar effects have been observed by Byfield *et al.*(1981). These studies indicate caffeine to be of little clinical use as the concentrations of methylxanthines required for sensitisation would be clinically intolerable. In addition, caffeine potentiated the cytotoxicity of 6-TG in MAC16, MAC13 and CHO cells, although the degree of potentiation did not correlate closely with those for temozolomide. Such enhancement has been ascribed to the inhibitory effect of caffeine on the gap-filling process of post-replication repair (Lehmann, 1974). In this regard, Roberts and Basham (1990) have reported caffeine to increase the toxicity of MNU in MNU-tolerant HeLA/A22 (Mer-) cells but not in parental MNU-sensitive HeLa (Mer-). However, more recently the enhancement produced by caffeine has been attributed to its ability to reverse DNA damage-induced G_2 delay (Painter, 1980). By reducing the G_2 delay caffeine appears to lower the time available for repair, causing the cells to arrive at mitosis with unrepaired DNA damage. Other studies are consistent with this model (Lau *et al.*, 1982, Das *et al.*, 1982, Fingert *et al.*, 1986, Roberts and Basham, 1990). In addition, this may explain the dramatic caffeine-induced potentiation of temozolomide in the MAC13 cell line, compared with only moderate sensitisation to 6-TG. Thus, loss of G_2 delay may prevent efficient alkyltransferase repair of O^6-methylguanine. Cytotoxicity and strand-breakage studies in UV sensitive CHO/UV20 cells have suggested 6-TG to be repaired by excision repair, in addition to post-replicative repair (Christie *et al.*, 1984). Consequently, the potentiation of 6-TG by caffeine may in part be due to a reduction in the time available for this excision repair. As such, the multiple effects of caffeine in cells

prevents specific determination of the role of post replication repair in resistance 6-TG and temozolomide.

In addition to DNA repair, other metabolic processes involve the production of DNA breaks (review Eastman and Barry, 1992). Topoisomerases induce transient DNA breaks followed by unwinding of the helix, and religation of the DNA. This is associated with chromosome segregation at mitosis, and in release of torsion in DNA induced by replication and transcription. Except indirectly by topoisomerases, the process of DNA replication itself does not lead to production of strand breaks. However, it involves discontinuous synthesis of short sections of DNA known as Okazaki fragments on the lagging strand. If unligated, these fragments would be detected as strand breaks in the alkaline elution protocol. DNA breaks may also result from the digestion of DNA by endogenous deoxyribonucleases. The intracellular function of these endonucleases is uncertain. Some are thought to be involved in lysosmal degradation, while others could function in DNA repair or Ca^{2+} activated degradative processes. These degradative enzymes include Ca^{2+}/Mg^{2+}-dependent endonucleases which are thought to function in the production of DNA double strand breaks during apoptotic cell death, a process that can be induced by DNA damage (Marks and Fox, 1991).

The growth suppressive activity of DNA extracted from cells exposed to temozolomide and introduced into MAC13 cells has suggested that sites after DNA are ultimately responsible for cytotoxicity (Hepburn and Tisdale, 1991). It was envisaged that further studies might elaborate on the correlation between alkyltransferase repair capability and the growth suppressive effect of DNA extracted from temozolomide treated cells. However, attempts at repeating these studies failed to substantiate the results of Hepburn and Tisdale (1991). It is plausible that differences in technique could account for the discrepancies between the results, although the efficiency of the calcium phosphate DNA transfection technique was confirmed. As such, differences may have occurred in the DNA extraction procedure and the length of the DNA, or purity from RNA or protein may prove important to the effect. Studies were repeated in the CHO (Mer-) cell line, which it was hoped might be more sensitive to the growth suppressive effect than the MAC13 cell line due to its lower alkyltransferase repair capability. However, these along with further studies in which a liposome DNA transfection technique was used to introduce DNA into MAC13 and GM892A (Mer-), proved

similarly disappointing.

Since it is unlikely that much of the alkylated DNA is actually incorporated into cellular DNA, if correct, the results of Hepburn and Tisdale (1990) may imply that a controlled form of cell division or cell death could exist and could be responsible for the cytotoxicity of the imidazotetrazinones. In view of the suggestion that the DNA strand break activated enzyme ADPRT could cause lethal depletion of NAD^+ in response to extensive DNA damage, cellular adenine nucleotide levels have been determined following exposure of Raji and GM892A cells to temozolomide. Tisdale (1985b) found a delayed dose dependent elevation of ADPRT activity , with maximal activity (3.5 fold at 73.5 μM) after 48 hr following treatment of K562 cells with temozolomide. Consequently, nucleotide levels were monitored for up to 96 hr after exposure to the cytotoxic. Comparison was made with MNNG an agent which at supratoxic concentrations (100μM) has been demonstrated in this and previous studies (Berger *et al.*, 1986) to cause rapid and sustained depletion of NAD^+ and hence ATP, resulting in loss of cell viability. However, at a concentration of temozolomide (500μM) that caused greater than 90% inhibition of cell population growth in both cell lines there was no depletion in the NAD^+ pool size, although an equitoxic concentration of MNNG (6μM) caused a transient decrease in NAD^+ and ATP. Lunn and Harris (1988) have shown MTIC to produce an NAD^+ drop, although this was confined to extreme MTIC levels (>1mM), far in excess of those attainable *in vivo*. Thus, the postulated ADPRT induced 'suicide' mechanism appears unlikely to be relevant to the chemotherapeutic action of temozolomide or MNNG.

