STUDIES ON THE MODE OF CYTOTOXICITY OF IMIDAZOTETRAZINONES.

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Studies on the mode of cytotoxicity of imidazotetrazinones.
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Thesis Summary.

The imidazotetrazinones are a novel group of antitumour agents which have demonstrated good activity against a range of murine tumours and human xenografts. They possess a structure activity relationship similar to the antitumour triazenes, with the chloroethyl (mitozolomide) and methyl (temozolomide) analogues being active antitumour agents, whilst the ethyl (CCRG 82019) and higher homologues are inactive. This thesis attempts to elucidate the biological mechanisms responsible for the strict structure-activity relationship observed amongst the imidazotetrazinones.

Mitozolomide is the only agent chemically capable of cross-linking DNA, which has been suggested to be responsible for the cytotoxicity of this group of agents. Only mitozolomide and temozolomide exhibit a marked differential toxicity towards the O^6-alkylguanine-DNA alkyltransferase deficient GM892A (Mer-) cell line rather than the proficient Raji cell line (Mer+). The rate of uptake of imidazotetrazinones into cells is similar for all three agents in both cell lines, and does not explain the differing sensitivities to these agents.

The effect of drug treatment on the incorporation of precursors into macromolecules, and their pool sizes, was examined. Temozolomide administration was found to alter de novo protein synthesis in both GM892A and Raji cells. Flow cytometric analysis revealed that temozolomide and CCRG 82019 block cells in late $S/G_2/M$ phase of the cell cycle, similar to that observed with mitozolomide.

The extent of reaction of all three drugs with isolated macromolecules and cellular macromolecules was determined, and differences found, with cellular repair processes influencing the number of alkyl lesions remaining bound to macromolecules. The specific bases formed in calf thymus DNA after treatment with either temozolomide and CCRG 82019 was measured, and it was found that the types and relative amounts of lesions formed, differed, as well as the total level of alkylation.

Whereas DNA extracted from imidazotetrazinone treated cells is not affected in its ability to support RNA polymerase activity, an effect is observed on the ability to extract DNA polymerase from drug treated cells. This may suggest that the alkylated DNA must be in intact chromatin for the lesion to manifest its effects. Temozolomide and methyl methanesulphonate do not appear to act with a synergistic mode of action. The O^6-position of guanine is suspected to be a critical site for the action of these types of drugs.

Key Words.
DNA; DNA alkylation; DNA repair; O^6-alkylguanine; imidazotetrazinone.

To my parents.

This is not the end. It is not even the beginning of the end. But it is, perhaps, the end of the beginning.

Winston Churchill.

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LIST OF ABBREVIATIONS.

ATP	Adenosine 5'-triphosphate
BCNU	1,3-bis(2-chloroethyl)-1-nitrosourea.
CCNU	1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea.
CNS	Central nervous system.
CTP	Cytosine 5'-triphosphate
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytosine 5'-triphosphate
dGTP	2'-deoxyguanosine 5'-triphosphate
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetra-acetic acid.
ETIC	5-(3-ethyl-1-triazenyl)imidazole-4-carboxamide.
GTP	Guanosine 5'-triphosphate
i.p.	intra-peritoneally
MCTIC	5-(3-[2-chloroethyl]-1-triazenyl)imidazole-4-carboxamide.
meCCNU	1-(2-chloroethyl)-3-(4-methylcyclohexyl)-1-nitrosourea.
MMS	methyl methanesulphonate.
MNNG	1-methyl-3-nitro-1-nitrosoguanidine.
MNU	1-methyl-1-nitrosourea.
MTIC	5-(3-methyl-1-triazenyl)imidazole-4-carboxamide.
MW	Molecular weight
O^6-AGAT	O^6-alkylguanine-DNA alkyltransferase
O^6-MeG	O^6-methylguanine.
PBS	Phosphate-buffered saline
PPO	2,5-diphenyloxazole.
RNA	Ribonucleic acid

RPMI	Rosewell Park Memorial Institute (medium)
s.c.	sub-cutaneous.
SDS	Sodium dodecylsulphate
SDS-PAGE	Sodium dodecylsulphate-polyacrylamide gel electrophoresis
TEMED	N,N,N',N'-tetramethylethylenediamine
Tris	Tris(hydroxymethyl)aminomethane base
TTP	Thymidine 5'-triphosphate
UTP	Uridine 5'-triphosphate

1. INTRODUCTION.

1.1 General introduction.

In 1971, President R. Nixon announced that a cure for cancer would be found, as part of the federal War on Cancer. The belief was based on the same premise that had previously seen President J. F. Kennedy state in 1962 that there would be an American on the moon by the end of the decade. Both declarations were made in the conviction that if enough money and resources were made available, then any problem was surmountable. However, over 20 years later, cancer is still one of the major causes of death in Western society.

The quest for drugs to treat cancer can be traced back to at least 1865 when physicians administered arsenic to patients in an attempt to treat leukaemia, and since then has often been characterised by a hit and miss type approach. A more rational approach was taken, following the observation that exposure to sulphur mustard caused a marked depletion in bone marrow cells (the observation was made following an explosion on board a ship unloading the chemical, which was to have been deployed as a war gas), and led to the use of the related chemicals nitrogen mustard and tris-chloroethylamine in the treatment of bone-marrow malignancies.

Many of the clinically used drugs have one thing in common- they are typically cytodestructive towards any proliferating tissue, with little or no intrinsic selectivity towards tumour cells. The bone marrow,

gastro-intestine and basement membrane of the skin all contain rapidly proliferating cells which are often damaged by these agents, and such toxicity is often a limiting factor of drug treatment. The ultimate anti-cancer drug, therefore, is one which selectively destroys cancer cells with no deleterious effect on normal tissues. Advances have been made in the treatment of some kinds of cancer (for example leukaemias, lymphomas, ovarian, cervical, breast and testicular cancers), but many others (e.g. head, neck, and lung cancers, colorectal carcinoma and malignant melanomas) remain incalcitrant to treatment.

With many tumours the first line of therapy is surgery, followed by radiotherapy. Unfortunately, all too often by the time of presentation the tumour has metastasised, so that chemotherapy is required as an adjunct to these procedures, or is used as a first line therapy in its own right, with the almost inevitable consequent side-effects.

One of the commonly encountered problems is that tumours may be a heterogeneous mixture of cells, with some cells being drug resistant, with the consequence that resistant sub-populations of the tumour are able to re-establish the tumour mass after the sensitive population has been destroyed, but this may be overcome in part by the use of combination chemotherapy. Furthermore, it is likely that a proportion of tumour tissue will be quiescent and as such will remain unaffected by drug

treatment, but which may be recruited to proliferate later. Poor vascularisation of many solid tumours may present as an almost impenetrable barrier for drugs to reach the cells within the tumour.

The major problem in anti-cancer chemotherapy, unlike for example, anti-bacterial chemotherapy, is that there are few defined biochemical differences between normal and cancer cells which can be exploited to kill the aberrant cells. A greater understanding of the biochemistry of both normal and tumours cells is required if more effective and selective drugs are to be designed to treat cancer. Furthermore, a deeper understanding of why currently used drugs are ineffective in many patients may also provide a route to uncover more effective drugs, and maybe equally importantly, aid identification of those patients for whom certain drugs will not help, in an effort to improve their quality of life.

The imidazotetrazinones are a novel group of antitumour agents which have demonstrated good activity against a range of murine tumours and human xenografts. They possess a structure-activity relationship similar to the antitumour triazenes, with the chloroethyl (mitozolomide) and methyl (temozolomide) analogues being active antitumour agents, whilst the ethyl (CCRG 82019) and higher homologues are inactive (Stevens et al, in press). This thesis attempts to elucidate the biological mechanisms responsible for the strict structure-activity relationship observed amongst the imidazotetrazinones.

1.2.1 The rationale for the synthesis and screening of imidazotetrazinones.

The synthesis and screening of the novel agent mitozolomide (8-carbamoyl- 3(2-chloroethyl)imidazo [5,1-d] -1,2,3,5-tetrazin-4(3H)-one) and subsequently its 3-methyl (temozolomide, CCRG 81045) and 3-ethyl (CCRG 82019) analogues, stemmed from an interest in small molecules bearing "NNN" linkages in either cyclic (e.g. 1,2,3-triazines) (Stevens, 1976) or acyclic (e.g. triazenes) (Vaughan and Stevens, 1978) arrangements and bicyclic heterocycles with bridgehead nitrogen atoms (Baig et al, 1982).

Although mitozolomide and its 3-alkyl substituted analogues are derivatives of a novel ring system (Stevens et al, 1984), the "NNN" linkage is a feature of other compounds which are of interest in cancer chemotherapy. The demonstration that 3,3-dimethyl-1-phenyltriazene (PDMT) was active against the mouse sarcoma 180 (Clarke et al, 1955) prompted the synthesis of other triazenes including phenyl derivatives (Lin and Loo, 1978), benzene analogues (Shealy et al, 1971) and imidazotriazenes (Shealy, 1970), many of which were shown to be active against murine tumours.

Initially it was suggested that PDMT's activity was due to the formation of a reactive benzenediazoium ion which inhibited cell proliferation (Clarke et al, 1955), but a demonstration of the metabolism of PDMT in vitro by

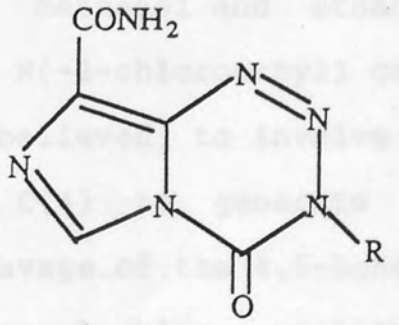
rat liver microsomes (Preussman et al, 1969) suggested that it may act as an alkylating agent in vivo, through the production of carbonium ions after metabolism. This prompted the synthesis of 5-diazo-imidazole-4-carboxamide (Diazo-IC) by Shealey et al (1961), via the diazotisation of 5-aminoimidazole-4-carboxamide (AIC). It was known then that AIC occupies a central position in the de novo purine synthetic pathway, and is utilised by tumour cells more rapidly than normal tissue (Hano and Akashi, 1964). It was thus hoped that Diazo-IC would act not only as a tumour selective antimetabolite of AIC, but also cause cellular damage via production of the diazonium ion. Diazo-IC was found to exhibit antitumour activity both in vitro and in vivo (Shealy et al, 1961, Hano et al, 1968).

The antitumour activity of Diazo-IC was limited by a tendency to cyclise under aqueous conditions to yield 2-azahypoxanthine, which does not possess antitumour activity (Hano et al, 1968). Attempts were made to improve the stability of Diazo-IC by coupling with secondary amines (Shealy et al, 1962) which resulted in the production of the clinically active compound 5- (3,3-dimethyltriazenyl) imidazo-4- carboxamide, more commonly known as DTIC.

1.2.2 The synthesis of imidazotetrazinones.

Ege and Gilbert (1979) described the synthetic route to pyrazolo [5,1-d]-1,2,3,5-tetrazin-4(3H)-one, which until its synthesis had been thought to be inherently unstable (Wiley,1978). Stevens et al (1984) synthesised a series of 8-carbamoyl-3-substituted-imidazo[5,1-d]- 1,2,3, 5-tetrazin-4(3H)-ones in an analogous manner, based on the interaction of 5-diazoimidazole-4-carboxamide and a series of alkyl and aryl isocyanates in either ethyl acetate or dichloromethane.

Figure 1.
Structures of the imidazotetrazinones used in this study.



	R=
MITOZOLOMIDE	CH_2CH_2CL
TEMOZOLOMIDE	CH_3
CCRG 82019	CH_2CH_3

1.3 The chemical decomposition of imidazotetrazinones.

Studies of the chemistry of 1,2,3-benzotriazin-4(3H)-ones(1 Stevens,1976) and imidazo[5,1-c]-1,2,4-triazin-4(3H)-ones (Baig *et al*, 1982), suggested that mitozolomide might either revert to the diazoimidazole and 2-chloroethyl isocyanate, or undergo hydrolytic attack at C(4) to ring open, with subsequent decarboxylation, to yield 5-[3-(2-chloroethyl)- triazen-1-yl] imidazole- 4-carboxamide, (MCTIC). Evidence confirming the existence of both decomposition routes was found, but under different conditions (Stevens *et al*, 1984). Indeed the nature and rate of decomposition of mitozolomide and its 3-methyl and 3-ethyl homologues has been shown to be very dependent on the reaction conditions (Baig and Stevens, 1987).

Mitozolomide has been shown to be unstable in both hot methanol and ethanol, and affords 2-azahypoxanthine and N(-2-chloroethyl) carbamate(s), the mechanism of which is believed to involve initial attack by the nucleophile at C(4) to generate hemiacetals which ring open by cleavage of the 4,5-bond to yield unstable triazenes. An intramolecular cyclization then occurs to yield 2-azahypoxanthine, with loss of the isocyanate moiety. In contrast both the 3-methyl and 3-ethyl analogues were found to be more stable in hot methanol and ethanol than mitozolomide, and after 10 days yielded 2-azahypoxanthine (80%) and a colourless solid (20%).

Thus there are apparent differences in the chemistry of mitozolomide and its 3-methyl and 3-ethyl analogues. Breakdown of hemiacetals involves fission of the 4,5 bond when the 3-substituent is a chloroethyl moiety, whereas cleavage of the 3,4 bond may occur when the 3-substituent is either methyl or ethyl, and thus 1,4,5-trisubstituted imidazoles are formed to a minor extent.

When mitozolomide and the 3-alkyl homologues are boiled in water, the main product is AIC. The rate of decomposition of mitozolomide (and presumably the 3-alkyl analogues) are profoundly influenced by pH, with mitozolomide being stable even in hot concentrated sulphuric acid, but has a half life of only 0.15hr at pH 9.0 in Tris buffer (Goddard, 1985).

Decomposition of mitozolomide in 0.2M phosphate buffer over the more physiologically relevant range of pH 7.21 to pH 7.81 shows a decrease in half life from 1.59hr to 0.45hr. For comparative purposes, the half life of mitozolomide at pH 7.4 in 0.2M phosphate buffer is some 0.9hr, while the half live for temozolomide under the same conditions is 1.24hr. The slightly greater stability of temozolomide as compared to mitozolomide is also observed in physiological fluids (Goddard, 1985).

Mitozolomide has been shown to ring open to form MCTIC in an aqueous 5% sodium carbonate solution (Stevens et al, 1984), and more recently temozolomide and CCRG 82019 have been shown to convert to MTIC and ETIC (the methyl and ethyl analogues of MCTIC, respectively) under

similar conditions (Baig and Stevens, 1987).

Shealy (1975) had previously found AIC and a similar range of minor components from the decomposition of MCTIC. A consideration of the potential degradation pathways reveals structural similarities to the triazenoimidazoles and the chloroethylnitrosoureas.

1.4 Pharmacokinetics of mitozolomide.

The pharmacokinetics of mitozolomide in humans is similar to that predicted from animal studies, with a half life of between 1 and 1.3hr, and being undetectable 12hr after administration (Slack et al, 1983, Goddard et al, 1985). Mitozolomide is well absorbed after oral administration, with about 95% bioavailability, which suggests that absorption takes place through the stomach, given the compound's lability at higher pH (Slack et al, 1983). The pharmacokinetics of mitozolomide suggest a one-compartment model: In contrast, the pharmacokinetics of the clinically used nitrosoureas BCNU and CCNU suggest a two-compartment model, with plasma concentrations rapidly declining from an initial peak (Levin et al, 1979, Levin, 1981, Lee and Workman, 1983). The pharmacokinetic differences observed between these nitrosoureas and mitozolomide may be of therapeutic value if they represent a relatively high sustained tumour concentration.

Brindley et al (1986), using a murine model,

established the plasma and tissue distribution of mitozolomide, and found appreciable drug concentrations in both brain and tumour tissue. The dose-limiting side effect of mitozolomide has been found to be myelo-suppression, with thrombocytopenia being more pronounced than leukpoenia, which showed a delayed recovery of 6-8 weeks (Newlands et al, 1985).

1.5. Nitrosoureas.

The history of the nitrosoureas dates back to 1956 when 1-methyl-3-nitro-1-nitrosoguanidine (MNNG) was submitted to the National Cancer Institute for screening. It had been synthesised by McKay & Wright (1947), and was entered into the programme for screening as a randomly allocated compound. It was selected for clinical development in 1958 and received abbreviated clinical trials in 1960, but was supplanted by more active derivatives. MNNG was reported to be active, when injected intra-peritoneally (i.p.) against i.p. inoculated ascites leukaemia L1210 (Greene and Greenberg, 1960), but its activity was reduced when administered subcutaneously (s.c.) or orally (Schepartz, 1976).

A second drug, 1-methyl-1-nitrosourea (MNU), was found to be active against the i.p. inoculated L1210 leukaemia, not only after i.p. injection, but also after s.c. and oral administration. MNU also exhibited activity

against intracranially inoculated L1210 leukaemia (Skipper et al, 1961), indicating that the drug was able to cross the blood brain barrier.

The ability of MNU to penetrate the blood-brain barrier was a significant finding, since the majority of drugs available at that time did not achieve cytotoxic concentrations in the central nervous system (CNS). It was also recognised that the CNS acted almost as a sanctuary for leukaemia cells, and was the site of relapse for many patients in apparent remission. The ability of MNU to cross the blood-brain barrier depended on its lipid solubility, and led to the development of a series of chloroethyl derivatives, including 1,3-bis (2-chloroethyl)-1-nitrosourea (BCNU), which demonstrated curative activity against L1210 leukaemia cells inoculated both intra-peritoneally and intra-cranially in mice (Johnston et al, 1966).

More than 200 analogues of the original MNU compound were synthesised, but only the specific structural type N-(2-haloethyl)- N-nitrosourea proved superior to MNU. The 2-iodoethyl compounds lacked antitumour activity, while the 2-bromoethyl derivatives were not as effective as 2-chloroethyl compounds. Although the 2-fluoroethyl analogues showed similar activity to 2-chloroethyl compounds, their tendency to decompose to fluoroacetate at physiological pH , both in vitro and in vivo, and the resultant toxicity to the nervous system, limited any further development (Johnson et al, 1963). Further

research on variations of the 2-chloroethyl and the nitrogen group led to the synthesis of 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU) and 1- (2-chloroethyl)- 3- (4-methylcyclohexyl)- 1- nitrosourea (meCCNU).

Streptozotocin is a naturally occurring nitrosourea, being a fermentation product of Streptomyces achromogenes, and is composed of MNU plus an aminoglucose carrier. Streptozotocin has a relatively reduced myelosuppressive activity, but retains its antitumour properties in experimental systems. It does however destroy pancreatic ß-cells in the islet of Langerhans, which induces a diabetic state in animals (Schein et al, 1967).

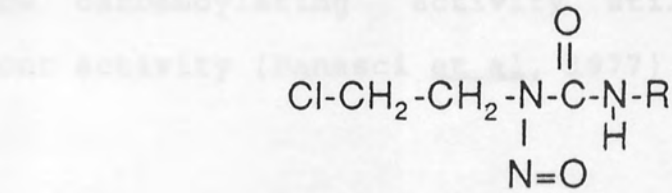
Chlorozotocin was synthesised by Johnson et al, (1975) for the further investigation of the importance of the aminoglucose carrier in reducing myelosuppression. Chlorozotocin has significant experimental antitumour activity, and exerts only a minor degree of inhibition of mouse and human bone marrow DNA synthesis as compared to BCNU (Anderson et al, 1975, Schein et al, 1976), and has the advantage that, unlike Streptozotocin, is non-diabetogenic.

The 2-cloroethylnitrosoureas, including BCNU, CCNU, meCCNU and chlorotozocin have found widespread clinical application in the treatment of various human malignancies, including gliomas, lymphoproliferative diseases, melanoma and gastrointestinal cancer (Wasserman et al , 1975).