The exact role of ADPRT is poorly understood. It is thought to participate in cellular processes in which cleavage and rejoining of DNA may be required. Thus, it appears important to differentiation, DNA recombination, and also in DNA replication and DNA repair (review Boulikas, 1993). The effect of 3-AB, an inhibitor of ADPRT, on cell line sensitivity to temozolomide has been determined, and cytotoxicity found to be potentiated in the Raji (Mer+) cell line but not GM892A (Mer-) cells. Studies by Lunn and Harris (1988) have shown a similar differential sensitisation to the cytotoxic effects of MTIC between A549 (Mer+) and VA13 (Mer-) cells in the presence of an alternative

inhibitor 3-acetamidobenzamide. In addition, the enhancement in cell killing was directly proportional to an increase in DNA strand breaks (Lunn and Harris, 1988). This may suggest repair of O^6-methylguanine lesions to be linked in some way with ADPRT repair activity, and may further imply a connection between O^6-guanine methylation and strand-break formation. However, it is apparent that the effect on DNA repair is not specific to O^6-methylguanine repair, since ADPRT inhibition has also been shown to sensitise cells to cisplatin (Chen and Zeller, 1990) and to DMS (James and Lehmann, 1982). Enhanced O^6-AGAT activity has been found in response to ADPRT inhibitors in an MNNG treated rat hepatoma (H4) cell line (Lefebvre and Laval, 1989) and this was suggested to imply poly(ADP-ribose) to be involved in the induction of O^6-AGAT. However, an alternative explanation could be that O^6-AGAT is increased as part of a general DNA damage response that is perhaps induced by the elevated DNA strand-breaks which accompany DNA damage in the presence ADPRT inhibitors.

ADPRT is entirely dependent on DNA strand breaks for its activity, and might be important in regulating the activity of nuclear enzymes involved in the metabolism of DNA strand breaks, such as ligase II and topoisomerases I and II (review Boulikas, 1993). An increase in DNA ligase II, an enzyme involved in the final step of strand break repair, has been shown following exposure of mouse leukaemia cells to DMS, an agent known to stimulate poly(ADP-ribose) synthesis. In addition, inhibitors of ADPRT prevented the elevation in DNA ligase II activity (Creissen and Shall, 1982). Thus, poly(ADP-ribosyl)ation of ligase II may constitute a mechanism of modulating repair of strand breaks. However, *in vitro* experiments by Yoshihara *et al*. (1985) found that ligase II activity was, paradoxically, inhibited after its ADP-ribosylation.

A second possibility is that DNA repair may be controlled via poly(ADP-ribosyl)ation of topoisomerases or DNA and RNA polymerase. In damaged cells modification of these enzymes, resulting in their inactivation, could function to shut down transcription and replication, giving priority to DNA repair (review Boulikas, 1993). Inhibition of ADPRT resulting in the loss of an extended period for repair before replication would account for the potentiation of temozolomide shown in the alkyltransferase proficient Raji cell line, while the deficient GM892A cell line remained unsensitised. However, DNA polymerase ß, an enzyme mainly involved in DNA repair

synthesis is inhibited by poly(ADP-ribosyl)ation *in vitro* (Yoshihara *et al.*, 1985) and a mechanism by which the cell could manage to circumvent replication yet maintain the repair of strand breaks is unclear.

An alternative proposal is that poly(ADP-ribosyl)ation may inhibit chromatin condensation, and might function to increase the accessibility of DNA repair enzymes to transcriptionally inactive genes (review Boulikas, 1993). Other than ADPRT itself histones are the main molecules upon which poly(ADP-ribose) molecules are built, and *in vitro* poly(ADP-ribosyl)ated H1 protein has been shown to prevent the condensation of chromatin into higher order structures (Poirier *et al.*, 1982). Furthermore, depletion of cellular ADPRT by antisense RNA expression results in hypersensitivity of chromatin to DNAaseI digestion (Ding *et al.*, 1992). Consequently, it has been speculated that poly(ADP-ribose) can induce free DNA domains by removing histones from specific nucleosomes whose DNA has been damaged. Gene expression is associated with decondensed chromatin, and actively transcribed genes are known to be repaired more readily and at higher rates than inactive genes (Palombo *et al.*, 1992, Boffa *et al.*, 1992). Thus, inhibition of poly(ADP-ribosyl)ation may hinder the access of O^6-AGAT to DNA damage in regions of condensed DNA. The exact details of the role of ADPRT are ill defined, however, the results obtained following exposure of GM892A and Raji cells to temozolomide in the presence of 3-AB are compatible with such a hypothesis. Consequently, ADPRT activity could play an important role in regulating DNA repair and the cytotoxicity of temozolomide.