The chloroethylnitrosoureas, however, whilst being one of the most effective classes of antitumour compounds so far tested in animal models, have unfortunately proved disappointing in the clinic.

Figure 2:

Structures of some derivatives of chloroethylnitrosoureas.



R=

BCNU CH_2-CH_2-Cl

CCNU

me-CCNU CH_3

CHLOROZOTOCIN D-glucopyranose

1.6 Isocyanates.

Certain nitrosoureas, for example BCNU and CCNU, cleave spontaneously to yield, in this instance, a 2-halogenoethyl diazo hydroxide, and an isocyanate; isocynates are thought to react preferentially with cellular proteins by carbamoylation (Cheng et al, 1972). The role of isocyanates in the cytotoxicity of nitrosoureas remains largely speculative, but may be considered to be of minor importance, since nitrosoureas such as ACNU or chlorozotocin which have been designed to have low carbamoylating activity still retain their antitumour activity (Panasci et al, 1977)

1.7 Structure-activity relationship of the imidazotetrazinones and triazenes.

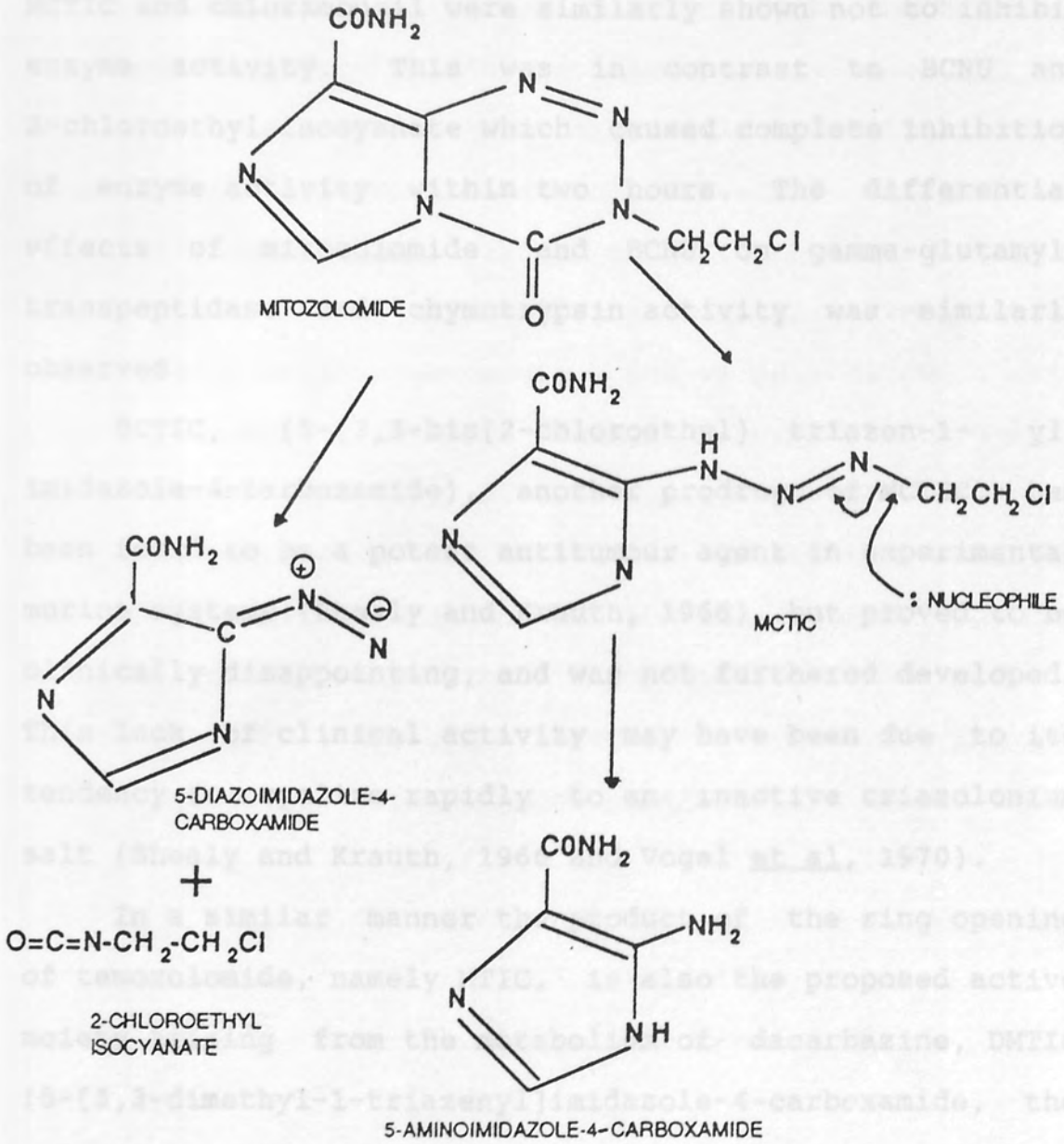
Both chemical (Stevens et al, 1984) and biological studies (Horgan and Tisdale, 1984) suggest that mitozolomide may act as a prodrug for MCTIC, an acyclic triazene, with ring opening occurring under alkaline conditions. Analogously the 3-methyl and 3-ethyl analogues of mitozolomide were shown to ring open to yield MTIC, (5-[3-methyl-1-triazenyl]imidazole-4-carboxamide) and ETIC (5-[3-ethyl-1-triazenyl]imidazole-4-carboxamide) in a 5% aqueous solution of sodium carbonate (Baig and Stevens, 1987).

There is also a similar structure-activity relationship between the imidazotetrazinones and triazenes in that the chloroethyl and methyl substituted compounds are active as antitumour agents, whilst the ethyl substituted compounds are inactive (Stevens et al, in press). Furthermore, a TLX5 lymphoma cell line with induced cross-resistance to triazenes, an L1210 leukaemia cell line resistant to BCNU and another resistant to BCTIC were shown to be cross-resistant to mitozolomide (Hickman et al, 1985). However, similar cross-resistance was not demonstrated by an L1210 leukaemia cell line resistant to cyclophosphamide (Hickman et al, 1985), nor by the Walker carcinoma either sensitive or resistant to alkylating agents (Horgan and Tisdale, 1984).

Two potential chemical decomposition pathways for mitozolomide may be considered:- (figure 3).

Figure 3.

Two potential chemical decomposition pathways for mitozolomide.



Most of the species that are generated by these schemes are potentially toxic. However Horgan and Tisdale (1984) showed that the isocyanate moiety was not formed significantly. This was demonstrated by the inability of mitozolomide to specifically inhibit glutathione reductase extracted from TLX5 cells treated with mitozolomide. MCTIC and chlorambucil were similarly shown not to inhibit enzyme activity. This was in contrast to BCNU and 2-chloroethyl isocyanate which caused complete inhibition of enzyme activity within two hours. The differential effects of mitozolomide and BCNU on gamma-glutamyl-transpeptidase and chymotrypsin activity was similarly observed.

BCTIC, (5-[3,3-bis{2-chloroethyl} triazen-1- yl] imidazole-4-carboxamide), another prodrug of MCTIC, had been found to be a potent antitumour agent in experimental murine systems (Shealy and Krauth, 1966), but proved to be clinically disappointing, and was not furthered developed. This lack of clinical activity may have been due to its tendency to cyclize rapidly to an inactive triazolonium salt (Shealy and Krauth, 1966 and Vogel et al, 1970).

In a similar manner the product of the ring opening of temozolomide, namely MTIC, is also the proposed active moiety arising from the metabolism of dacarbazine, DMTIC (5-[3,3-dimethyl-1-triazenyl]imidazole-4-carboxamide, the only clinically used triazene , first developed by Shealy et al (Shealy et al, 1961, Shealy et al, 1962).

The mechanism of action of DMTIC is thought to

involve metabolic N-demethylation, via the N-hydroxymethyl triazene (5- [3-hydroxymethyl- 3- methyl- 1- triazenyl] imidazole-4-carboxamide (HMTIC) to give the cytotoxic monomethyltriazene, (5-3-methyl-1-triazenyl)imidazole- 4-carboxamide) (MTIC), which is known to methylate N^7-sites of guanine residues in DNA (Preussman & von Hodenberg, 1970). Metabolic activation of DMTIC by N-demethylation has been demonstrated in rats and man (Skibba et al, 1970), but disputes over whether MTIC is the active moiety have arisen due to the demonstration of mutagenicity (Bartoli-Klugman et al, 1982), and apparent anti-metastic activity (Giraldi et al, 1978, Sava et al, 1982), which were evident without the requirement of metabolism.

Significantly the 3,3- diethyl- 1- aryltriazenes lack any antitumour activity (Connors et al, 1976). Although the diethyltriazene is as readily dealkylated as the dimethyl triazene (Vaughan and Stevens, 1978), and the monoethyl triazene can ethylate guanine (Preussman and von Hodenberg, 1970), diethyl triazenes are not active in vivo against rodent tumours (Connors et al, 1976).

1.8.1 The DNA damage caused by alkylating agents.

The main target for the imidazotetrazinones, the chemically related triazenes, and the nitrosoureas, is thought to be nuclear DNA. Drug induced DNA damage can interfere with DNA function in two general, but not mutually exclusive ways. In the first instance chemical alterations, such as base alkylation, may affect the direct interaction of the affected sites with enzymes or other molecules. In the second instance changes may be induced which alter the topological nature of the DNA, which may be exemplified by strand breaks, inter- and intra-DNA cross-links, and DNA-protein cross-links. The first damage category includes actions that are considered to lead to mutation and carcinogenesis, while the second is more associated with cytotoxicity and anti-cancer activity.

1.8.2 DNA single strand breaks.

Single strand breaks may result as an immediate consequence of chemical effects on nucleic acids, as a delayed consequence of a slow spontaneous decomposition process, or as a result of enzymatic repair processes. It is known for example that N^7-guanine alkylation labilises the glycosidic bond between purine and sugar to such an extent that considerable spontaneous depurination occurs

even at normal physiological pH. (Lawley, 1976, Pegg, 1977, Margison and O'Connor, 1979). In the case of human fibroblasts treated with MNU, Medcalf and Lawley (1981) found that the rate of removal of 7-methylguanine and 3-methyladenine bases was considerably accelerated by the enzymatic activities of 7-methylguanine glycosylase and 3-methyladenine glycosylase.

Although single strand breaks have not been implicated in cell killing, they may be responsible for a transient inhibition of DNA replication initiation following exposure of cells to ionizing radiation and methylating agents (Painter and Young, 1976, Painter, 1977, Povirk, 1977). Gibson et al, (1984a) however found that mitozolomide, MCTIC and CNU (1-[2-chloroethyl]- 1-nitrosourea) (all agents considered to possess low biological carbamoylating activity), caused negligible single strand breaks up to nine hours after drug removal. Drugs such as BCNU, which possess high carbamoylating activity, do cause single strand breaks (Sariban et al, 1987).

1.8.3 DNA alkali-labile sites.

Most of the methods used to detect strand breaks in cells require alkaline conditions, which can convert certain types of chemical damage to strand breaks. Two types of alkali-labile sites have been identified, one being base-free sites, the other being alkylated DNA phosphate groups (Verly, 1974, Schooter, 1976). Therefore it is sometimes not clear to what degree single strand breaks observed following drug treatment reflect pre-existing breaks as opposed to alkali-labile sites.

1.8.4 DNA interstrand cross-links.

Chloroethylnitrosoureas can alkylate the N^7-position of guanine, and this reaction may lead to cross-linking between adjacent guanines in the same strand (Tong and Ludlum, 1981).

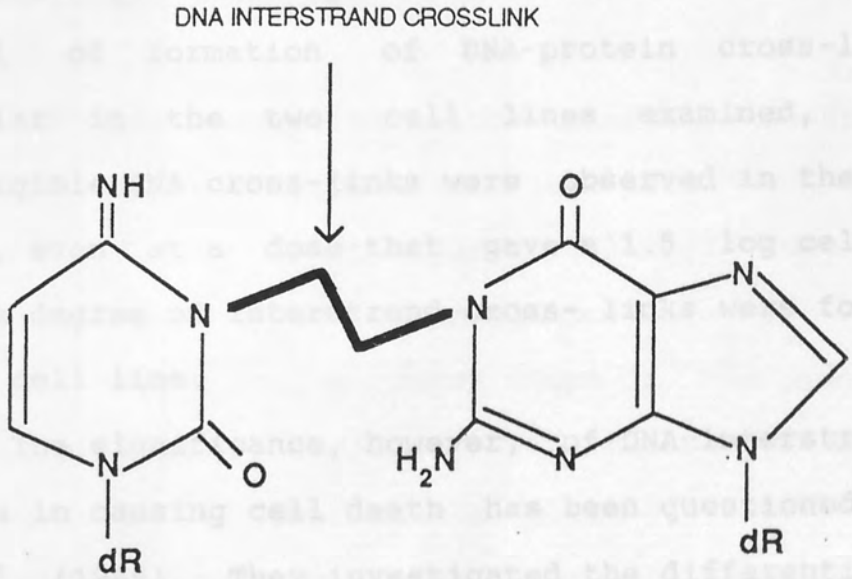
The mechanism by which chloroethylating agents kill tumour cells is thought to be due to their ability to cross-link DNA (Kohn, 1977). It is thought that initial alkylation occurs at the O^6 position of guanine followed by internal rearrangement to the N^1 position of guanine, followed by the the completion of the interstrand link to the N^3 position of the complementary cytosine residue.

This cross-link has been demonstrated in BCNU treated DNA (Tong et al, 1982). Mitozolomide has also been shown

to produce DNA interstrand cross-links in the murine L1210 leukaemia cells (Gibson et al, 1984a).

Figure 4.

Diagrammatic representation of one postulated DNA interstrand cross-link.



Diagramatic representation of the postulated DNA interstrand cross-link between the N^1-position of the guanine base and the N^3-postion of the complementary cytosine base.

It is thought that the initial alkylation occurs at the O^6-position of guanine,followed by internal rearrangment to the N^1-position of the guanine.

Erickson et al (1980) showed that normal Mer+ (IMR-90) and SV40- transformed Mer- (VA-13) human embryo cells differed in their response to chloroethyl nitrosoureas, possibly resulting from a difference in their ability to repair O^6-alkylguanine lesions. Gibson et al (1984b) demonstrated a similar differential response to the same cell lines with both mitozolomide and MCTIC, where the Mer- cell line was about five to seven fold more sensitive to these agents than the Mer+ cell line. The level of formation of DNA-protein cross-links was similar in the two cell lines examined, but while negligible DNA cross-links were observed in the Mer+ cell line, even at a dose that gave a 1.5 log cell kill, a large degree of interstrand cross- links were found in the Mer- cell line.

The significance, however, of DNA-interstrand cross-links in causing cell death has been questioned by Gibson et al, (1986). They investigated the differential effects of a series of alkyltriazenyl imidazoles on the HT-29 colon carcinoma cells (Mer+) and BE colon cells (Mer-) and demonstrated that while the chloroethyl triazene was able to cross-link DNA in Mer- cells, the monomethyl and monoethyl analogues were unable to produce cross-links in either cell line.

The chloroethyl and monomethyl triazenes showed a differential cytotoxicity between the two cell lines, but the monoethyl compound did not. This, they argued, suggested that DNA cross-linking is not the only cause of

cytotoxicity, as the monofunctional methylating agent from the same series as a bifunctional alkylating agent would not be expected to produce a differential cytotoxicity between Mer+ and Mer- cells. However cross-linking may indeed be the cause of cytotoxicity in the Mer- cell line by the chloroethylating agent.

1.9 DNA repair processes.

With the exception of the naturally occurring bases thymine and 5-methylcytosine, the presence of alkylated bases in DNA is deleterious to cellular functioning and may cause cytotoxicity or mutagenicity.

All oxygen and nitrogen atoms in the polynucleotide structure can be modified, with the exception of those nitrogen atoms forming a glycosyl bond with the deoxyribose sugar, and the exocyclic amino groups. On exposure of DNA to alkylating agents, the relative types and amounts of modified bases formed is dependent on the chemical used, and varies markedly between agents. For example, alkyl sulphates predominantly alkylate at nitrogen atoms, while the N-nitroso compounds show a broader specificity, and will alkylate at both oxygen and nitrogen residues (Karran and Lindahl, 1985).

Furthermore, the reaction products in DNA treated with monofunctional methylating and ethylating agents of the same class may differ, in both the total amount of DNA

alkylation products formed, and the proportion of each product to the total. Beranek et al (1980) comprehensively analysed the reaction products of salmon sperm DNA alkylated with either methyl or ethyl nitrosourea or methyl or ethyl methanesulphonate.

They found that after methyl methanesulphonate treatment, about 81% of the total DNA alkylated was at the N^7-position of guanine and about 11% at the 3-position of adenine, but there was negligible formation of O^6-methylguanine. In contrast, 58% of the total alkylated products after ethyl methanesulphonate treatment was 7-ethylguanine, 4% was 3-ethyladenine and about 2% was O^6-ethylguanine.

Following MNU treatment, 66% of the total alkylated products was 7-methylguanine, about 8% was 3-methyladenine, 12% was phosphotriesters, and 5-6% was O^6-methylguanine. However only about 11% of the total alkylated products after ethylnitrosourea treatment was as 7-ethylguanine, 3% was 3-ethyladenine, 8-9% O^6-ethylguanine, but some 55% of the total products was phosphotriesters. These results must be also tempered in the knowledge that monoethylating agents are approximately 10-fold less reactive than their monomethylating counterparts (Pegg, 1973, Frei et al, 1978).

As mentioned previously, both 7-alkylguanine and 3-alkyladenine are subject to repair by specific glycosylases, and their repair by enzymatic means would appear to occur more rapidly than loss by spontaneous

hydrolysis (Medcalf and Lawley, 1981).

The significance of the presence of 7-alkylguanine residues in DNA is uncertain, as methyl and ethyl adducts do not apparently change the coding specificity of the guanine base (Karran and Lindahl, 1985), but its rate of loss from human fibroblasts is relatively slow, with a half life of approximately 30 hours (Medcalf and Lawley, 1981). However, DNA methylases would appear not to bind to a methyl accepting polymer in which the N^7-position of guanine is alkylated (Drahovsky and Morris, 1972).

3-alkyladenine would appear to be a more toxic lesion, and protrusion of the alkyl group into the minor groove of the DNA helix is thought to act as a block to DNA polymerases (Medcalf and Lawley, 1981, Karran and Lindahl, 1985), and therefore in the absence of repair would be potentially a cytotoxic lesion. The ability of cells to remove 3-methyladenine has been shown to be associated with the recovery from the cytotoxic action of methylating agents (Harris et al, 1981). It is removed from the DNA of human fibroblasts, with a half life of about 2hr (Medcalf and Lawley, 1981).

1.10 The significance of the O^6-alkylguanine lesion.

The O^6-alkylguanine lesion is of prime interest, and is thought by some to be the lesion responsible for the mutagenic and carcinogenic effects of alkylating agents (Doniger et al, 1985, Newbold et al, 1980). Day et al, (1980a) demonstrated that human cells differed in their ability to reactivate adenovirus which had been treated in vitro with MNNG. They designated cells which were capable of reactivating the damaged virus as Mer+ (methylation repair) and cells deficient as Mer-. Day et al (1980b) further showed that Mer+ cells were capable of removing O^6-methylguanine lesions from their DNA, but that Mer- cells are incapable of this function. Similar observations were later reported by Sklar and Strauss (1981), who found that 20-30% of human tumour cell lines appeared unable to remove O^6-alkylguanine from their DNA, and described such cell lines as being Mex-.