Polyamines are ubiquitous cellular cations that bind tightly to nucleic acids, exerting a stabilising influence on DNA structure (review Pegg, 1986). Depletion of cellular polyamines using DFMO, an inhibitor of ornithine decarboxylase, the first and rate limiting step in the biosynthesis of polyamines, has been reported to increase the cytotoxicity of L-phenylalanine mustard (Ducore and McNamara, 1986) and also of BCNU (Seidenfeld *et al.*, 1987). BCNU cytotoxicity was significantly enhanced in HT-29 (Mer+) cells but not BE (Mer-) and consequently the observed potentiation appears to be at least in part due to altered DNA repair. Spermine has also been shown to inhibit MNU alkylation of chromatin DNA at the N^7- and O^6-positions of guanines and the N^3-position of adenine (Rajalakshmi *et al.*, 1978), and consequently it remains in question whether DFMO-mediated potentiation occurs via increased DNA damage. Depletion of

polyamines causes perturbations of the cell cycle and changes in DNA conformation which could affect DNA repair. As regards this, polyamine depleted cells have been shown to be deficient in repair of x-ray induced DNA damage repair (Snyder *et al.*, 1989), although the interaction of Polyamines and DNA repair pathways is poorly understood. In the present study, a similar protocol of DFMO treatment to that used by Seidenfeld *et al*. (1987) was employed, although the depletion of cellular polyamines was not confirmed. However, DFMO pretreatment of Raji (Mer+) as well as GM892A (Mer-) cells failed to potentiate the cytotoxic effects of temozolomide. Interpretation of these studies is complicated by evidence, in contrast to Seidenfield *et al.* (1987), that rodent L1210 (Mer-) (Cavanaugh *et al.*, 1984) and 9L (Mer-) cells (Hung *et al.*, 1981) are sensitised to BCNU by DFMO. The lack of potentiation of temozolomide in DFMO treated cells may, however, suggest polyamines to be of little importance in alkyltransferase repair.

In summary, while alkylation at the O^6-position of guanine has been correlated with the formation of DNA single-strand breaks whether this represents a cause of cytotoxicity or is simply a feature of DNA repair remains unclear. It appears unlikely that such a cytotoxic mechanism could involve depletion of cellular adenine nucleotide levels initiated in a 'suicide' type response by ADPRT. However, this enzyme would seem important in modulating DNA repair, and may be of particular significance in alkyltransferase repair, since inhibition of ADPRT sensitises Raji but not GM892A cell lines to the cytotoxicity of temozolomide. In addition to O^6-AGAT, a mechanism for tolerance of O^6-methylguanine may contribute to cellular resistance to temozolomide whilst providing a potential link between alkylation at the O^6-position of guanine and DNA strand-breakage. Tolerance may be associated with mismatch repair activity, or post-replication repair allowing DNA replication to proceed on a damaged template. Alternatively, strand-breaks may arise due to an as yet unidentified O^6-methylguanine excision repair mechanism, or are perhaps related to an effect of DNA damage on other enzymes involved in metabolism of DNA strand breaks. Given this uncertainty and the complexity of the mechanisms underlying cell death the relationship between alkylation at the O^6-position of guanine, DNA strand breaks and cytotoxicity should be viewed as a tentative one.

Chapter Seven

General Discussion

General Discussion.

The studies undertaken in this thesis have attempted to elucidate the biochemical mechanisms underlying the chemotherapeutic activity of the imidazotetrazinones. It is envisaged that such knowledge might aid, not least, the development of more specific agents but also provide an increased understanding of DNA repair processes, inhibition of which may offer therapeutic potential by possibly overcoming drug resistance.

Reaction of imidazotetrazinones with DNA *in vitro* produces a spectrum of alkylated bases, of which the major products are N^7-, O^6-alkylguanine, N^3-alkyladenine and, in addition alkylphosphotriesters (Bull, 1988). Countering the deleterious effects of these alkylations cells have evolved specific DNA-repair mechanisms. Consequently, although 3-methyladenine is of known lethality in bacteria (Lindahl and Karran, 1983), following temozolomide treatment of a range of human and murine tumour cell lines, excision repair of this lesion appeared to be sufficient to prevent it from being a cytotoxic lesion (Deans and Tisdale, 1992). In contrast, the major product of temozolomide-DNA alkylation, 7-methylguanine, has been reported as only slowly repaired (Medcalf and Lawley, 1981), a characteristic that was evident in the range of human and murine tumour cell lines examined. Thus, as 7-methylguanine appears to be tolerated by cells sensitive and resistant to temozolomide there is little evidence to suggest a lethal role for this lesion. A salient feature of these cell lines is, however, a correlation between the cellular levels of the enzyme O^6-alkylguanine alkyltransferase (O^6-AGAT) and sensitivity to temozolomide and mitozolomide (Tisdale, 1987). O^6-AGAT repairs the O^6-alkylguanine lesion by transferring the alkyl group to a cysteine-thiol group on the protein resulting in regeneration of unmodified guanine and in inactivation of the protein (Pegg and Byers, 1992). Unequivocal proof for the importance of this enzyme in cytotoxicity, and therefore of alkylation at O^6-guanine, has been provided by transfection of plasmids expressing mammalian O^6-AGAT into alkyltransferase deficient human cell lines. This has been reported to elicit resistance to the cytotoxic effects of MNNG and ACNU (Hayakawa *et al.*, 1990) and CNU (Wu *et al.*, 1991).

The results generated during the course of this thesis, and considerable previous data have further correlated an ability to repair the O^6-alkylguanine lesion with the

cytotoxicity of the imidazotetrazinones (Tisdale, 1987), triazenes (Gibson *et al.*, 1986), and nitrosoureas (Scuderio *et al.*, 1984a). Here we report the resistance of a cell line proficient in O^6-AGAT (Raji) to MNNG, MNU, MTIC, mitozolomide and temozolomide (but not ethazolastone) in comparison with an alkyltransferase deficient cell line (GM892A). Similar differential effects were not evident on exposure to MMS, a methylating agent that produces few alkylations at the O^6-position of guanine (Beranek *et al.*, 1980). Additional evidence for the importance to cytotoxicity of O^6-guanine alkylation has been provided by depletion of the 'suicide' O^6-AGAT repair enzyme using the free base O^6-methylguanine, a weak substrate for the enzyme. This has been shown to sensitise Mer+ cell lines to cell killing by CNU, MNNG (Dolan *et al.*, 1985, Yarosh *et al.*, 1986), mitozolomide and temozolomide, although not to ethazolastone (Tisdale, 1987). While O^6-methylguanine has been found to deplete alkyltransferase levels in the Raji (Mer+) cell line, sensitisation to these agents in this cell line has been reported as atypical, a feature that Karran and Williams (1985) have proposed to refute adducts at the O^6-atom of guanine as potentially cytotoxic lesions. However, in our hands, alkyltransferase depletion in Raji cells using a 5-fold higher concentration of O^6-methylguanine (0.5 mM) resulted in potentiation of the growth inhibitory effects of temozolomide. Cell extracts containing O^6-AGAT activity have little capacity to repair methylphosphotriesters or O^4-methylthymine lesions (Yarosh, 1985) which, since it is unlikely that O^6-methylguanine treatment could alter repair of these sites, would appear to exclude these other lesions from considerations as alternative targets for lethality. Moreover, as might be expected for inhibition specific to O^6-methylguanine repair, this technique did not sensitise the GM892A (Mer-) cell line to temozolomide, nor either cell line to MMS.

The ineffectiveness of ethazolastone against both Mer+ and Mer- cell lines, despite ethyl-groups at the O^6-position being subject to alkyltransferase repair, although at reduced efficiency (Pegg *et al.*, 1984), may suggest this lesion not to be cytotoxic. However, given the apparent toxicity of methylations at this position, a more probable explanation is that O^6-ethylguanine is subject to an alternative repair mechanism, possibly

involving nucleotide excision repair (Bronstein *et al.*, 1992). Additionally, ethazolastone appears more reactive to protein yet less DNA reactive than temozolomide, while the spectrum of DNA lesions produced by these agents differs considerably (Bull, 1988), and ethylation at positions other than O^6-guanine may prove cytotoxic.

Further experiments have attempted to relate observations on O^6-AGAT repair to the clinical situation. Three glioma cell lines isolated from a single human brain tumour have been shown to correlate in their alkyltransferase repair capacity and sensitivity to temozolomide. In addition, O^6-methylguanine-mediated depletion of O^6-AGAT was shown to sensitise the alkyltransferase proficient glioma cell lines (GL16 and GL18) to the cytotoxic effects of temozolomide. Hence, O^6-AGAT appears important to the sensitivity of these glioma cell line to temozolomide; findings that are of particular interest given the current clinical success of temozolomide in the treatment of glioma patients (O'Reilly *et al.*, 1993). Furthermore, the observation that many glial tumours have low alkyltransferase levels (Mineura *et al.*, 1991) appears to provide a biochemical basis for clinical response to temozolomide.

The nature of the alkyltransferase enzyme may also account for the marked dose schedule dependency of the antitumour activity of temozolomide in both murine and clinical studies (Stevens *et al.*, 1987, Newlands *et al.*, 1992). In *in vitro* studies with the GM892A (Mer-) and Raji (Mer+) cell lines no improvement was found on the toxicity of a single dose of temozolomide. However, toxicity towards the Mer+ cell line, but not the Mer- cell line, was reduced on dose division. This appears to be a feature of the suicide nature of O^6-AGAT, and would imply scheduling of the drug to improve tumour selectivity by reducing the toxic effects to normal alkyltransferase proficient tissue. The lack of mitozolomide schedule dependency, as well as the severity of the toxic side effects of this agent, are probably attributable to the resilience to DNA repair of cross-links, formed following chloroethylation at O^6-guanine (Gibson *et al.*, 1984b), which are then no longer subject to O^6-AGAT repair.