This repair process is mediated by a repair protein (variously named, but typically called) O^6- alkylguanine DNA-alkyltransferase (O^6-AGAT), which has been purified from bacterial (Demple et al, 1982), rat liver (Pegg et al, 1983) and human cells (Harris et al, 1983). All normal cells and cell lines have been found to be proficient in O^6-AGAT (Harris et al, 1983 and Yarosh et al, 1983). Its method of action in these cell types may be summarised as follows:-

(a)The alkyl group is enzymatically transferred from the O^6- alkylguanine residue to the protein molecule with the regeneration of the unsubstituted guanine base.

(b)No release of the O^6-alkylguanine occurs in the form of the free base or nucleotide, and there is no generation of apurinic sites or chain breaks.

(c)The alkyl group is transferred to one of four cysteine thiol residues in the protein thereby producing S-alkylcysteine.

(d)Each alkyltransferase molecule can stoichometrically accept only a single alkyl group, thereby inactivating the protein.

(e)The transfer reaction is rapid for a methyl group, but takes progressively longer, the longer the alkyl or halogeno-alkyl chain, and occurs by suicide kinetics, reflecting the lack of regeneration of the protein. Once the O^6-AGAT in a cell has reacted, only RNA and protein synthesis restore the original levels of O^6-AGAT.

(f)Both E. coli and mammalian O^6-AGAT would appear to function similarly, but the two proteins do not appear to be identical, (Yarosh, 1985).

Inter-strand cross-linking of DNA by a variety of chloroethylnitrosoureas may be prevented by the O^6-AGAT repair protein. It is thought that for this to occur then the chloroethyl group must be removed from the O^6-position of guanine, prior to it internally rearranging to the N^1-position (Robins et al, 1983, Brent, 1984).

It is known that pre-treatment of Mer+ cells with MNNG results in a depletion of the O^6-AGAT with a resultant synergistic increase in cell kill by CNU (Zlotogorski and Erickson, 1984, Gibson et al, 1985), but not other types of bifunctional agents, such as nitrogen mustard and cis-diamminedichloroplatinum (II) (Gibson et al, 1985). Brent (1986) has also showed that O^6-AGAT could be inactivated with methylating and chloroethylating agents, both directly and by DNA treated with these agents, but their efficacies varied considerably. This, he suggested, indicated that depletion of the transferase by reaction with O^6-methylguanine in DNA may not be the only method for sensitizing cells to these agents.

Dolan et al (1985a) demonstrated that exposure of HeLa cells to free O^6-methylguanine base for four hours or longer led to a 70-80% loss in the activity of O^6-AGAT. Exposure of cells to other O^6-alkylguanines also caused a depletion in O^6-AGAT activity, but they were less effective. Yarosh et al (1986) further demonstrated the depletion of O^6-AGAT by O^6-methylguanine in a variety of cell lines, and their subsequent sensitisation to the cytotoxic effects of chloroethylnitrosourea.

These results would appear to be in conflict with those of Karran and Williams (1985), who demonstrated the complete depletion of O^6-AGAT in Raji (Mer+) cells by exposure of to O^6-methylguanine (as free base), but found that this did not sensitize the cells to MNNG, BCNU or CNU. This they suggested indicated that O^6-alkylguanine adducts in DNA are not lethal, and that some other lesion(s) may be responsible.

An explanation of the observations of Karran and Williams (1985) may be offered by Tisdale (1987), who investigated the response of a range of cell lines, with various intrinsic levels of O^6-AGAT, to chlorambucil and three imidazotetrazinone analogues. He observed the effect of the drugs on the depletion of O^6-AGAT by free O^6-methylguanine base, and was able to show that Raji cells were atypical in their sensitization response after O^6-AGAT depletion.

This study also observed that all the cell lines were about equally sensitive towards the nitrogen mustard-type alkylating agent, but there was a wide variation in their sensitivities to the 3-(2-chloroethyl)- and 3-methyl-imidazotetrazinones, which correlated well with the intrinsic cellular levels of O^6-AGAT. In contrast, the cytotoxicity produced by the 3-ethyl analogue did not vary greatly between cell lines, and did not correlate with repair protein levels.

The O^6-AGAT levels in the Mer+ cell lines were shown to be depleted by incubation with free O^6-methylguanine,

which in all cases (except the Raji cell line) sensitized them to to the effects of mitozolomide and its 3-methyl, but not 3-ethyl analogue. These results suggest that alkylation of the O^6-position of guanine is important in the mode of cytotoxicity of mitozolomide and temozolomide, but its role in the cytotoxicity of CCRG 82019 is less clear.

1.11 O-alkyl pyrimidines.

Much interest has been shown recently over the possible role of O-alkyl pyrimidines (principally O-alkyl thymidine) in carcinogenesis and mutagenesis. They contribute much less than 1% to the total DNA alkylation produced *in vitro* by MMS, EMS or MNU, but about 10% of the total alkylation produced *in vitro* by ENU (Beranek *et al*, 1980). However, *in vivo*, several workers have found that on continuous administration of methylating or ethylating agents which induce hepatocellular tumours, O^6-alkylguanine is rapidly lost, whilst O^4-alkylthymidine accumulates (Swenberg *et al*, 1984, Richardson *et al*, 1985, Dyroff *et al*, 1986). It has also been shown that O-alkylated thymidine can cause both miscoding and mispairing in synthetic polynucleotides *in vitro* (Singer, 1986). The significance of O-alkylated pyrimidines is, however, the subject of much discussion at this point in time.

1.12 DNA methylation.

Mammalian DNA contains about 3% 5-methylcytosine, and it is thought that DNA methylation may play an important role in the governing of gene function and expression, and in the differentiation of cells (Razin and Riggs, 1980, Razin and Cedar, 1984). It has been found that 5-methylcytosine is present in those genes in cells in which the gene is not expressed, but that the same sequence is hypomethylated in cells in which the gene is active (McGhee and Ginder, 1979, Compere and Palmiter, 1981). It is thought that methylation is maintained during DNA replication by the enzyme DNA (cytosine-5)-methyltransferase,which uses S-adenosyl methionine as the methyl donor, acting at hemimethylated sites containing 5-methylcytosine in the parent strand (Drahovsky and Boehm, 1980, Razin and Riggs, 1980).

This would provide a mechanism by which methylation is conserved during cell division, but can be removed for succeeding generations if the methylation process is ever inhibited.

There is evidence that exposure to carcinogens reduces the methylation of DNA. MNNG is thought to act by direct inhibition of the methylase enzyme (Cox, 1980), whereas MNU did not affect the enzyme directly, and is thought to act by altering the ability of DNA to act as a substrate for the methylase enzyme (Drahovsky and Boehm, 1980). Inhibition of the DNA (cytosine-5)-

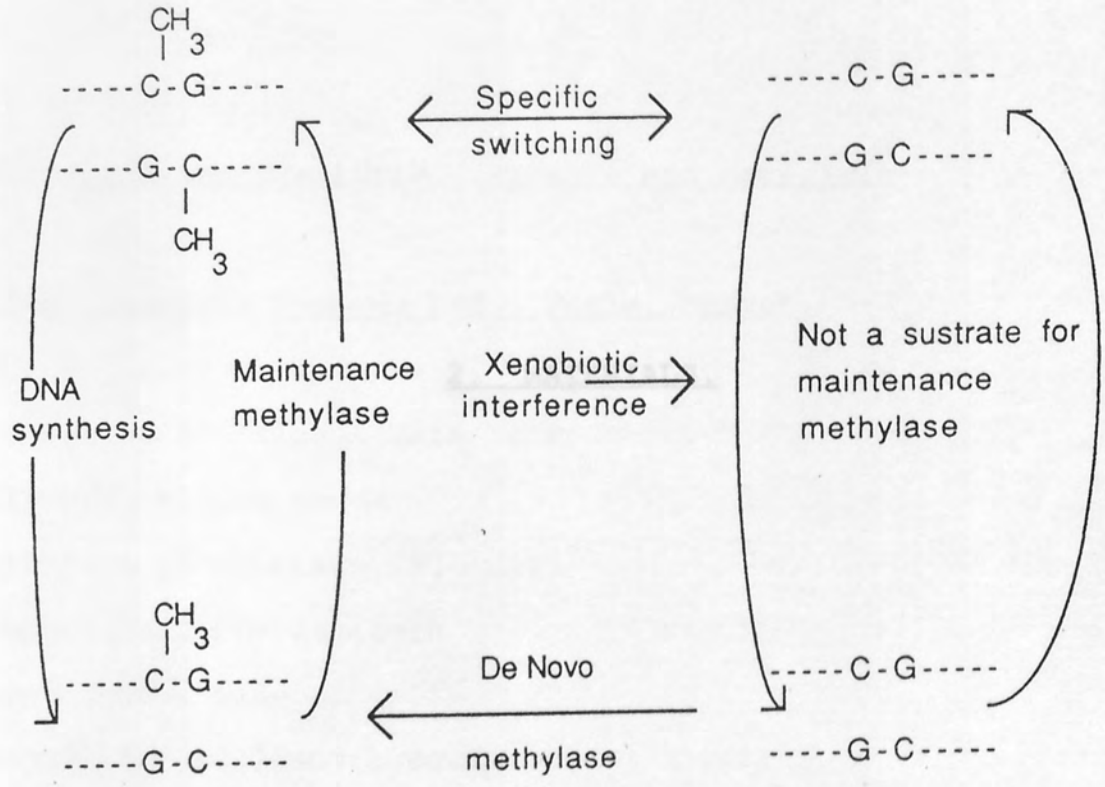
methyltransferase by exposure of cells to 5-azacytidine (Freidman, 1981) results in hypomethylation of the DNA which persists through many generations and is associated with changes in the differentiation of cells (Jones and Taylor, 1980).

Recent work has demonstrated that temozolomide and a methyltriazene induced both differentiation and hypomethylation in the K562 human erythroleukaemia cell line, whereas their ethyl analogues did not (Tisdale, 1985, Tisdale, 1986). It is known that guanine alkylated at the O^6-position may be mispaired during replication, with subsequent insertion of thymidine in place of cytosine in newly replicated DNA (Nyce et al, 1983).

Thus a possible outcome of such a transition may be the loss of critical cytosine residues involved in maintenance of gene function, as well as a subsequent G-C to A-T mutational transition

Figure 5.

Representation of a postulated mechanism for the maintenance of DNA methylation.



2. MATERIALS.

2.1 Cell lines used.

GM892A human lymphoblastoma cells (Mer-) and the Burkitt lymphoma derived Raji (Mer+) cell lines were obtained from Dr. P. Karran of the Mill Hill Laboratories, London, and are as described by Harris et al, (1983).

2.2 Purchased chemicals, reagents and materials

Sigma Chemical Company Ltd., Poole, Dorset.

adenosine 5'-triphosphate (ATP)
albumin, bovine serum
alkaline phosphatase, E. coli
ampholines, pH9-11, 3-10
Bromophenol blue
Coomassie brilliant blue R
cytidine 5'-triphosphate (CTP)
2'-deoxyadenosine 5'-triphosphate (dATP)
2'-deoxycytidine 5'-triphosphate (dCTP)
2'-deoxyguanosine 5'-triphosphate (dGTP)
deoxyribonuclease I, (bovine pancreas)
dithiothreitol
DNA, calf thymus
glycerol
glycine
guanosine 5'-triphosphate (GTP)

8-hydroxyquinoline

magnesium acetate

methyl methanesulphonate (MMS)

molecular weight markers (SDS-6H)

Nonidet NP-40

phenol

phenylmethylsulphonyl fluoride

propidium iodide

rhodamine-123

ribonuclease, type 1-A

RNA, calf liver

RNA polymerase (type I, E coli, strain K-12)

snake venom diesterase (from Crotalus adamateus)

sodium borate

sodium citrate

N,N,N',N'-tetramethyl-ethylenediamine (TEMED)

thymidine 5'-triphosphate (TTP)

Tris(hydroxymethyl)aminomethane (Tris) base

triton X-100

trypan blue

uridine 5'-triphosphate (UTP)

BDH Chemicals Ltd., Poole, Dorset.

acetic acid, glacial (analar)

4-aminosalicylic acid

ammonia (analar)

ammonium persulphate (electran)

m-cresol

dimethyl sulphoxide (DMSO) (analar)

diphenyloxazole (PPO) (scintran)

dipotassium hydrogen phosphate

2-ethoxyethanol

ethylenediaminetetra-acetic acid (EDTA) (disodium salt)

hyamine hydroxide (scintran)

2-mercaptoethanol

3-methyladenine

N,N'-methylenebisacrylamide (electran)

7-methylguanine

perchloric acid

potassium dihydrogen phosphate

sodium acetate

sodium cacodylate

sodium chloride

sodium dodecyl sulphate (biochem. grade, specially pure)

Fisons Scientific Eqiupment, Loughborough, Leics.

acetonitrile

acrylamide (ultrapure for electrophoresis)

agarose LE

boric acid

chloroform

formic acid

hydrochloric acid

potassium chloride

potassium hydroxide

toluene (scintillation grade)

trichloroacetic acid

urea

May and Baker, Loughborough, Leics.

Luma-Gel scintillation fluid

methanol (HPLC grade)

Hopkin and Williams, Chadwell Heath, Essex.

Dow-Corning silicon oil (550)

Gibco, Paisley, Scotland.

foetal calf serum

L-glutamine (for tissue culture)

RPMI 1640 (with 25mM HEPES and L-glutamine)

methionine free RPMI 1640 (with 25mM HEPES and L-glutamine)

Difco Laboratories Ltd., East Molesley, Surrey.

Noble agar

Amersham International, Bucks.

5-[methyl ^{3}H]-thymidine (sp.act. 5Ci/mmol)

[5-^{3}H]-uridine (sp.act. 29Ci/mmol)

L-[4,5-^{3}H] leucine (sp.act. 67Ci/mmol)

[methyl ^{3}H]thymidine 5'-triphosphate (sp.act. 42Ci/mmol)

[5-^{3}H]uridine 5'-triphosphate (sp.act. 13.5Ci/mmol)

inulin-[^{14}C]carboxylic acid (sp.act. 5mCi/mmol)

[^{3}H]-water (sp.act. 5mCi/ml)

New England Nuclear, Du Pont (UK) Ltd, Southampton.

L-[^{35}S]-methionine (>800Ci/mmol)

Biomedical Laboratory Supplies.

400µl and 1.5ml microfuge tubes

Ceaverken AB, Strägäs, Sweden.

CEA Singul non-screen medical X-ray film X-RP

Kodak Ltd., Hemel Hempstead, Herts.

Kodafix

D-19 developer

Millipore UK Ltd., Harrow, Middx.

Millipore 0.45µm filters

Whatman Labsales Ltd., Croydon, Surrey.

Whatman 3MM paper

Whatman GF/C glass microfibre filters, 2.5cm

2.3 Synthesised chemicals.

O^6-methylguanine and O^6-ethylguanine were synthesised as described by Warren (1984).

O^2-, 3- and O^4-methylthymidine and O^2-, 3- and O^4-ethylthymidine were synthesised as described by Kusmierek and Singer (1976) and Farmer et al (1973).

dTp(methyl)dT and dTp(ethyl)dT were synthesised as described by Swenson and Lawley (1978).

2.4 Donated chemicals.

Mitozolomide, temozolomide and CCRG 82019 were synthesised and donated by May and Baker, Dagenham, England. Drugs were dissolved in DMSO at a concentration of 20mg/ml, and aliquots stored frozen at -20°C.

8-carbomyl-3-[^{14}C]-(2-chloroethyl)imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one, (sp.act. 15.8mCi/mmol)

8-carbomyl-3-[^{14}C]-methylimidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one, (sp.act. 20.2mCi/mmol)

8-carbomyl-3-[^{14}C]-ethylimidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one, (sp.act.12.9mCi/mmol)

were synthesised by ICI and donated by May and Baker Ltd., Dagenham, England. Radiolabelled imidazotetrazinones were dissolved in DMSO at a concentration of 20mg/ml, and aliqouts stored frozen at -20°C.

7-ethylguanine and 3-ethyladenine were gifts from Dr. P.

Lawley, Institute of Cancer Research, London.

2.5 Preparation of molecular weight standards for SDS-PAGE.

A 3mg vial of high molecular weight standards (Sigma, SDS-6H) containing carbonic anhydrase (mw 29K), ovalbumin (mw 45K), bovine albumin (mw 66K), phosphorylase b subunit (mw 97.4K), ß-galactosidase subunit (mw 116K) and myosin subunit (mw 205K) were dissolved in 1ml of SDS sample buffer (omitting the 2-mercaptoethanol), and 25µl aliqouts frozen at -20°C.

Immediately prior to use, a further 25µl of SDS sample buffer (with twice the quantity of 2-mercapto-ethanol) was added to each aliqout, and the sample heated at 100°C for 10min. 20µl of this mixture was used as marker compounds.

2.6 Stock solutions and buffers.

Reticulocyte standard buffer.

Tris base	1.211g
sodium chloride	0.584g
magnesium chloride	0.143g
hydrochloric acid	to pH 7.4
distilled water	to 1000ml

(plus phenylmethylsulphonyl flouride, 20µg/ml, as a protease inhibitor).

0.9% PPO/toluene.

2,5-diphenyloxazole	9g
toluene	to 1l

10% SDS.

Sodium dodecylsulphate	10g
distilled water	to 100ml

10% TCA.

trichloroacetic acid	10g
distilled water	to 100ml

50% TCA.

trichloroacetic acid	50g
distilled water	to 100ml

2M ammonium formate stock solution.

(as described by Warren, 1984).

Add 140ml of 0.88 ammonia to 700ml of distilled water, slowly add 120ml of formic acid, allow the mixture to cool to room temperature and continue to add formic acid until the pH is 3.95. Adjust the volume to 1000ml

1M borate buffer

(as described by Warren, 1984).

Make up 1M H_3BO_4 solution and add conc. NaOH until the pH of a 10% solution is 8.5. This is equivalent to a 1M solution of approx pH 8.0. Adjust the volume to give 1M, and re-check the pH of a 10% solution.

Acrylamide Stock I solution.

Acrylamide	44g
N,N'-methylenebisacrylamide	0.8g
distilled water	to 100ml

(and filtered through a Millipore 0.45μM filter).

Acrylamide Stock II solution.

Acrylamide	30g
N,N'-methylenebisacrylamide	0.8g
distilled water	to 100ml

(and filtered through a Millipore 0.45μM filter).

SDS-PAGE electrode buffer.

Tris base	6g
glycine	28.8g
10% SDS solution	20ml
Distilled water	to 2000ml

SDS-PAGE sample buffer.

10% SDS	5ml
0.5M Tris HCl (pH 6.8)	2.5ml
distilled water	5ml
glycerol	5ml
2-mercaptoethanol	2.5ml
5% bromophenol blue	200ul

5% bromophenol blue.

bromophenol blue	5g
distilled water	to 100ml

Gel stain.

Coomassie brilliant blue R	1.37g
methanol	250ml
distilled water	250ml
glacial acetic acid	50ml

Gel storage solution.

acetic acid	70ml
distilled water	to 1000ml

Gel destain.

acetic acid	70ml
methanol	500ml
distilled water	to 1000ml

(Gel destain could be recovered and decolourised for further use by the addition of approx. 2.5g of activated charcoal per litre of solution for a few hours, followed by filtration).

DNA polymerase cell lysis buffer.

Tris-HCl, pH 7.3	10mM
EDTA, disodium salt	10mM
Nonidet NP-40	0.5%

Nuclear proteins extraction buffer.

magnesium acetate	6mM
dithiothreitol	2mM
sodium chloride	0.5M
phenylmethylsulphonylfluoride	0.2mM

in 50mM potassium phosphate buffer, pH 7.1

RNase solution.

RNase type 1-A	1mg
PBS	to 1ml

DNA polymerase buffer.

magnesium acetate	6mM
dithiothreitol	2mM
dATP	100µM
dGTP	100µM
dCTP	100µM
dTTP	100µM

in 50mM potassium phosphate buffer, pH7.1

(plus 2µCi/400ul of methyl(5-^{3}H)-dTTP)

Phenol reagent.

phenol	500g
distilled water	62ml
m-cresol	62ml
8-hydroxyquinoline	620mg

Cell solubilising buffer (for IEF).

urea	54.05g
Nonidet NP-40	2ml
ampholines, pH9-11	2ml
2-mercaptoethanol	1ml
distilled water	to 100ml

Propidium iodide staining solution.

propidium iodide	2mg
sodium citrate	10mg
triton X-100	50µl
sodium chloride	90mg
distilled water	to 10ml

RNA polymerase assay mixture.

ATP	0.2mM
CTP	0.2mM
GTP	0.2mM
UTP	0.2mM
Bovine serum albumin	100µg
5-^{3}H-uridine triphosphate	2.5µCi
DNA (from treated cells)	5µg

each sample made up to a total volume of 245µl with RNA polymerase incubation buffer.

3. METHODS.

3.1 Cell lines used.

3.1.1 Raji cells.

Raji is a Burkitt lymphoma derived cell line. This cell line was classified as O^6-alkylguanine-DNA alkyltransferase proficient (ie Mer+/Mex+) (Harris et al, 1983).

3.1.2 GM892A cells.

The GM892A cell line is a human lymphoblastoma cell line derived from a normal individual. This cell line was classified as O^6-alkylguanine-DNA alkyltransferase deficient (ie Mer-/Mex-) (Harris et al, 1983).

3.1.3 Maintenance of cell lines.

Both Raji and GM892A cells were maintained in exponential growth at a density of between 0.8 and $8x10^5$ cells/ml. Both cell lines were grown in RPMI 1640 medium (with 25mM HEPES and L-glutamine), supplemented with 10% foetal calf serum, under an atmosphere of 5% CO_2 in air. Under these conditions, the cells were found to have a doubling time of between 22 and 26hr.

3.1.4 Cell counting.

Cell counting was performed using a Coulter Counter (model ZM) (Coulter Electronics, Bedfordshire), using the following settings:-

	Cell line	
	GM892A	Raji
Current	200	200
Attenuation	16	8
T_L	10	12
T_U	OUT	OUT

3.1.5 In vitro growth inhibition studies.

Cells growing in exponential phase were seeded at 8 x 10^4 cells/ml in Nunc 24 well plates, and drug solutions added as appropriate. After a 72hr incubation period the cells were counted, and inhibition of growth of the treated cell population as compared to control cell population was determined.

3.2.1 Colony formation in agar.

The technique was a modification of that described by Tew and Wang (1982). Attempts were made to clone single cells in RPMI 1640 medium (with and without HEPES and L-glutamine) semi-solidified by the addition of Noble agar in 4.5cm Petri dishes. After the agar had set, incubation was carried out at 37°C in a humidified atmosphere of 5% CO_2 (in air) for 9-12 days.

A variety of supplements were added, both singly and in combination, to the basic mixture described above. These included 5-20% foetal calf serum, 10-50µM 2-mercaptoethanol, 5-20mM L-glutamine and pre-conditioned medium. The final concentration in the medium was also varied between 0 and 0.5%.

3.2.2 Measurement of cell viability by trypan blue exclusion.

To 100µl of a cell suspension was added 50µl of 0.1% trypan blue in PBS, mixed, and a drop of the suspension placed on an haemocytometer and viewed under a microscope. Non-viable cells take up the dye and appear blue in colour, while the viable cells are able to exclude the dye. At least 200 cells were counted, and the percentage of viable cells calculated.

3.2.3 Measurement of cell viability using rhodamine 123.

To 800µl of a cell suspension was added rhodamine 123 (dissolved in water) to give a final concentration of between 1 and 10µg/ml. The samples were incubated at 37°C for 10min, then washed twice with PBS. The samples were placed in an haemocytometer and viewed under a Nikon Optiphot epifluorescence microscope, with an excitation wavelength of 485nm.

3.3.1 Determination of the purity of radiolabelled imidazotetrazinones

The purity of the radiolabelled 3-^{14}C temozolomide and 3-^{14}C CCRG 82019 were assessed by comparison with pure unlabelled drug by thin layer chromatography, using 0.2mm silicic acid plates (Merck Kieselgel 60 F_{254}), and a solvent system composed of Chloroform: Acetonitrile: Acetic acid, 80: 30: 5. The plates were then cut into strips and counted in 0.9% PPO in toluene scintillation fluid.

Greater than 95% of the radioactivity was found to be associated with the authentic unlabelled compound, and only one spot was found for each sample, which again corresponded to the authentic compound (the Rf values obtained for the compounds were:- Rf 0.44 for temozolomide, and Rf 0.51 for CCRG 82019).

3.3.2 Drug uptake into cells.

Exponential phase GM892A and Raji cells were centrifuged at 500g for 5min in a bench- top Labofuge centrifuge and resuspended in fresh RPMI 1640 medium (supplemented with 25mM HEPES and L- glutamine, and 10% foetal calf serum), to give a cell density of $5x10^6$ cells/ml. The cells were then equilibriated for 10min at 4^oC in an ice-water bath, under an atmosphere of 5% CO_2 in air.

Uptake was initiated by the addition of 0.1mM 3-^{14}C labelled drug. At the specified time points 200µl samples of cells were drawn in triplicate, and removed to an Eppendorf 400ul tube. The tube contained 100µl of Dow Corning silicon oil: Mazda corn oil (10:3) and 50µl of 90% formic acid. After centrifugation at 900g for 30 seconds in a microfuge, the tubes were frozen at the oil/acid boundary. Each portion was placed in scintillation vial, allowed to thaw and mixed with 5ml of Luma-Gel scintillation fluid, and the radioactivity determined. The oil layer contained the extracellular pool, whilst the acid layer contained the intracellular radioactivity.

The cell volume was established by treating a further sample of cells from both cell lines with ^{14}C inulin and [^3H] H_2O, and once equilibrium had been established, the samples were treated as for the experimental tubes.

3.4 Measurement of radiolabelled precursor incorporation into acid-insoluble material.

Cells at a density of 3×10^5 cells/ml were incubated with drug as indicated in the figure legends. One hour prior to the time stated in the figure legends, 2.5µCi of radiolabelled precursor was added to 1ml of the cell suspension and incubated at 37°C for 1hr. Incorporation of the labelled precursors into acid-insoluble material was determined by washing the cell suspension onto a Whatman GF/C glassfibre filter with 3ml of PBS, precipitated and washed further with 3 x 3ml of ice-cold 5% TCA and 3ml of ice- cold absolute alcohol. The filters were dried at room temperature overnight, and the radioactivity was determined in a 0.9% PPO in toluene scintillation fluid.

3.5 Measurement of precursor pool sizes.

Cells at a density of 3×10^5 cells/ml were incubated with drug as indicated in the figure legends. One hour prior to the time stated in the figure legends 2.5µCi of radiolabelled precursor was added to 1ml of the cell suspension. At the appointed time the cells were rapidly sedimented in a microfuge. The cell pellet was washed twice with 1ml of ice-cold PBS, and then lysed with 500µl of ice-cold 0.5M perchloric acid and left for one hour at

4°C. The acid-insoluble material was then sedimented by centrifugation for 10min in a microfuge, and the supernatant collected. The pellet was then washed with a further 500µl of ice cold 0.5M perchloric acid, resedimented, and the supernatants were combined, neutralized with 5M aqueous potassium hydroxide solution, and after the removal of the insoluble potassium chlorate, the radioactivity was determined in Luma-Gel scintillation fluid.

3.6 Reactions of radiolabelled imidazotetrazinones with isloated macromolecules.

Radiolabelled drugs were incubated with 600µg of calf thymus DNA, calf liver RNA or bovine serum albumin in 300µl of 50mM KCl plus 700µl of 50mM Tris-HCl, pH7.8 and 10mM EDTA for 2hr at 37°C. The reactions with DNA and RNA were terminated by chilling on ice, adding sodium acetate to give a 2% solution, followed by precipitation of the macromolecules with 3vol of ice-cold absolute ethanol. The DNA and RNA were collected by centrifugation, and the non-reacted drug removed by two washes with ice-cold absolute ethanol. The reaction with protein was terminated by chilling the solution on ice followed by the addition of 3ml of ice-cold 10% trichloroacetic acid. The protein was collected by centrifugation and washed and recentrifuged twice in 3ml of ice-cold 10% trichloroacetic

acid.

The DNA and RNA precipitates were dissolved in 150µl of 50mM KCl and the radioactivity determined in Luma-Gel scintillation fluid, using a Packard Tri-carb 2000 CA scintillation analyser. A portion was assayed for DNA content by the method of LePecq and Paoletti (1966) and RNA was determined by the absorption at 260nm. The protein was dissolved in 150µl of 0.01M NaOH and the concentration determined by the method of Lowry et al (1951). The solution was neutralised with 1M HCl prior to radioactivity detection. All solutions of macromolecules were solubilised with 200µl of hyamine hydroxide prior to counting.

To establish that the radioactivity was covalently attached to the macromolecules, portions of the DNA, RNA and protein after incubation with 0.1mM drug for 2hr at 37°C were precipitated and washed as above, then redissolved in 50mM KCl (DNA and RNA) or 0.01M NaOH (protein). A portion of each sample was dialysed against water for 24hr at 4°C. The undialysed and dialysed samples were then reprecipitated, washed and the macromolecular bound radioactivity was determined. After dialysis the percentage of radioactivity remaining bound to DNA, RNA and protein was for mitozolomide, 130, 99, and 88, for temozolomide, 93, 108 and 120 and for CCRG 82019 97, 94 and 98 respectively. This suggests that all of the radioactivity was covalently bound to macromolecules.

3.7 Assessment of specific base alkylations in calf thymus DNA after treatment with either temozolomide or CCRG 82019.

3.7.1 Alkylation of the calf thymus DNA.

Calf thymus DNA was dissolved to give a concentration of 10mg/ml in 300µl of 50mM potassium chloride solution, and 700µl of a 50mM Tris-HCl, (pH 7.8), 10mM EDTA solution added. This mixture was incubated with either 0.5mM 3-[^{14}C]-labelled temozolomide or CCRG 82019 for 2hr at 37^{o}C. The reaction was terminated by chilling on ice, the addition of sodium acetate to give a final concentration of 2%, followed by the precipitation of the DNA with 3 volumes of ice-cold absolute ethanol. The DNA was collected by centrifugation at 5000g for 5min and non-reacted drug removed by three washes in ice-cold absolute alcohol, and dried at room temperature.

3.7.2 Hydrolysis of the alkylated calf thymus DNA.

3.7.2.1 Neutral hydrolysis.

The treated DNA was redissolved to give a concentration of 5mg/ml in 10mM sodium cacodylate buffer (pH 7.0), and heated at 100°C for 30min. The solution was rapidly cooled on ice, and the unhydrolysed DNA was precipitated by the addition of 0.1vol of ice-cold 1M HCl, centrifuged at 5000g for 10min, and the supernatant removed. The precipitate was washed with 500µl of ice-cold 1M HCl, centrifuged at 5000g for 10min, and the supernatant again removed. The two supernatants were combined, lypholised and redissolved in 1.4ml of water, and filtered through a Millipore 0.45µm filter immediately prior to HPLC analysis.

3.7.2.2 Acid hydrolysis of partially apurinic DNA.

A portion of the precipitate after the neutral hydrolysis procedure was dissolved in 1ml of 0.1M HCl, and the mixture hydrolysed at 70°C for 30min. The mixture was allowed to cool and filtered through a Millipore 0.45µm filter prior to HPLC analysis.

3.7.2.3 Enzymatic hydrolysis of partially apurinic DNA.

A portion of the precipitate after the neutral hydrolysis procedure was dissolved in 900µl of water and 100µl of 1M sodium borate buffer. To this was added 100µl of DNase I, and the mixture was incubated at 37°C for 1hr. After 1hr 100µl of snake venom diesterase (0.04 units) and 2.5 units of E.coli alkaline phosphatase was added, and the mixture incubated at 37°C for 24hr. Hydrolysis was terminated by the addition of 40µl of 1M formic acid, the pH adjusted to pH 4.0 with the further addition of 1M formic acid, and the solution filtered through a Millipore 0.45µM filter prior to HPLC analysis.

3.7.3.1 HPLC analysis of alkylated DNA products following neutral or neutral and acidic hydrolysis.

HPLC separations were performed on a system consisting of an Altex model 420 microprocessor controller/programmer, two Altex 100A pumps, a Water Lambda-Max model 480 UV detector (at either 254 or 280nm) and an LKB Broma 2210(1) channel recorder.

Methylated products from the neutral thermal hydrolysis or the neutral and acid hydrolysis fraction of the DNA were separated with a Partisil 10 SCX column, and eluted with a buffer, the composition of which changed from Buffer A (8% methanol) to Buffer B (1M ammonium

formate, pH 4.0,in 8% methanol) in the following manner:-

Time after injection (min)	% composition of buffer	
	Buffer A	Buffer B
0 to 20min	98%	2%
20 to 40min	98 to 60%	2 to 40%
40 to 60min	60%	40%
60 to 70min	60 to 98%	40 to 2%

Ethylated products were similarly separated, except the buffer composition changed from Buffer A (12% methanol) to Buffer B (0.4M ammonium formate, pH 4.0, in 12% methanol) in the following manner:-

Time after injection (min)	% composition of buffer	
	Buffer A	Buffer B
0 to 5min	96%	4%
5 to 30min	96 to 55%	4 to 45%
40 to 60min	55%	45%
60 to 70min	55 to 96%	45 to 4%

The flow rate used was 2ml/min. All buffers were degassed under vacuum immediately prior to use. 50µl of a solution of marker compounds (containing O^6-(methyl or ethyl) guanine, 3-(methyl or ethyl) adenine and 7-(methyl or ethyl) guanine, dissolved in 0.1M HCl at 1mg/ml) was added prior to injection. Fractions were collected every 30sec directly into liquid scintillation vials, and the

counts determined in Luma-Gel. The amounts of each product formed were determined by the radioactivity which co-eluted with the marker compounds.

3.7.3.2 HPLC analysis of alkylated DNA products following enzymatic hydrolysis.

The enzyme hydrolysed samples were adjusted to pH 4.0 with formic acid, filterd through a Millipore 0.45µm filter and eluted from a Partisil 10 SCX column with a buffer which changed in composition from Buffer A (distilled water), Buffer B (0.4M ammonium formate, pH 4.0) in the following manner:-

Time after injection (min)	% composition of buffer	
	Buffer A	Buffer B
0 to 15min	100%	0%
15 to 35min	100 to 60%	0 to 40%
35 to 45min	60%	40%

The material which eluted in the first 10min was collected and neutralised with 1M sodium borate (pH 8.0) (approx 0.05 vol). The mixture was lypholised, redissolved in 1.8ml of water and neutralised with 1M formic acid (approx 40µl). To 450µl was added 50µl of a mixture of marker compounds (1mg/ml of O^2-, 3, and O^4-(methyl or ethyl) thymidine, O^6-(methyl or ethyl) guanine,

and dTp(methyl or ethyl)dT in 0.1M HCl). This mixture was then chromatographed on a Radial pak 10um C_{18} column, and eluted with a buffer the composition of which changed from Buffer A (0.05M ammonium formate, pH4.0) to Buffer B (methanol) in the following manner:-

Time after injection (min)	% composition of buffer	
	Buffer A	Buffer B
0 to 20min	95 to 45%	5 to 55%
20 to 30min	45%	55%

Fractions were collected every 30sec directly into liquid scintillation vials, and the counts determined in Luma-Gel, as described elsewhere. The amounts of each product formed were determined by the radioactivity which co-eluted with marker compounds.

3.8 Extent of reaction of imidazotetrazinones with macromolecules in intact cells.

Both Raji and GM892A cells were routinely grown in RPMI 1640 medium containing 10% foetal calf serum under an atmosphere of 5% CO_2 in air. $8x10^6$ cells were treated with sodium formate to give a final concentration of 20mM 30min prior to the addition of 0.1mM (final concentration) of 3-^{14}C side-chain labelled imidazotetrazinones at the specific activities indicated in the materials section. At time intervals a portion of the cell suspension was removed, sedimented by centrifugation and washed three times in 1ml of PBS. The cell pellet was lysed in 0.5ml of ice-cold 0.2M perchloric acid and sedimented by centrifugation at 4°C. The precipitate was washed twice with 0.5ml of ice-cold 0.2M perchloric acid and sedimented by centrifugation at 4°C, and the washings plus the original supernatant were used to measure the acid-soluble pool. The radioactivity associated with the RNA in the cell pellet was determined by hydrolysis in 0.7ml of 0.3M KOH for 1hr at 37°C followed by precipitation with 0.5ml of perchloric acid at 4°C. The precipitate was collected by centrifugation and washed twice with 0.2M perchloric acid. The supernatant and washings were neutralised and the radioactivity determined in Luma-Gael scintillation fluid.

The radioactivity in the pellet from the RNA determination was assayed by hydrolysis (twice) in 0.75ml

of 0.5M perchloric acid for 30min at 80°C. On cooling the protein precipitated and the supernatants when neutralised were used for the determination of radioactivity associated with the DNA. The remaining acid-insoluble fraction was dissolved in 1ml of 0.01M NaOH and the radioactivity determined after the addition of 0.2ml of hyamine hydroxide. The concentration of DNA was determined by the reaction with diphenylalanine and RNA by reaction with orcinol, according to the method of Munro and Fleck (1966). To determine the efficiency of extraction of macromolecules and the extent of contamination of the various macromolecules with one another, both Raji and GM892A cells were incubated with 1μCi/ml of either 5[methyl-^{3}H]-thymidine, [5-^{3}H]-uridine or L-[4,5-^{3}H]-leucine, and the DNA, RNA and protein extracted as above. In both cell lines 96% of the 5[methyl-^{3}H]-thymidine was associated with DNA fraction, 97% of the [5-^{3}H]-uridine was associated with the RNA and 94% of the L-[4,5-^{3}H]-leucine with the protein fraction.

3.9 The ability of DNA extracted from Raji and GM892A cells treated with imidazotetrazinones to support RNA polymerase activity.

3.9.1 Extraction of DNA from cells

The extraction of DNA from cells was based on the method described by Warren (1984).

Raji cells were treated either 20μM mitozolomide, 206μM temozolomide or 361μM CCRG 82019, and GM892A cells were treated with either 2μM mitozolomide, 10μM temozolomide or 245μM CCRG 82019. After the stated incubation period, $2x10^7$ cells were pelleted by centrifugation at 500g for 5min in a bench-top Labofuge centrifuge, and washed twice with 5ml of PBS. The pellet was homogenized in 2ml of 6% 4-aminosalicylic acid, and to this was added 200μl of 10% SDS. As cell lysis proceeds the solution clarifies and becomes more viscous. When lysis was complete, 2.2ml of phenol reagent was added, and the mixture stirred for 30min. It was then centrifuged at 3000g in a bench- top Labofuge centrifuge for 30min. The upper, aqueous layer was removed and the DNA precipitated from it by the addition of 1.5vols of 2-ethoxyethanol. The DNA was washed three times by sedimentation and resuspension in 3ml of a solution of 70% ethanol and 2% sodium acetate, and dried at room temperature.

The DNA was dried overnight at room temperature and

redissolved in 500µl of 10mM Tris-HCl buffer, pH 8.0. The concentration of DNA in solution was determined by the method of Le Pecq and Paoletti (1966) and the concentration adjusted with 10mM Tris-HCl buffer, pH 8.0, to give a volume of 0.5mg/ml.