In vivo murine studies in established MAC13, MAC16 and M5076 tumours have failed to convincingly substantiate a correlation between alkyltransferase repair capability and the response of these tumours to temozolomide. Studies of O^6-AGAT levels in the

MAC13 tumour have suggested that tumour resistance may in part be achieved by induction of this repair enzyme, perhaps as part of a general DNA damage response. In addition, since M5076 tumours respond well to temozolomide if treated from implantation, it appears that tumour vascularisation, and hence drug penetration, will influence the chemotherapeutic activity of this agent.

Within the studies performed in this thesis there are, in some instances, discrepancies between the determined level of cellular alkyltransferase and cell line sensitivity to temozolomide cytotoxicity. The differential response of the GL 7 and GL 16 glioma cell lines is far less dramatic than that between the GM892A and Raji cells despite a similar variation in O^6-AGAT levels. Furthermore, the CHO (Mer-) cell line is 12-fold more resistant to temozolomide than GM892A (Mer-) cells. With regards to this, several other studies have identified cancer cell lines whose response to the toxic effects of MNU or MNNG lacks correlation with cellular O^6-AGAT levels (Goldmacher *et al.*, 1986, Ishida and Takahasi, 1987, Ikenaga *et al.*, 1987, Walker *et al.*, 1992, Lefebvre and Laval, 1993). Cell lines have been described that are Mer- and yet resistant to cell killing by MNU and MNNG. Consequently, the straightforward account of how cells deal with O^6-methylguanine lesions is clearly incomplete. Explanations of this phenomenum are essentially speculative, although it appears that rather than preventing alkylation of DNA or removing alkylated adducts, this resistance is related to an ability to tolerate O^6-methylguanine adducts (Goldmacher *et al.*, 1986). One possible mechanism of such tolerance could involve error-prone post-replication repair or, alternatively loss of an incorrect mismatch repair activity (Karran *et al.*, 1993). Either of these processes of repair could account for observations of a correlation between O^6-methylguanine lesions and DNA strand-breakage (Kalemegham *et al.*, 1988, Taverna *et al.*, 1992).

It appears that other cellular processes also influence DNA repair and cell survival. Exposure to temozolomide results in the arrest of cells in the SG_2M phase of the cell cycle (Bull, 1988). This is a common response to DNA damage and appears to be a positively regulated process that provides increased time for repair of DNA damage before mitosis (Burt *et al.*, 1991). While it is a possible inhibitor of post-replication repair, it appears that potentiation of temozolomide toxicity in rodent cell lines by caffeine may result from abolition of the G_2 delay induced by DNA damage, as has been

suggested for MNU (Roberts and Basham, 1990). This DNA damage-induced delay would therefore seem critical for efficient alkyltransferase repair.

ADPRT is a DNA strand break activated enzyme that has been speculated to inactivate transcription and DNA replication through ADP-ribosylation of RNA and DNA polymerase, and so allow time for DNA repair (Boulikas, 1993). Inhibition of the enzyme potentiated the toxicity of temozolomide in Raji (Mer+) although not GM892A (Mer-), and hence ADPRT may be important to alkyltransferase activity. The exact role of this enzyme in modulation of RNA and DNA polymerases is, however, uncertain and an attractive alternative hypothesis is that poly(ADP-ribosyl)ation may control chromatin condensation and the accessibility of DNA repair enzymes to damage in transcriptionally inactive genes (Boulikas, 1993). In addition, it has been suggested that this enzyme may function in a 'suicide' response to DNA damage, causing lethal cellular NAD+ depletion. However, while this appears feasible at high concentrations of MTIC (Lunn and Harris, 1988) and MNNG, at chemotherapeutic concentrations this represents an unlikely pathway for the cell death caused by these agents or temozolomide.

The question remains as to the mechanisms by which O^6-guanine alkylations cause cell death. The cytotoxicty of mitozolomide has been attributed to its ability to cross-link DNA via this lesion (Gibson *et al.*, 1984), however, methylations at this position are similarly cytotoxic. Cytotoxicity may be due to the presence of the methyl group itself, or perhaps due to the formation of single-stranded DNA breaks. Correlations between DNA breaks and the O^6-methylguanine lesion have been reported (Kalemegham *et al.*, 1988, Taverna *et al.*, 1992), and are supported by the observation of an increased alkaline elution rate of the DNA of alkyltransferase depleted Raji cells, in comparison with normal cells, on exposure to temozolomide. These strand breaks may simply result from DNA repair activity, perhaps involving post-replication repair or mismatch repair activity (Godfrey *et al.*, 1992). However, if strand breaks are a cause of cytotoxicity those elicited by O^6-methylguanine would seem to be distinguished from those produced from N-methylpurine-glycosylase repair. One possible explanation of this phenomena is that long lived strand breaks arise from improper action of mismatch repair activity excising the DNA base opposite O^6-methylguanine (Karran and Bignami, 1992). Cycling of this system could ultimately result in cell death.