3.9.2 RNA polymerase assay.

The method employed is based on that described by Marushige and Marushige (1983b).

The following reaction mixture was employed which contained:-

ATP	0.2mM
CTP	0.2mM
GTP	0.2mM
UTP	0.2mM
Bovine serum albumin	100ug
5-^{3}H-uridine triphosphate (sp. act. 13.5Ci/mmol)	2.5µCi
DNA (from treated cells)	5µg

made up to a volume of 245µl with RNA polymerase incubation buffer.

The reaction was initiated by the addition of 1 unit of RNA polymerase (Type I, E coli, strain K-12), and the mixture incubated at 37^{o}C for 10min. The reaction was terminated by rapidly chilling the mixture in an ice-bath, and the addition of 0.5ml of ice-cold 5%

trichloroacatic acid.

The precipitate from each tube was collected on a Whatman GF/C filter, washed three times with 3ml of ice-cold 5% trichloroacetic acid, and further rinsed three times with ice-cold absolute ethanol. The filter was dried at room temperature overnight, and the radioactivity determined in 5ml of 0.9% PPO in toluene.

3.10 DNA polymerase activity in nuclear protein extracts from Raji and GM892A cells treated with imidazotetrazinones.

3.10.1 Extraction of nuclear proteins

Drug treated cells ($2x10^7$) were sedimented by centrifugation at 500g for 5min in a bench-top Labofuge centrifuge and washed twice by resuspension and resedimentation in 5ml of PBS. The nuclei were prepared by treating the cell pellet with 1ml of cell lysis buffer and vortexed for 10min. The nuclei were sedimented by centrifugation for 5min at 1500g, and the supernatant discarded. The pellet was resuspended in 0.5ml of nuclear protein extraction buffer, vortexed, and left to stand on ice for 1hr. The suspension was then resedimented by centrifugation at 1500g for 5min, and the supernatant assayed.

3.10.2 Preparation of activated DNA.

Calf thymus DNA was dissolved to give a concentration of 2mg/ml in a solution of 0.01M Tris-HCl (pH 7.5), and 5mM magnesium acetate. To one portion of the DNA solution was added DNase to give a concentration of 4µg/ml, and the mixture incubated at 37°C for 40min. The mixture was then heated at 77°C for 5min, rapidly cooled in an ice bath, and used directly.

The degree of DNase digestion was determined by comparing the hyperchromic changes at 260nm of DNA samples prepared in this method, to an undigested sample, and a further sample which had been hydrolysed in 3ml of 1M perchloric acid for 15min at 100°Cto render the sample 100% acid soluble, as described by Oleson and Koerner (1964). It was found that 17% of the DNA had been rendered acid soluble by this method.

3.10.3 DNA polymerase assay.

The DNA polymerase assay was performed in a similar manner to that described by Tisdale (1980).

To 400µl of the DNA polymerase buffer was added 50µl of the nuclear protein extract and 0.1mg of activated DNA, and incubated at 37°C for 30min. The reaction was terminated by chilling the mixture on ice and the addition of 500µl of ice-cold 1M perchloric acid. The mixture was

poured onto a Whatman GF/C filter, washed three times with 3ml of ice-cold 1M perchloric acid and rinsed three times with ice-cold absolute ethanol. The filter was allowed to dry overnight at room temperature and counted in 5ml of 0.9% PPO in toluene scintillation fluid.

3.11 Investigation of a possible synergistic mode of action between temozolomide and methyl methanesulphonate.

The experiment was carried out as described in above section(3.1.5),the only difference being that cells were treated with a range of concentrations of temozolomide and methyl methanesulphonate, both singly and in combination, to investigate possible synergism.

3.12 Analysis by SDS-PAGE of de novo protein synthesis in Raji and GM892A cells treated with temozolomide.

3.12.1 Dialysis of foetal calf serum.

Foetal calf serum was dialysed against a 0.9% sodium chloride aqueous solution over 48hr at 4°C, changing the solution several times. The serum was then sterilised by filtration through a 0.45μm Millipore filter, and frozen in aliquots until required.

3.12.2 Labelling of cell proteins with methionine

Cells were incubated with appropriate drug treatment, and prior to the stated time point $5x10^6$ cells from each sample were centrifuged at 500g in a bench top labofuge for 5 min, and washed twice with methionine-free RPMI 1640 medium, which had been pre-warmed to 37°C. Each pellet was resuspended in 1ml of methionine-free RPMI 1640 medium supplemented with 10% dialysed foetal calf serum and 10μCi of L-^{35}S methionine, the samples were gassed with 5% CO_2 in air and incubated in a gently shaking water bath for 2hr at 37°C

The cells were then resedimented by centrifugation in a bench top Labofuge at 500g for 5min, washed twice with 5ml of methionine-free RPMI 1640 medium and once with

reticulocyte standard buffer. The cell pellet was then resuspended in 1ml of the reticulocyte standard buffer and sonicated three times for 10min, cooling on ice in between. A small portion of the sample was removed and assayed for protein content by the method of Lowry et al (1951). A further portion was dissolved in Luma-Gel scintillation fluid and the radioactivity determined. The rest of the sample was frozen at -20°C until required.

3.12.3 Preparation of SDS-polyacrylamide gels for electrophoresis.

The method employed was adapted from that described by Lugtenberg et al (1975).

The running gel was prepared by mixing in the following order:-

Acrylamide Stock I solution	12.5ml
10% SDS	1.5ml
1.5M Tris-HCl buffer (pH 8.8)	18.75ml
distilled water	26ml

This mixture was degassed (under vacuum) and 140µl of TEMED and 200µl of a 10% ammonium persulphate aqueous solution added and mixed well. The mixture was poured immediately (by syringe) between the plates of a Biorad Protean 16cm electrophoresis system and left for 1hr to polymerize.

The stacking gel was prepared by mixing in the

following order:-

Acrylamide Stock II solution	7.5ml
10% SDS	4.5ml
0.5M Tris-HCl buffer (pH6.8)	11.25ml
distilled water	24ml

This mixture was degassed (under vacuum) and 120µl of TEMED and 150µl of a 10% aqueous solution of ammonium persulphate was added and mixed well. The mixture was poured immediately (by syringe) onto the running gel almost to the top of the plates. A comb (with 5mm wide and 15mm long teeth) was inserted between the plates to provide wells. The gel was left for a further hour to polymerize. The comb was removed, the gel covered with running buffer and left at 4°C until required (but not longer than 18hr).

3.12.4 Preparation and running of protein samples on SDS-PAGE.

The volume of each protein sample containing 80,000 dpm was drawn and mixed with an equal volume of sample buffer. The mixture was heated for 10 min at 100°C in a boiling water bath to denature the proteins.

The samples were loaded onto the gel (together with 20µl of molecular weight standard markers for comparison) and electrophoresed at a constant voltage of 100mV for 8hr or until the blue dye front was 1cm from the bottom of the gel.

3.12.5 Gel staining.

After the electrophoresis was completed, the gel was immediately removed and fixed in 50% trichloroacetic acid solution for about 10min (or until the blue dye front turned yellow), stained for 1hr in gel stain, rinsed briefly in water to remove excess stain, and destained in gel destain with several changes of solution. Destaining was continued until the protein bands became apparant and the gel background was almost colourless.

Gels were occasionally stored in gel storage solution until required.

3.12.6 Drying and autoradiography of gels.

1ml of glycerol was added to the destain or storage solution and the gel left to soak in this for 15min to soften the gel and prevent cracking. The gel was dried under vacuum onto Whatman 3MM Chr chromatography paper using a Bio-rad Model 224 gel slab drier.

A CEA Singul X-RP non-screen medical plate was exposed to the dried gel in the dark for 9- 12 days. The autoradiograph was then developed by incubation for 5min in Kodak D-19 developer followed by 5min in Kodafix solution, and washed for 30min in distilled water.

3.12.7 Computerised scanning of autoradiographs.

The autoradiographs were analysed with the aid of an LKB ultrascan system.

3.13 Two dimensional gel electrophoresis

The methods used for two-dimensional gel electrophoresis was essentially similar to those described by O'Farrell (1975). Cell proteins were labelled and prepared as described earlier (3.12.2).

3.13.1 Preparation of gels for isoelectric focusing

The isoelectric focusing gel was prepared by dissolving 8.25g of urea in 6ml of water, 2ml of 30% acrylamide solution, and 750µl of an ampholine mixture (90% pH 3.5-10, 10% pH 3.5-5), and warmed at 37°C until the urea dissolved. The mixture was then filtered through a Millipore 0.45µm filter, and degassed under vacuum for 5min. Warmed Nonidet NP-40 (0.3ml) was added, followed by 70µl of 10% APS and 10µl of TEMED, and briefly stirred.

The mixture was drawn up to the 0.35ml mark of a 1ml serological pipette which had been previously cut at the 0.1ml mark, and the tips of the tubes embedded in plasticine and allowed to polymerise for at least 1hr.

The top of the gels were then washed with water and shaken dry, and placed in the isoelectric focusing apparatus, with the tips of the gels in the previously degassed anode buffer (0.01M H_3PO_4 solution), making sure that any air bubbles at the tip were removed. The top of the gel was blotted dry with tissue paper, and filled with overlay buffer (8M urea solution) ensuring that there were no trapped air bubbles. The top reservoir was then filled with previously degassed cathode buffer (0.2M NaOH solution), and the pH gradient aligned by electrophoresing at 200V for 1hr. Following gradient equilibriation, the cathode buffer was removed, and the gel overlay buffer carefully removed.

The protein samples, previously prepared, were thawed and diluted with solubilising buffer to 50µl, and applied to the top of the gel, and covered with overlay buffer One or two gels had no protein applied but were filled with overlay buffer (these were used to determine the pH gradient later). Degassed cathode buffer was then poured into the top reservoir, and the gels electrophoresed for 16hr at 400V. After electrophoresis the top reservoir was drained, the tubes removed, their tops wiped to remove any residual cathode buffer and the gels were extruded.

The gels could be either frozen in a 5ml solution of 10% glycerol, 5% 2-mercaptoethanol, 2.3% SDS, 0.0625M Tris-HCl (pH 6.8) or applied directly to the SDS-PAGE.

The pH gradient of the blank gels was determined by cutting them into 1cm lengths and dissolving each portion

in freshly boiled and cooled water, and measuring the pH of the resultant solution.

3.13.2 SDS-PAGE second dimensional gel.

The running gel was prepared as described for one-dimensional electrophoresis, and poured to the top of the glass plates and allowed to polymerise for 1hr. The upper reservoir was attached and a stacking gel was poured on top of the running gel and left to polymerise for 1hr. The tube gel was thawed and solubilised for 1hr in SDS sample buffer, with gentle agitation, then layered on to the stacking gel, and embedded in a molten 1% agarose solution (the agarose being dissolved in a solution containing 0.3% Tris base, 1.44% glycine, 0.1% SDS). The SDS-PAGE was electrophoresed at 40mA until the dye front was 1cm from the bottom of the gel, then removed, fixed, stained and dried as described for one-dimensional electrophoresis.

3.14 Preparation and processing of cells for cell cycle analysis.

Raji cells were treated with either temozolomide at a concentration of 2.06mM or CCRG 82019 at a concentration of 2.88mM. The Raji control cells were treated with drug vehicle (DMSO) alone at a concentration of 20µl/ml, which corresponded to the volume in which the temozolomide was dissolved. GM892A cells were treated with either temozolomide at a concentration of 103µM or CCRG 82019 at a concentration of 1.92mM. The GM892A control cells were treated with drug vehicle (DMSO) alone at a concentration of 1µl/ml, which also corresponded to the volume in which the temozolomide was dissolved.

For cell cycle analysis cells were fixed and stained essentially using the methods of Gray and Coffino (1979). $1x10^6$ cells were drawn per sample, centrifuged, the medium removed and the pellet washed once in PBS at 4^oC. Following recentrifugation, the cells were fixed by the dropwise addition of ice-cold absolute alcohol with constant agitation, and the fixed cells could be stored in the dark at 4^oC for up to two weeks prior to analysis.

Fixed cells were resuspended in 1ml of RNase solution and incubated at 37^oC for 20min. This procedure was to degrade the RNA, so that only the interaction between fluorochrome and DNA was recorded. The suspension was then centrifuged and the RNase solution removed. The cells were resuspended and stained with 1ml of propidium

iodide staining solution. Samples were processed on a FACS 440 flow cytometer using an argon laser operating at an excitation wavelength of 488nm. Propidium iodide emission fluorescence was recorded for $2x10^4$ cell per sample, and the data was accumulated and processed on a Consort 40 computer.

4. RESULTS.

4.1 The effect of imidazotetrazinone treatment on Raji and GM892A cell growth.

Two cell lines, Raji and GM892A, with different sensitivities to imidazotetrazinones were utilized for studies reported in this thesis. The differences in sensitivities is thought to result from differences in the constitutive levels of the DNA repair protein, O^6-alkylguanine alkyltransferase in the two cell lines.

It can be seen from figures 6 and 7, and table 1 that the GM892A cells, which have a very low level of the DNA repair enzyme O^6-alkylguanine-DNA alkyltransferase were 8- and 25-fold more sensitive to mitozolomide and temozolomide, respectively, than Raji cells which are proficient in the repair enzyme (Harris et al, 1983, Tisdale, 1987,). In contrast, CCRG 82019 showed relatively little differential toxicity between the two cell lines, and towards GM892A cells was about 15-fold less potent than temozolomide, and 30-fold less potent than mitozolomide.

The growth curves of Raji cells treated with the three imidazotetrazinones show a shoulder-type curve. In contrast only CCRG 82019 exhibits a shoulder-type curve in GM892A cells, with mitozolomide and temozolomide lacking such a feature in their growth curves.

Figure 6.

The effects of imidazotetrazinone treatment on Raji cell population growth.

Cells were plated and treated with drug and were counted 72hr after drug addition. The results are expressed as the growth of the treated cell population relative to the growth of the untreated control cell population.

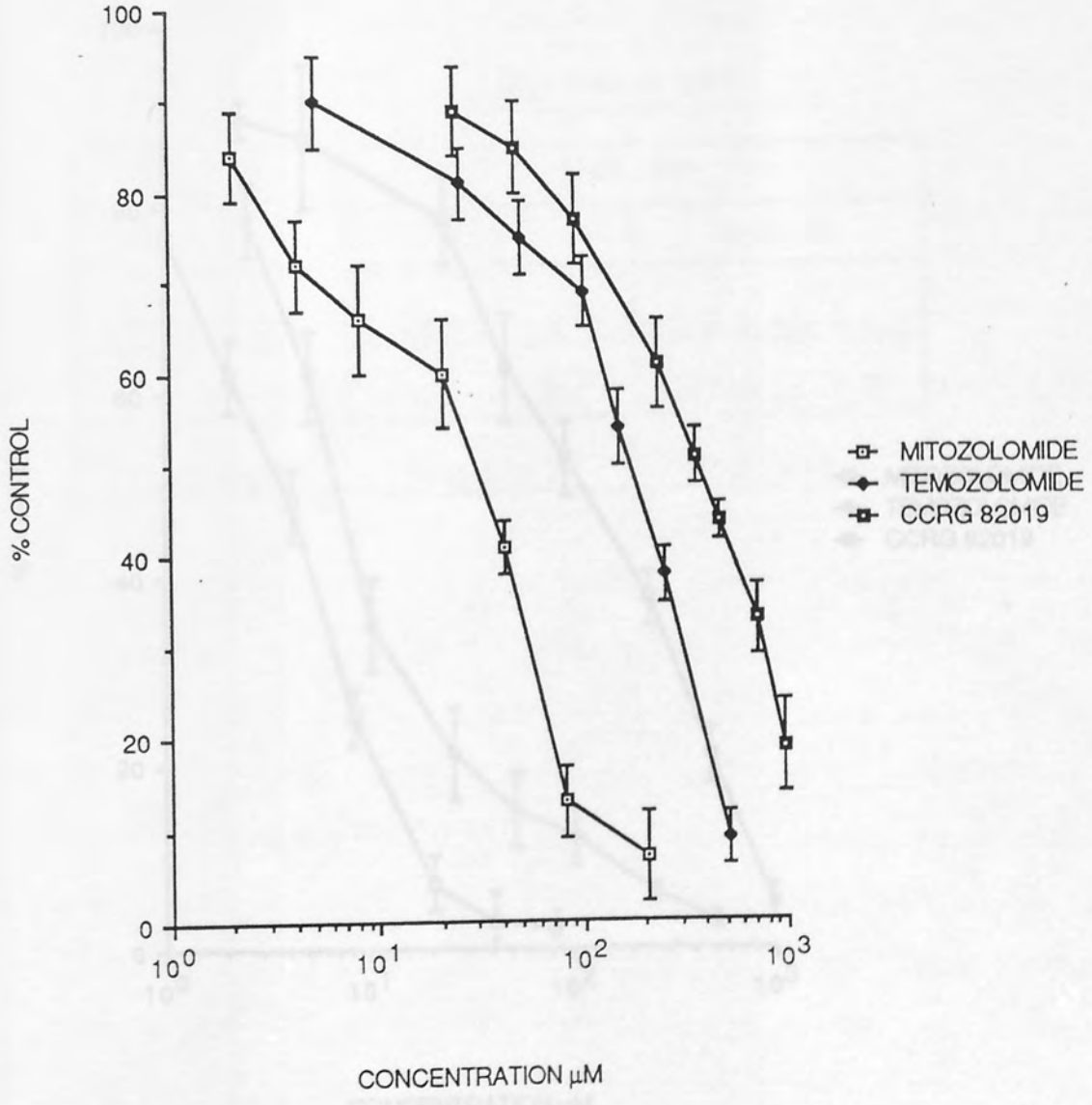


Figure 7.

The effects of imidazotetrazinone treatment on GM892A cell population growth.

Cells were plated and treated with drug and were counted 72hr after drug addition. The results are expressed as the growth of the treated cell population relative to the growth of the untreated control cell population.

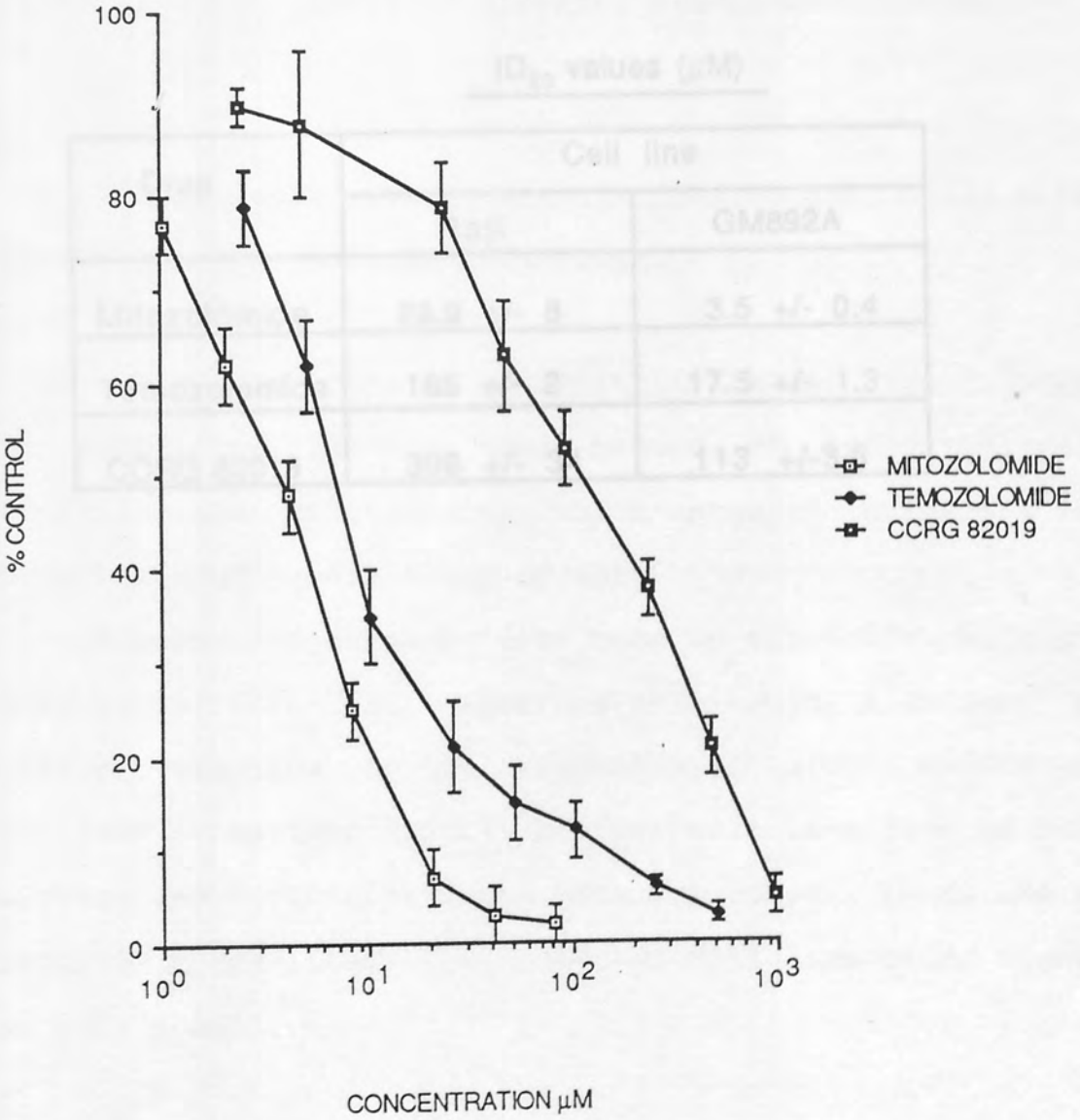


Table 1.

The effect of imidazotetrazinone treatment on Raji and GM892A cell growth.

The values stated in the table represent the uM concentration of each drug required to reduce cell population growth by 50%. Cells were plated and treated with drug, as described, and 50% growth inhibition calculated from the linear part of the growth curve.

ID_{50} values (μM)

Drug	Cell line	
	Raji	GM892A
Mitozolomide	28.9 +/- 8	3.5 +/- 0.4
Temozolomide	185 +/- 2	17.5 +/- 1.3
CCRG 82019	399 +/- 34	113 +/-3.6

4.2 Experiments to assess cell viability after treatment with imidazotetrazinones.

Observations made on cell growth after drug treatment does not necessarily reveal whether a drug has had a cytotoxic effect or whether it has merely caused some delay in cell growth. For this reason other methods of assessing cell viability were sought in an attempt to reveal the true cause of apparent growth inhibition.

4.2.1 Clonogenic assays of Raji and GM892A cells after treatment with imidazotetrazinones.

The ability of a cell to replicate and form colonies in soft agar is one such method of assessing cell viability and cytotoxicity, which normally by-passes the constraint of growth delay effects.

A number of attempts were made to clone both Raji and GM892A cells in soft agar. Even though a variety of changes were made to the original agar growth medium (as outlined in section 3.2.1), neither cell line grew to form clones, and further attempts were abandoned. There are no reports in the literature of either cell line being cloned in soft agar.

4.2.2 Assessment of cell viability by trypan blue exclusion.

The ability of cells to exclude trypan blue is a commonplace method of assessing cell viability, based on the premise that the cell membrane of only viable cells is competent to exclude the dye. However, in preliminary experiments it was noted that following drug treatment of cells, apparent cell viability remained high (usually greater than 85%), and was neither time nor dose related, and thus this technique was not considered further for assessment of cytotoxicity.

4.2.3 Assessment of cell viability by rhodamine 123 uptake into mitochondria

The uptake of the fluorescent dye rhodamine 123 into mitochondria has been reported to be strongly associated with cell viability: the mitochondrial membrane potential is apparently rapidly lost in dead cells, thus inhibiting the specific uptake of the dye (Bernal et al, 1982). The uptake of dye into cells is visualised by illumination of a cell suspension with fluorescent light at 485nm.

Trial experiments utilising this technique were performed, but were later abandoned due to an inability to distinguish between viable and non-viable cells with the equipment available.

4.3 Uptake of radiolabelled imidazotetrazinones into Raji and GM892A cells.

Differences in the rate of drug uptake may be the cause of observed differential toxicities of a variety of drugs in a given cell line, or of one drug in a variety of cell lines. Thus the uptake of mitozolomide, temozolomide and CCRG 82019 into Raji and GM892A cells was measured.

Uptake was established by the incubation of $5x10^6$ cells/ml with 0.1mM of each radiolabelled drug (dissolved in DMSO, without dilution of the initial specific activity), at 4^oC. It had been previously been shown that uptake of mitozolomide into TLX5 lymphoma cells was rapid at 37^oC, with equilibrium reached within 1min (Horgan and Tisdale, 1985). A lower temperature was therefore chosen to more accurately assess the initial rate of drug uptake. As can be seen from figures 8 and 9, and table 2, under the conditions used, equilibrium was reached some 4 to 8min after drug addition, and the peak value obtained was similar for all agents in both cell lines, except for temozolomide uptake into GM892A cells, the peak value of which was inexplicably slightly lower than for the other two agents. The cell/medium drug distribution ratio remained constant for 30min, at approximately 1.3 in Raji cells, and 1.8 in GM892A cells. Thus the difference in cytotoxicity for the three agents against the two cell lines would not appear to be due to differential accumulation.

Figure 8.

The uptake of imidazotetrazinones into Raji cells.

Cells were suspended in fresh growth medium, and equilibriated at 4°C for 10min. Uptake was initiated by the addition of 0.1mM 3-^{14}C labelled drug, and at the specified intervals, aliquots of the suspension were removed, and the drug uptake determined.

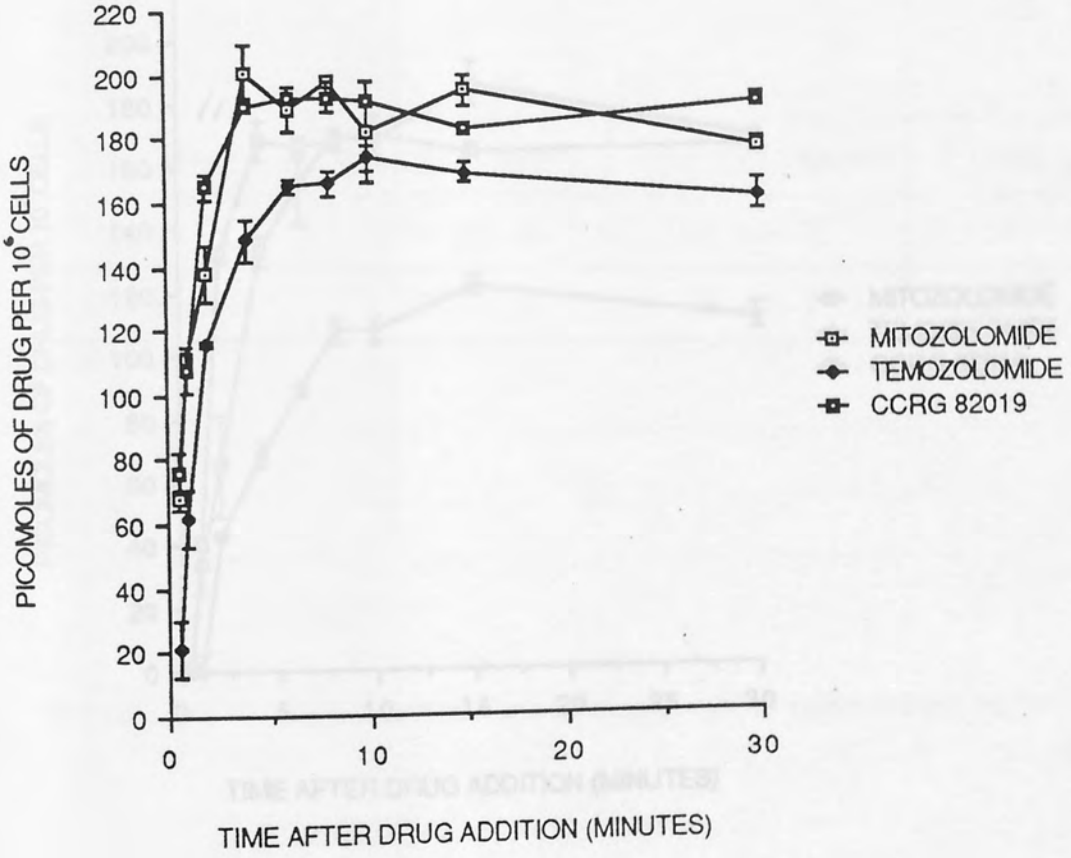


Figure 9.

The uptake of imidazotetrazinones into GM892A cells.

Cells were suspended in fresh growth medium, and equilibriated at 4°C for 10min. Uptake was initiated by the addition of 0.1mM 3-^{14}C labelled drug, and at the specified intervals, aliquots of the suspension were removed, and the drug uptake determined.

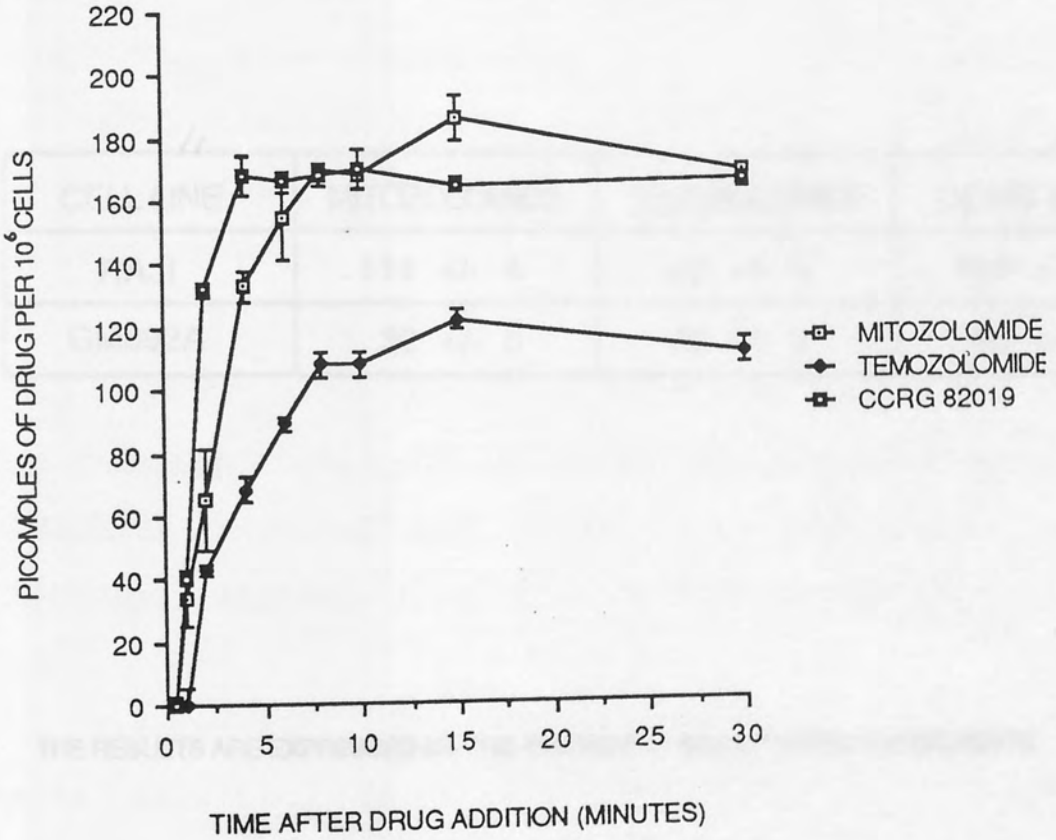


TABLE 2.

INITIAL RATES OF DRUG ACCUMULATION INTO RAJI AND GM892A CELLS

INITIAL VELOCITY pmol/10^6 cells/min

CELL LINE	MITOZOLOMIDE	TEMOZOLOMIDE	CCRG 82019
RAJI	111 +/- 4	62 +/- 9	108 +/- 7
GM892A	32 +/- 8	22 +/- 2	66 +/- 2

THE RESULTS ARE EXPRESSED AS THE AVERAGE +/- SEM OF THREE EXPERIMENTS.

4.4 Incorporation of radiolabelled precursors into the DNA, RNA and protein of Raji and GM892A cells after imidazotetrazinone treatment.

The effect of imidazotetrazinone treatment over a 24hr period on the incorporation of 5-[methyl-^{3}H]-thymidine , 5-[^{3}H]-uridine and 4,5-[^{3}H]-leucine into DNA, RNA and protein, respectively, was investigated in an attempt to elucidate possible differential effects by each drug. The time stated in figures 10 to 18 (inclusive) refers to the time after drug addition. The cells were pulse-labelled with radioactive precursor for 1hr immediately prior to the time point stated.

4.4.1 Incorporation of labelled thymidine into the acid-insoluble material of Raji and GM892A cells after imidazotetrazinone treatment. (Figures 10-12).

Mitozolomide at a concentration of 40μM caused a marked reduction in 5-[methyl-^{3}H]-thymidine incorporation into the acid-insoluble material of both Raji and GM892A cells 2hr after drug treatment. There was some recovery towards control levels of incorporation in both cell lines after 24hr, the level of recovery being greater in Raji cells.

Temozolomide at a concentration of 100μM similarly caused a marked reduction in ^{3}H-thymidine incorporation in

both cell lines 2hr after drug administration. There was a recovery in the level of incorporation towards control levels in Raji cells, but such a recovery was not observed in the GM892A cells.

Incorporation of ^{3}H-thymidine into the acid-insoluble material of both cell lines was maximally reduced 2hr after treatment with CCRG 82019, with a 500µM concentration causing a 70-80% inhibition of incorporation in both cell lines. The decrease in incorporation in Raji cells was less marked than that caused by either mitozolomide or temozolomide, with incorporation recovering to almost control values after 24hr. In contrast to the results observed with Raji cells, CCRG 82019 caused a marked reduction in the incorporation of ^{3}H-thymidine in GM892A cells, to a similar degree as to that effected by mitozolomide and temozolomide in this cell line (but requiring a higher drug concentration), with little recovery towards control levels after 24hr.

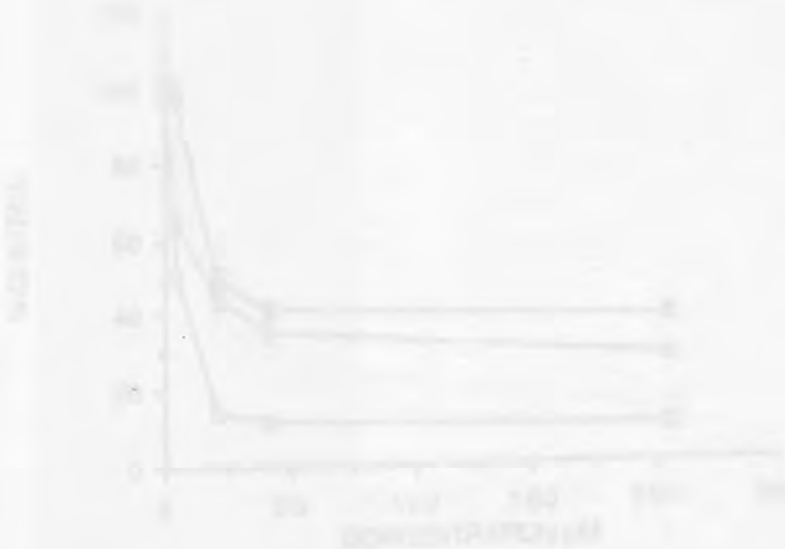
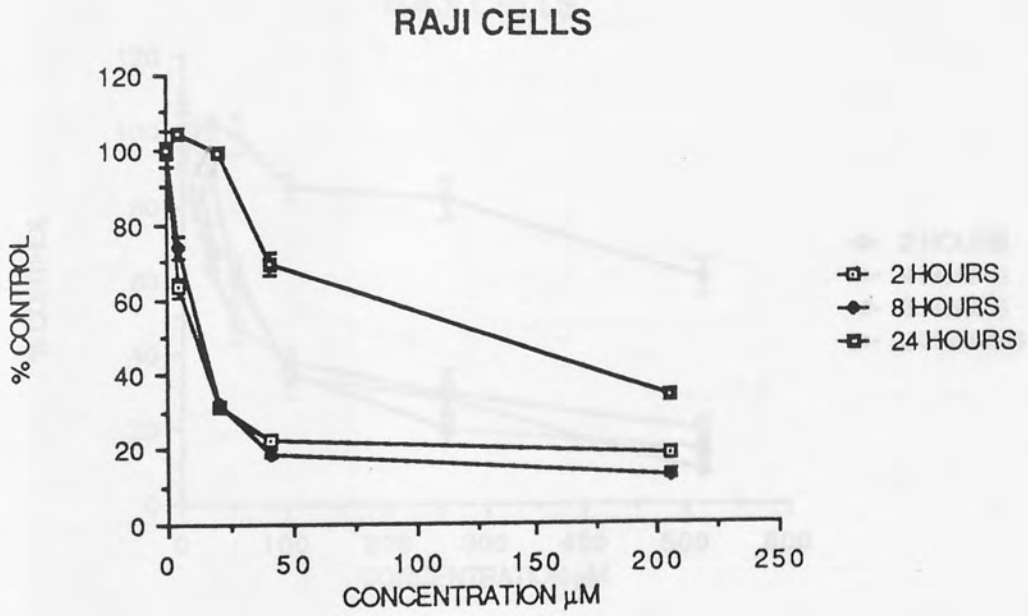


Figure 10.

The incorporation of labelled thymidine into the acid-insoluble material of Raji and GM892A cells treated with mitozolomide.

Cells at a density of $3x10^5$/ml were treated with mitozolomide at the concentrations indicated in the figure. 1hr prior to the stated time-point, 1ml of the suspension was incubated with 2.5µCi of ^{3}H-thymidine at 37°C for 60min, and the incorporation into acid-insoluble material determined.