Either the alkyl group or strand break damage may be cytotoxic as a consequence of interference with DNA-protein interactions or perhaps, in theory at least, as a controlled effect mediated by DNA damage recognition proteins involved in cell-cycle control or cell death. If we consider DNA-protein interactions, while single-strand breaks have been shown to cause transient inhibition of DNA synthesis (Painter and Young, 1976), a direct effect for the alkyl group on DNA polymerase appears suspect. O^6-methylguanine has been reported not to efficiently terminate DNA chain elongation *in vitro* (Larson et al., 1985), although it does slow replication fork progression (Dosanjh *et al.*,1991). Recent reports, however, may conflict with these studies as they indicate that O^6-methylguanine does block chain elongation *in vitro* (Voigt and Topal, 1993, Pfeiffer *et al.*, 1993). In this regard, post-replication repair tolerance to damage is generally conceived as a process by which cells overcome blocks to replication fork progression. A possible explanation that may reconcile these observations is that chain termination may result from O^6-guanine alkylation in particular sequence contexts. Alternatively, it has been suggested that the cytotoxic effect of O^6-methylguanine may be related to an ability to block replicon initiation rather than chain elongation (Karran and Bignami, 1992). In this regard, it is perhaps significant that O^6-methylguanine and the other tolerated base analogue 6-TG, both appear capable of inhibition of T-antigen binding and impairment of SV40 replication initiation (Bignami *et al.*, 1990, Maybaum *et al.*, 1987).

6-TG is more cytotoxic to an excision-repair defective CHO mutant than to parental CHO cells, and therefore it appears that 6-TG residues and UV-induced photoproducts are recognised by both excision-repair and post-replication repair. Consequently, Christie et al. (1984) have suggested that, while a single 6-TG residue was unlikely to influence DNA structure, incorporation of a series of residues might be sufficient to produce a helix distortion similar to that produced by a cyclobutane dimer. Analogously, it has been suggested that sequential O^6-methylguanine residues could arrest DNA synthesis (Toorchen and Topal, 1983). This proposal is of particular interest in the light of the results of the temozolomide sequence specificity studies reported in this thesis. These have shown temozolomide to preferentially alkylate runs of guanines, although these studies have only confirmed the specificity of guanine-N^7 alkylation.

However, a model has been proposed on the basis of molecular modelling studies by which a similar specificity may be extended to temozolomide alkylation of the guanine O^6-position (Clark *et al.*, 1990). In addition, Richardson *et al.* (1989) have described enhanced O^6-methylguanine alkylation in runs of guanines in DNA, while PCR studies have indicated preferential MNU alkylation of pairs of guanines in GGPy codons (Mironov *et al.*, 1993).

Temozolomide is capable of the disruption of other protein-DNA interactions. While it does not appear to cause a general disruption of gene expression (Bull 1988), some ability to alter expression of specific genes is indicated by the differentiation of K562 erythroleukaemia cells induced by temozolomide (Tisdale, 1985). Differentiation appears to result from inhibition of cytosine-C^5 methylation by 5-methyltransferase caused by O^6-methylguanine residues adjacent to these cytosines (Hepburn *et al.*, 1991). However, hypomethylation of these sites is normally related to increased oncogene expression and carcinogenesis (Diala *et al.*, 1983), and therefore this would seem unlikely to constitute a cytotoxic pathway. In addition, O^6-guanine methylation has been demonstrated to affect the binding of transcription factors, including the cellular sp1 factor, to regulatory sequences and thereby could possibly disrupt regulation of the expression of specific genes (Bonfanti *et al.*, 1991). The binding site for the cellular sp1 factor consists of a guanine rich regulatory sequence that is common to the promoter regions of oncogenes (Mattes *et al.*, 1988). This has provided the intriguing possibility that the sequence preference of temozolomide for runs of guanines may predispose these genes to alkylation by temozolomide and that the chemotherapeutic activity of temozolomide could involve inhibition of oncogene expression, and perhaps even correction of aberrant oncogene expression. However, while temozolomide alkylation of a plasmid expressing a reporter gene under the control of the G-rich promoter region of the Ha-ras1 gene appeared to inhibit its expression in CHO and MCF7 cell lines, this inhibition appeared unrelated to the ability of the cell lines to repair O^6-methylguanine. Consequently, it seems unlikely that this activity is related to the chemotherapeutic activity of temozolomide.

Some studies have tried to reconcile the sequence specificity of temozolomide with its structural properties (Lowe *et al.*, 1991, Clark 1991). These have proposed that

prior to the covalent bonding reaction an initial non-covalent binding step may allow sequence recognition. In the present study, DNA footprinting techniques have been used in attempts to determine whether imidazotetrazinones have preferred DNA binding sites. However, these studies along with attempts to inhibit temozolomide cytotoxicity using non-reactive analogues of temozolomide proved inconclusive. The fact that many agents differ significantly in the structure of their non-alkylating moieties yet show a similar sequence preference may, however, argue against a significant role for specific non-covalent binding interactions of the non-alkylating residues in influencing activity. Furthermore, the significance to cytotoxicity of a preference for G-rich sequences, if any, remains to be determined.