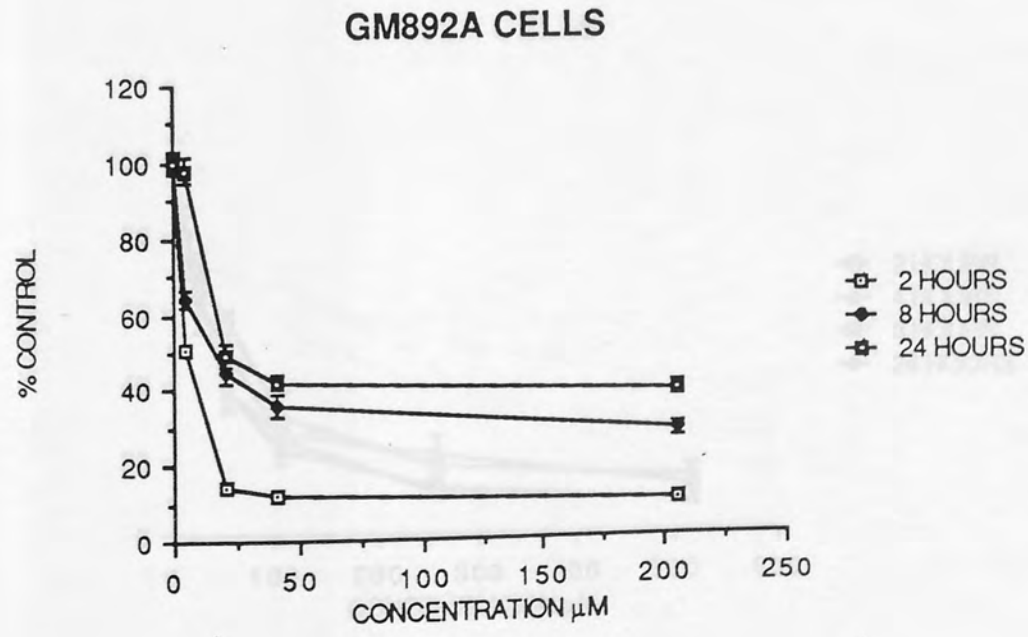
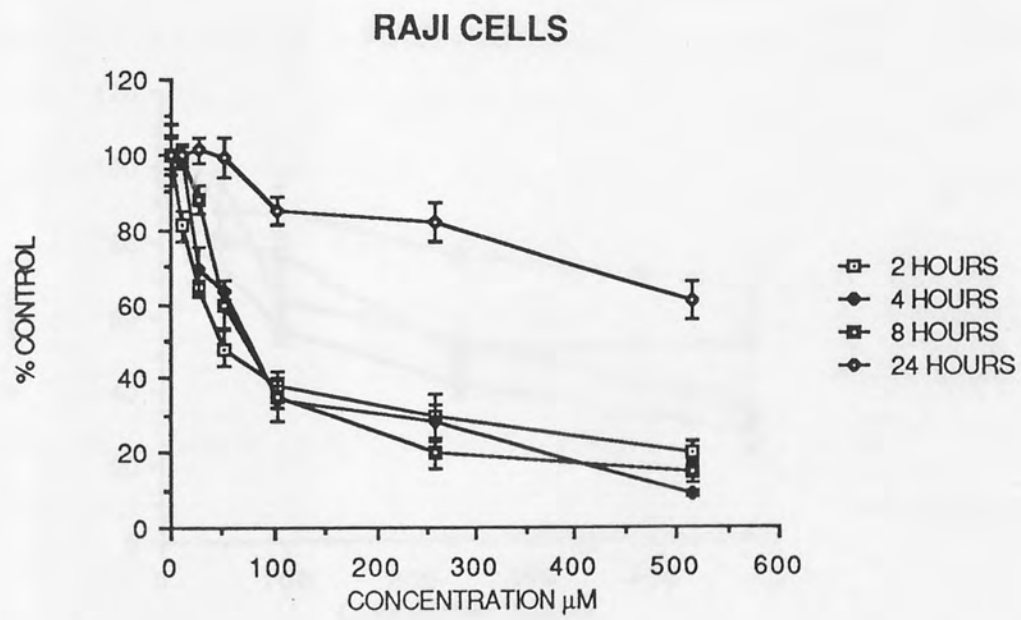


Figure 11.

<u>The incorporation of labelled thymidine into the acid-insoluble material of Raji and GM892A cells treated with temozolomide.</u>

Cells at a density of $3x10^5$/ml were treated with temozolomide at the concentrations indicated in the figure. 1hr prior to the stated time-point, 1ml of the suspension was incubated with 2.5µCi of ^{3}H-thymidine at 37°C for 60min, and the incorporation into acid-insoluble material determined.



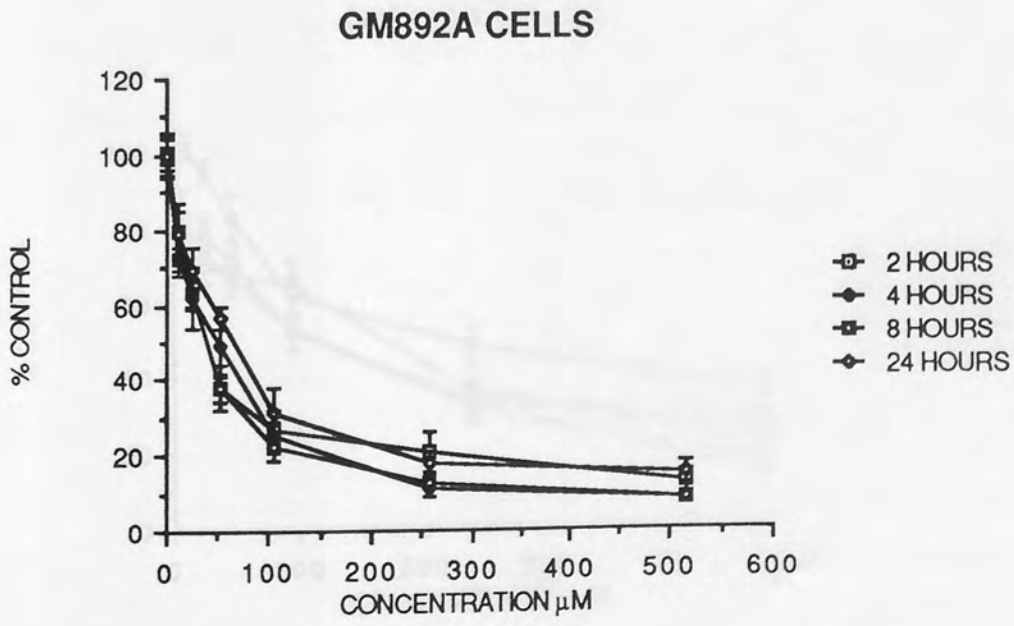
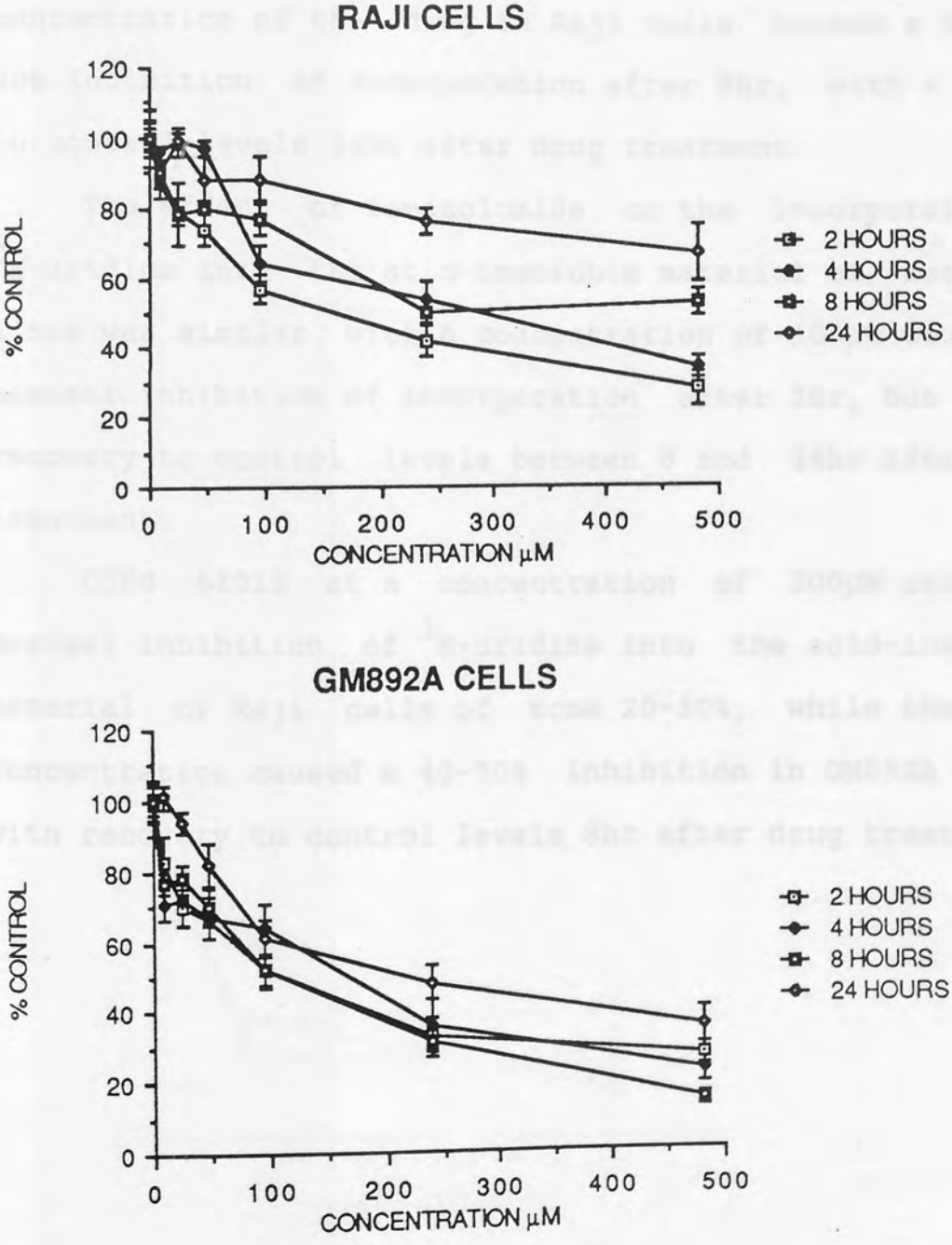


Figure 12.

The incorporation of labelled thymidine into the acid-insoluble material of Raji and GM892A cells treated with CCRG 82019.

Cells at a density of $3x10^5$/ml were treated with CCRG 82019 at the concentrations indicated in the figure. 1hr prior to the stated time-point, 1ml of the suspension was incubated with 2.5µCi of ^{3}H-thymidine at 37°C for 60min, and the incorporation into acid-insoluble material determined.



4.4.2 Incorporation of labelled uridine into the acid-insoluble material of Raji and GM892A cells after imidazotetrazinone treatment. (Figures 13-15).

Mitozolomide at a concentration of 40μM caused about a 70% reduction of ^{3}H-uridine incorporation in GM892A cells after 2hr, with virtually no recovery towards control levels after 24hr. In contrast, the same concentration of the drug in Raji cells caused a maximal 30% inhibition of incorporation after 8hr, with a return to control levels 24hr after drug treatment.

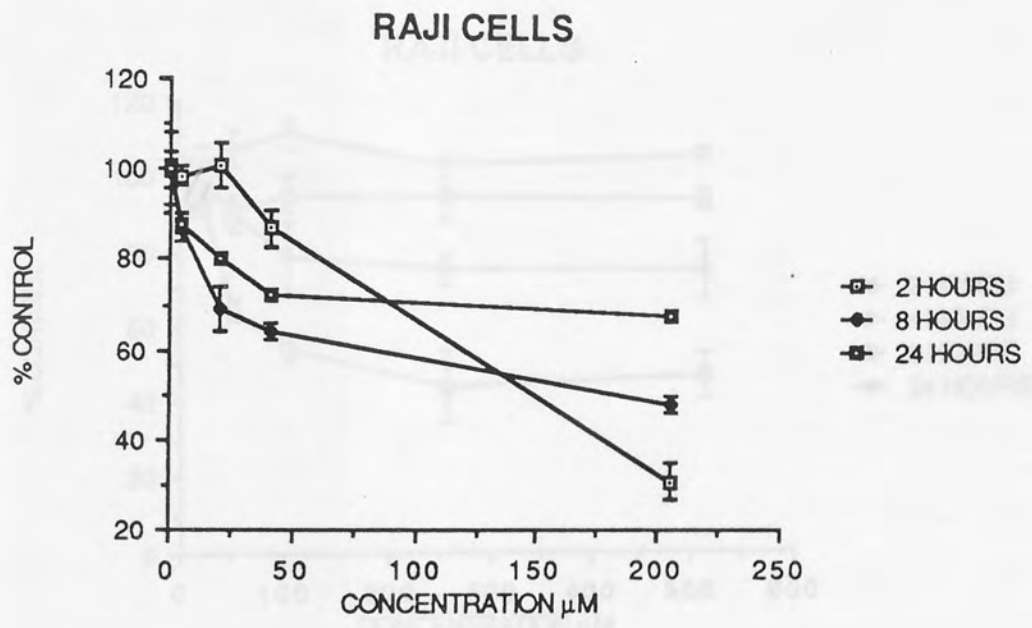
The effect of temozolomide on the incorporation of ^{3}H-uridine into the acid-insoluble material of both cell lines was similar, with a concentration of 100μM causing a maximal inhibition of incorporation after 2hr, but with a recovery to control levels between 8 and 24hr after drug treatment.

CCRG 82019 at a concentration of 200μM caused a maximal inhibition of ^{3}H-uridine into the acid-insoluble material of Raji cells of some 20-30%, while the same concentration caused a 40-50% inhibition in GM892A cells, with recovery to control levels 8hr after drug treatment.

Figure 13.

The incorporation of labelled uridine into the acid-insoluble material of Raji and GM892A cells treated with mitozolomide.

Cells at a density of $3x10^5$/ml were treated with mitozolomide at the concentrations indicated in the figure. 1hr prior to the stated time-point, 1ml of the suspension was incubated with 2.5µCi of ^{3}H-uridine at 37°C for 60min, and the incorporation into acid-insoluble material determined.



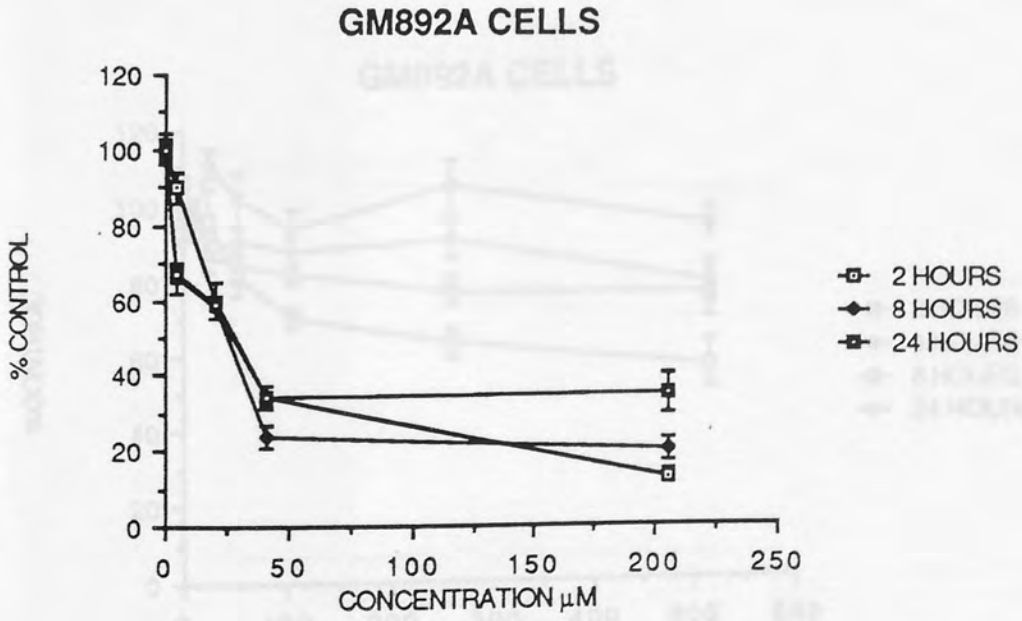


Figure 14.

The incorporation of labelled uridine into the acid-insoluble material of Raji and GM892A cells treated with temozolomide.

Cells at a density of $3x10^5$/ml were treated with temozolomide at the concentrations indicated in the figure. 1hr prior to the stated time-point, 1ml of the suspension was incubated with 2.5µCi of ^{3}H-uridine at 37°C for 60min, and the incorporation into acid-insoluble material determined.

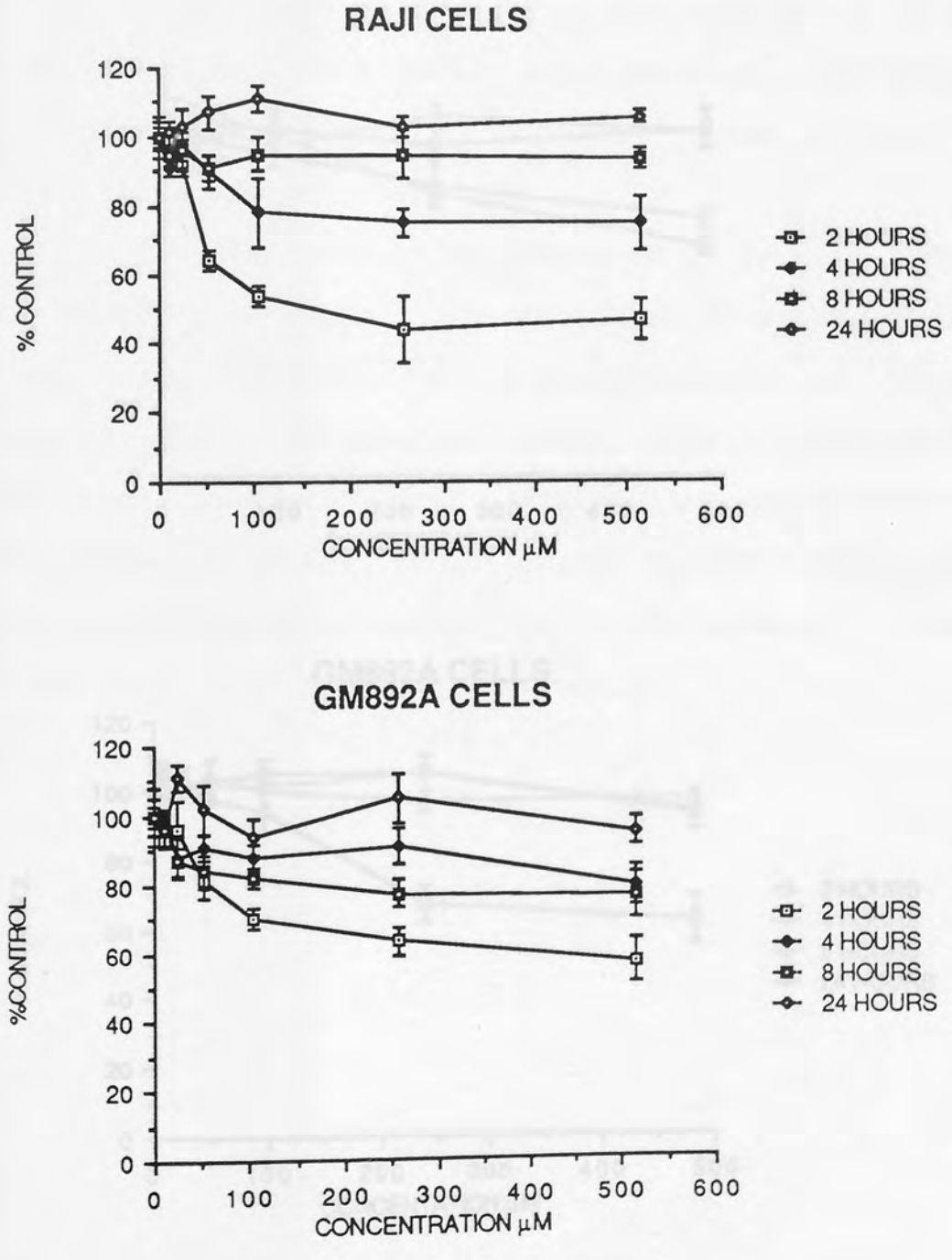
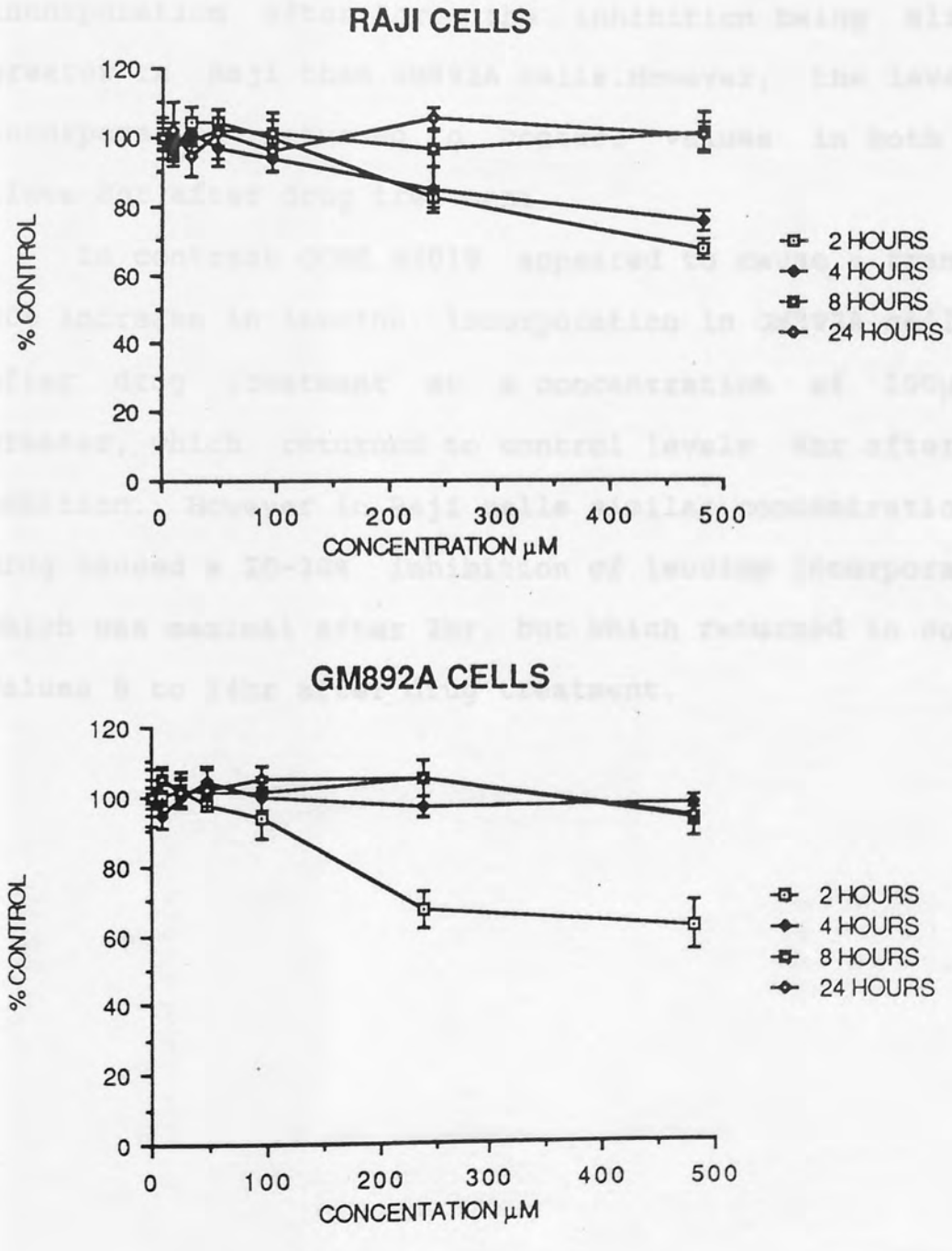