Despite the uncertainty concerning the mechanism by which O^6-guanine methylation may instigate cell death, during the research conducted in the course of this thesis, mounting evidence has suggested that resistance to temozolomide is determined by efficient repair of this lesion. In this regard, all normal cells tested appear proficient in alkyltransferase repair capacity (Harris *et al.*, 1983, Yarosh *et al.*, 1983), while 20-30% of tumour cell lines lack the O^6-AGAT enzyme (Sklar and Strauss, 1981), although this may in part be due to tissue culture conditions. Clearly other factors may influence tumour sensitivity, and response to temozolomide may also prove reliant on a poor tolerance of O^6-methylguanines. However, these observations would appear to offer considerable potential for exploitation, and given temozolomides clinical success it would appear significant that Mineura *et al.*(1991) have shown that tumours from 6 out of 17 glioma patients had low levels of O^6-AGAT (<100 fmol/mg, 4< 60 fmol/mg), whilst non-glial tumours appeared significantly higher. Furthermore, there may be potential for improving response to temozolomide by combining it with alkyltransferase lowering agents, since Dolan *et al.*(1990) have shown *in vitro* and in nude mice that human gliomas can be made more sensitive to nitrosoureas by treatment with O^6-benzylguanine, although it may prove that such depletion is also deleterious to non-tumour tissue.

This potential along with temozolomides already proven success with glioma, for which at present there is little alternative effective treatment, indicate it to be an exciting new agent whose further clinical progress will be followed with interest.

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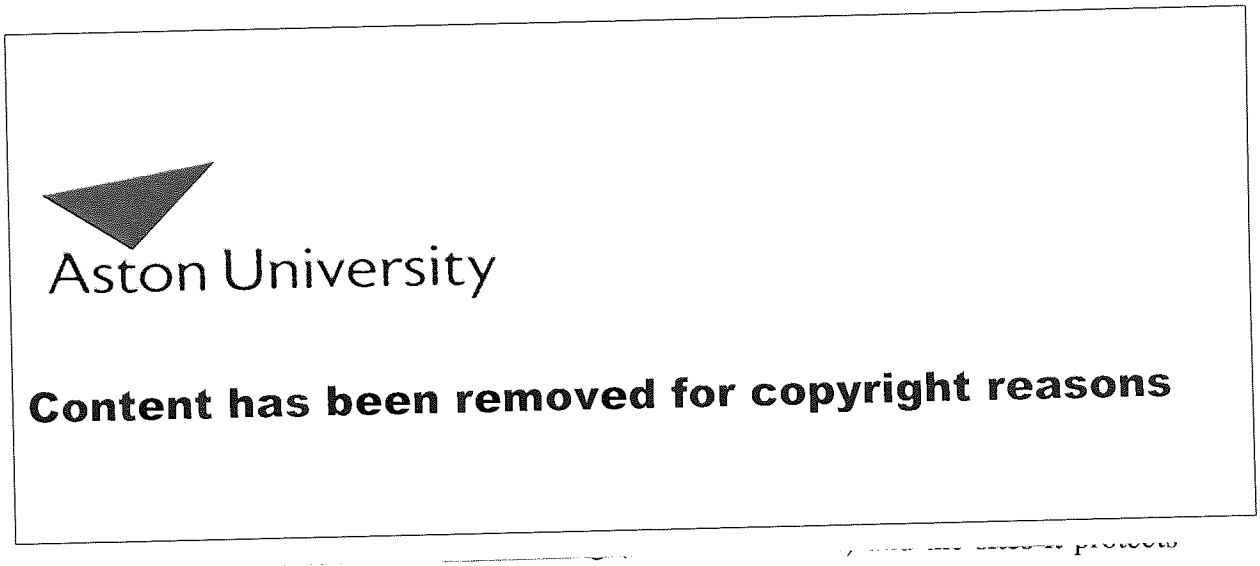
Appendix

Appendix A: Base sequence of *tyr*T DNA (160 bp) fragment.



from DNAase I digestion (Low *et al.*, 1984).

Appendix B: Base sequence of pBR322 DNA (bases 0-660).

HindIII

5′-TTCTCATGTT TGACAGCTTA TCATCGATAA GCTTTAATGC GGTAGTTTAT
3′-AAGAGTACAA ACTGTCGAAT AGTAGCTATT CGAAATTACG CCATCAAATA
10 20 30 40 50

CACAGTTAAA TTGCTAACGC AGTCAGGCAC CGTGTATGAA ATCTAACAAT
GTGTCAATTT AACGATTGCG TCAGTCCGTG GCACATACTT TAGATTGTTA
60 70 80 90 100

GCGCTCATCG TCATCCTCGG CACCGTCACC CTGGATGCTG TAGGCATAGG
CGCGAGTAGC AGTAGGAGCC GTGGCAGTGG GACCTACGAC ATCCGTATCC
110 120 130 140 150

CTTGGTTATG CCGGTACTGC CGGGCCTCTT GCGGGATATC GTCCATTCCG
GAACCAATAC GGCCATGACG GCCCGGAGAA CGCCCTATAG CAGGTAAGGC
160 170 180 190 200

ACAGCATCGC CAGTCACTAT GGCGTGCTGC TAGCGCTATA TGCGTTGATG
TGTCGTAGCG GTCAGTGATA CCGCACGACG ATCGCGATAT ACGCAACTAC
210 220 230 240 250

CAATTTCTAT GCGCACCCGT TCTCGGAGCA CTGTCCGACC GCTTTGGCCG
GTTAAAGATA CGCGTGGGCA AGAGCCTCGT GACAGGCTGG CGAAACCGGC
260 270 280 290 300

CCGCCCAGTC CTGCTCGCTT CGCTACTTGG AGCCACTATC GACTACGCGA
GGCGGGTCAG GACGAGCGAA GCGATGAACC TCGGTGATAG CTGATGCGCT
310 320 330 340 350

BamHI

TCATGGCGAC CACACCCGTC CTGTGGATCC TCTACGCCGG ACGCATCGTG
AGTACCGCTG GTGTGGCAG GACACCTAGG AGATGCGGCC TGCGTAGCAC
360 370 380 390 400

GCCGGCATCA CCGGCGCCAC AGGTGCGGTT GCTGGCGCCT ATATCGCCGA
CGGCCGTAGT GGCCGCGGTG TCCACGCCAA CGACCGCGGA TATAGCGGCT
410 420 430 440 450

CATCACCGAT GGGGAAGATC GGGCTCGCCA CTTCGGGCTC ATGAGCGCTT
GTAGTGGCTA CCCCTTCTAG CCCGAGCGGT GAAGCCCGAG TACTCGCGAA
460 470 480 490 500

GTTTCGGCGT GGGTATGGTG GCAGGCCCGT GGCCGGGGGGACTGTTGGGCG
CAAAGCCGCA CCCATACCAC CGTCCGGGCA CCGGCCCCCT GACAACCCGC
510 520 530 540 550

CCATCTCCTT GCATGCACCA TTCCTTGCGG CGGCGGTGCT CAACGGCCTC
GGTAGAGGAACGTACGTGGT AAGGAACGCC GCCGCCACGA GTTGCCGGAG
560 570 580 590 600

----------**Primer**----------

AACCTACTAC TGGGCTGCTT CCTAATGCAG GAGTCGCATA AGGGAGAGCG
TTGGATGATG ACCCGACGAA *GGATTACGTC CTCAGCGTAT* TCCCTCTCGC
610 620 630 640 650

SalI
TCGACCGATG-3′
AGCTGGCTAC-5′
660

Restriction sites utilised for the preparation of HindIII-BamHI and BamHI-SalI fragments are indiacted in bold characters. Runs of 3 or more guanine bases, corresponding to the sites of preferential alkylation for temozolomide as identified in chapter 4, are underlined. Italicised bases notate the labelled oligonucleotide primer sequence utilised in *Taq* polymerase stop assays (chapter 4).

Appendix C: Estimation of the No. temozolmide-induced O^6-guanine methylations per pHrasSVOCAT plasmid.

pHrasSVOCAT ≈ 4950 b.p

Average b.p Mw = 617.9 (given GC:AT ratio = 1:1)

Alkylation of pHrasSVOCAT with temozolomide (1mM) → 24.88 ± 1.44 pmoles methyl /μg plasmid DNA (Section 5.2.1.3b)

O^6-Methylguanine = 5.3% of temozolomide-induced calf thymus DNA alkylation products (Bull. 1988)

∴ No. moles plasmid DNA / μg plasmid

= $1 \times 10^{-6} / (4950 \times 617.9)$

= 3.27×10^{-13}

∴ No.temozolomide (1mM) methylations / plasmid

= $24.88 \times 10^{-12} / 3.27 \times 10^{-13}$

= 76.1 methyl groups / plasmid

∴ No. O^6-methylguanines / plasmid at 1mM temozolomide

= 76.1×0.053

= approx.4.0

Appendix D: Publications.

Deans,B. and Tisdale,M.J.

Antitumour imidazotetrazines XXVIII. 3-methyladenineDNA glycosylase activity in cell lines sensitive and resistant to temozolomide.

Cancer Letters, **63** (1992), 151-157.

Cancer Letters, 63 (1992) 151 – 157
Elsevier Scientific Publishers Ireland Ltd.

Antitumour imidazotetrazines XXVIII 3-methyladenine DNA glycosylase activity in cell lines sensitive and resistant to temozolomide

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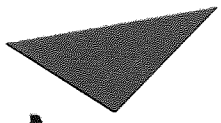
(Received 12 November 1991)
(Accepted 10 February 1992)



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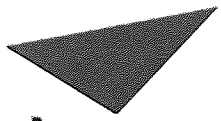

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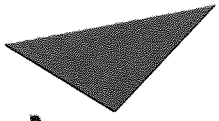

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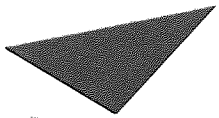

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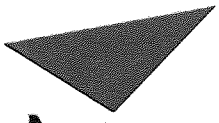

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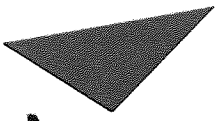

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