Figure 15.

The incorporation of labelled uridine into the acid-insoluble material of Raji and GM892A cells treated with CCRG 82019.

Cells at a density of $3x10^5$/ml were treated with CCRG 82019 at the concentrations indicated in the figure. 1hr prior to the stated time-point, 1ml of the suspension was incubated with 2.5µCi of ^{3}H-uridine at 37°C for 60min, and the incorporation into acid-insoluble material determined.



4.4.3 Incorporation of labelled leucine into the acid-insoluble material of Raji and GM892A cells after imidazotetrazinone treatment. (Figures 16-18).

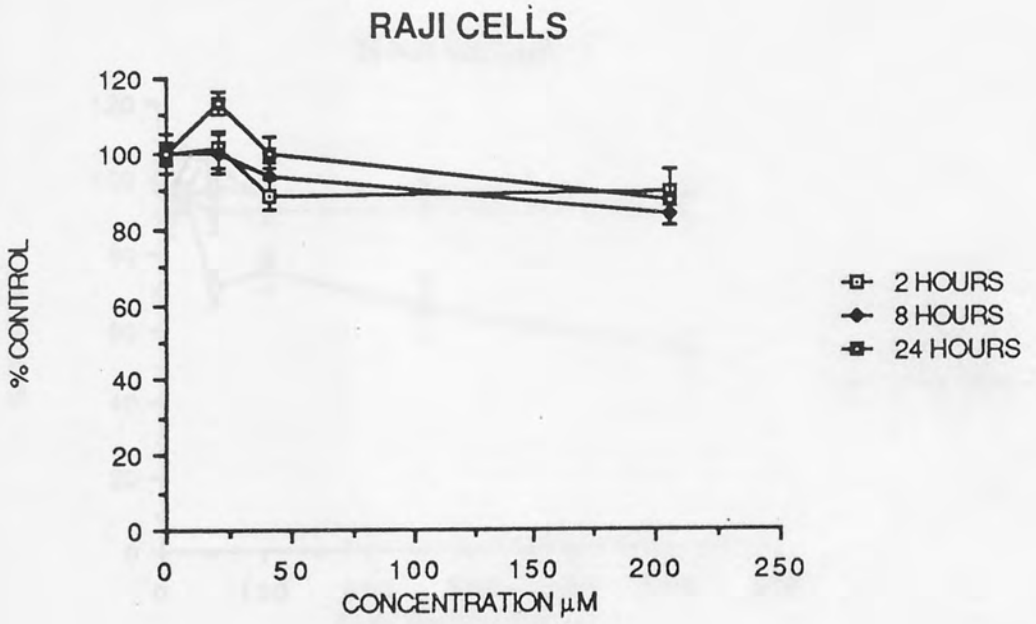
Whereas mitozolomide appeared not to affect leucine incorporation into the acid-insoluble material of either Raji or GM892A cells, temozolomide at a concentration of 100μM and greater caused a maximal 20-40% inhibition of incorporation after 2hr, the inhibition being slightly greater in Raji than GM892A cells.However, the levels of incorporation returned to control values in both cell lines 8hr after drug treatment.

In contrast CCRG 82019 appeared to cause a transient 40% increase in leucine incorporation in GM892A cells 2hr after drug treatment at a concentration of 100μM or greater, which returned to control levels 4hr after drug addition. However in Raji cells similar concentrations of drug caused a 20-30% inhibition of leucine incorporation, which was maximal after 2hr, but which returned to control values 8 to 24hr after drug treatment.

Figure 16.

<u>The incorporation of labelled leucine into the acid-insoluble material of Raji and GM892A cells treated with mitozolomide.</u>

Cells at a density of $3x10^5$/ml were treated with mitozolomide at the concentrations indicated in the figure. 1hr prior to the stated time-point, 1ml of the suspension was incubated with 2.5µCi of ^{3}H-leucine at 37^{o}C for 60min, and the incorporation into acid-insoluble material determined.



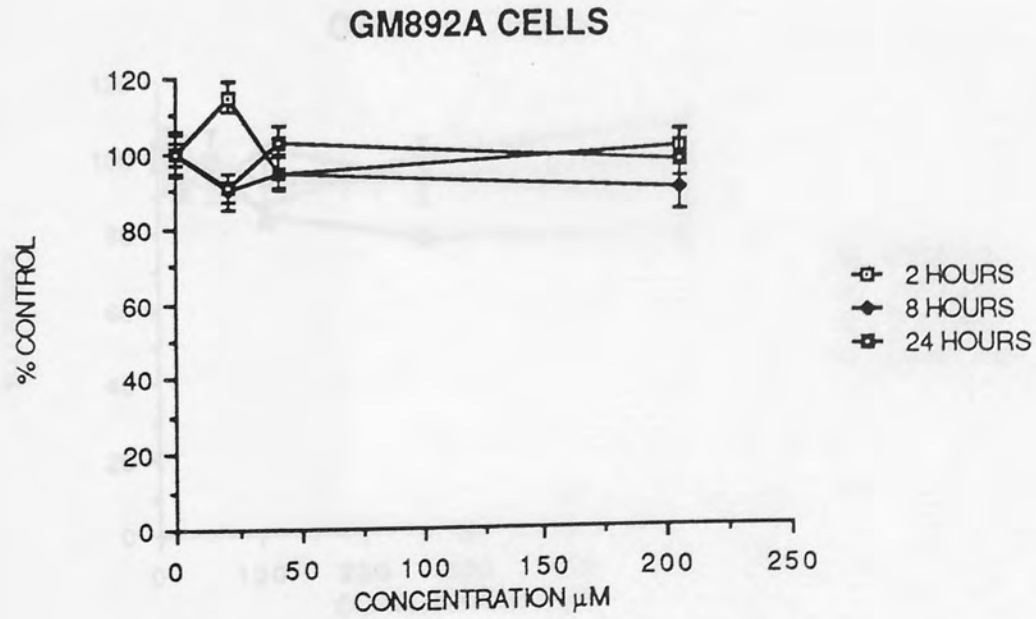


Figure 17.

The incorporation of labelled leucine into the acid-insoluble material of Raji and GM892A cells treated with temozolomide.

Cells at a density of $3x10^5$/ml were treated with temozolomide at the concentrations indicated in the figure. 1hr prior to the stated time-point, 1ml of the suspension was incubated with 2.5µCi of ^{3}H-leucine at 37°C for 60min, and the incorporation into acid-insoluble material determined.

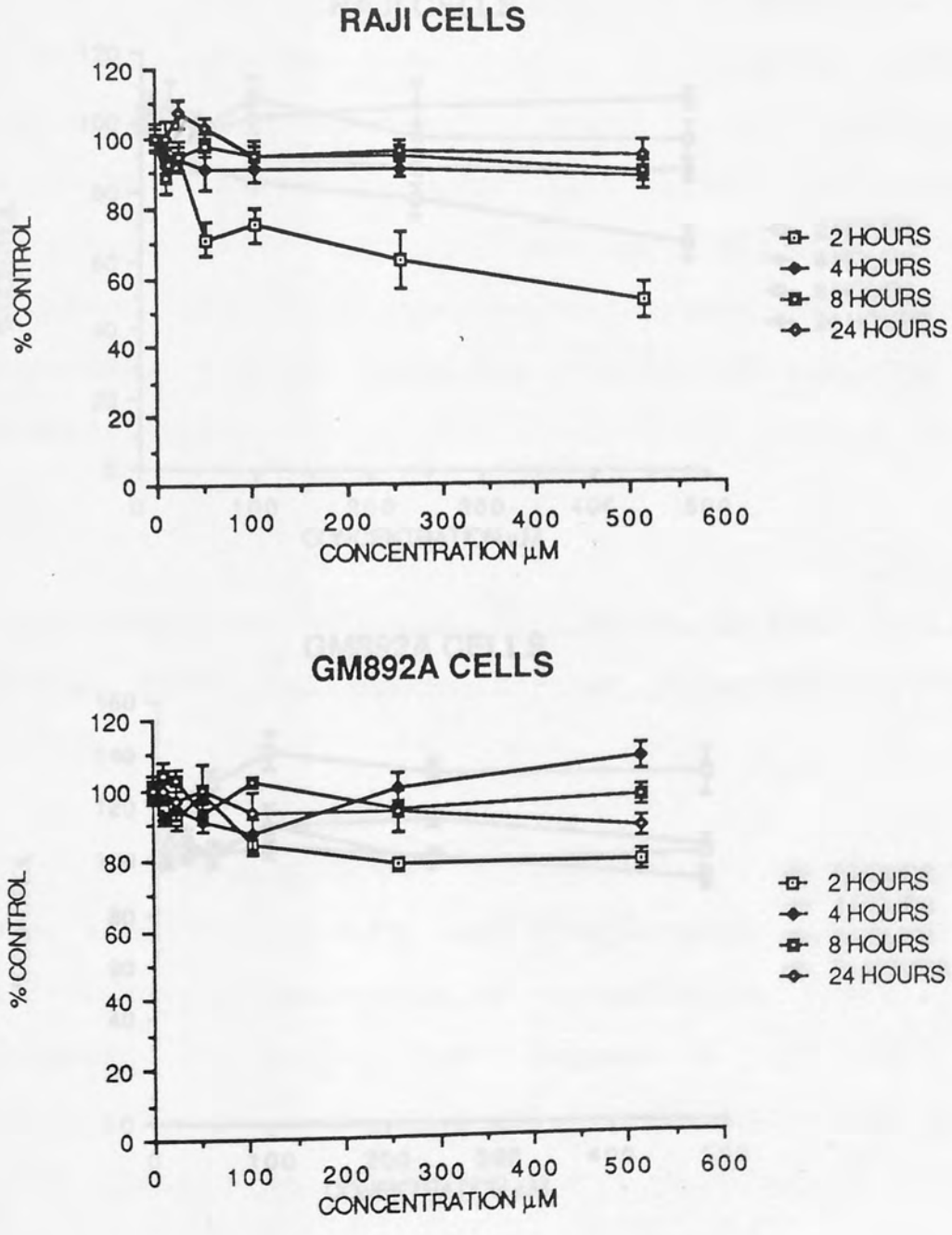
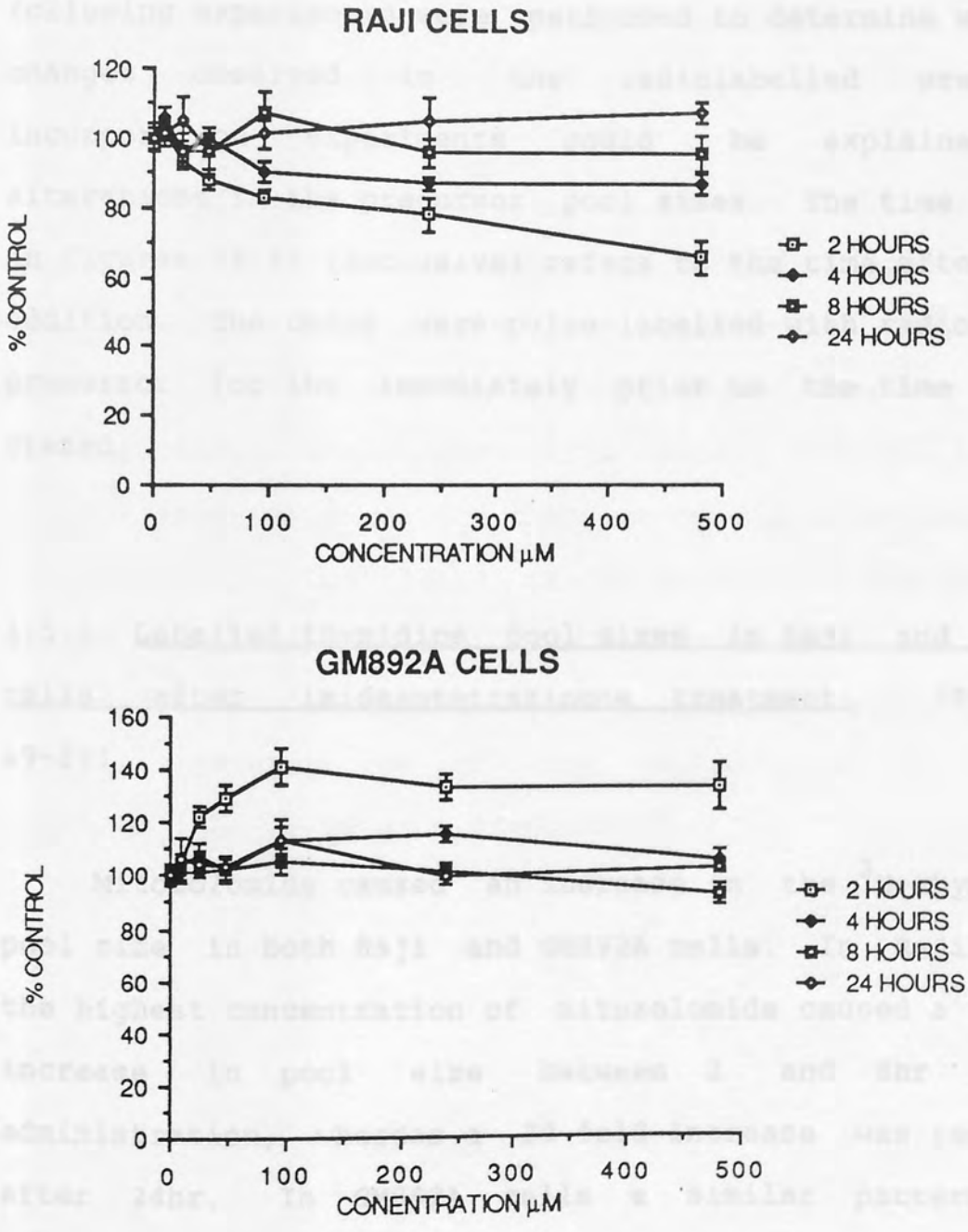


Figure 18.

The incorporation of labelled leucine into the acid-insoluble material of Raji and GM892A cells treated with CCRG 82019.

Cells at a density of $3x10^5$/ml were treated with CCRG 82019 at the concentrations indicated in the figure. 1hr prior to the stated time-point, 1ml of the suspension was incubated with 2.5µCi of ^{3}H-leucine at 37°C for 60min, and the incorporation into acid-insoluble material determined.



4.5 Precursor pool sizes in Raji and GM892A cells after imidazotetrazinone treatment.

Apparent changes in the incorporation of precursors into acid-insoluble material in cells after drug treatment may be effected by changes in the intracellular concentrations of the precursor, which in turn may be altered by drug damage to the the cell membrane. The following experiments were performed to determine whether changes observed in the radiolabelled precursor incorporation experiments could be explained by alterations in the precursor pool sizes. The time stated in figures 19-27 (inclusive) refers to the time after drug addition. The cells were pulse-labelled with radioactive precursor for 1hr immediately prior to the time point stated.

4.5.1 Labelled thymidine pool sizes in Raji and GM892A cells after imidazotetrazinone treatment. (Figures 19-21).

Mitozolomide caused an increase in the ^{3}H-thymidine pool size in both Raji and GM892A cells. In Raji cells the highest concentration of mitozolomide caused a 2-fold increase in pool size between 2 and 8hr after administration, whereas a 20-fold increase was recorded after 24hr. In GM892A cells a similar pattern was

evident, as the highest concentration of drug caused a 2-fold increase in pool size after 2hr, an 8-fold increase after 8hr and a 30-fold increase after 24hr.

The effect of temozolomide treatment on Raji cells was similar to that caused by mitozolomide, but less pronounced. In contrast, temozolomide caused an initial decrease in thymidine pool size in GM892A cells, which was most pronounced after 2hr, which recovered to almost control levels after 8hr, and a 12-fold increase in pool size was observed after 24hr of treatment with the highest concentration of drug.

The effects of CCRG 82019 on the two cell lines was similar to that elicited by temozolomide. There was a time dependent increase in pool size in the Raji cells apparent after 4hr, and which showed about a 4- to 5-fold increase after 24hr in the cells treated with the highest concentration of drug. In contrast to the effects seen in the Raji cells, CCRG 82019 caused an initial 80% decrease of the thymidine pool in the GM892A cells after 2hr, which returned to almost control levels after 8hr. However the highest concentration of drug caused a 4- to 5-fold increase 24hr after drug treatment.

Figure 19.

Measurement of the labelled thymidine pool size in Raji and GM892A cells treated with mitozolomide.

Cells at a density of $3x10^5$/ml were treated with mitozolomide at the concentrations indicated in the figure. 1hr prior to the stated time-point, 1ml of the suspension was incubated with 2.5µCi of ^{3}H-thymidine at 37°C for 60min, and the pool size determined.

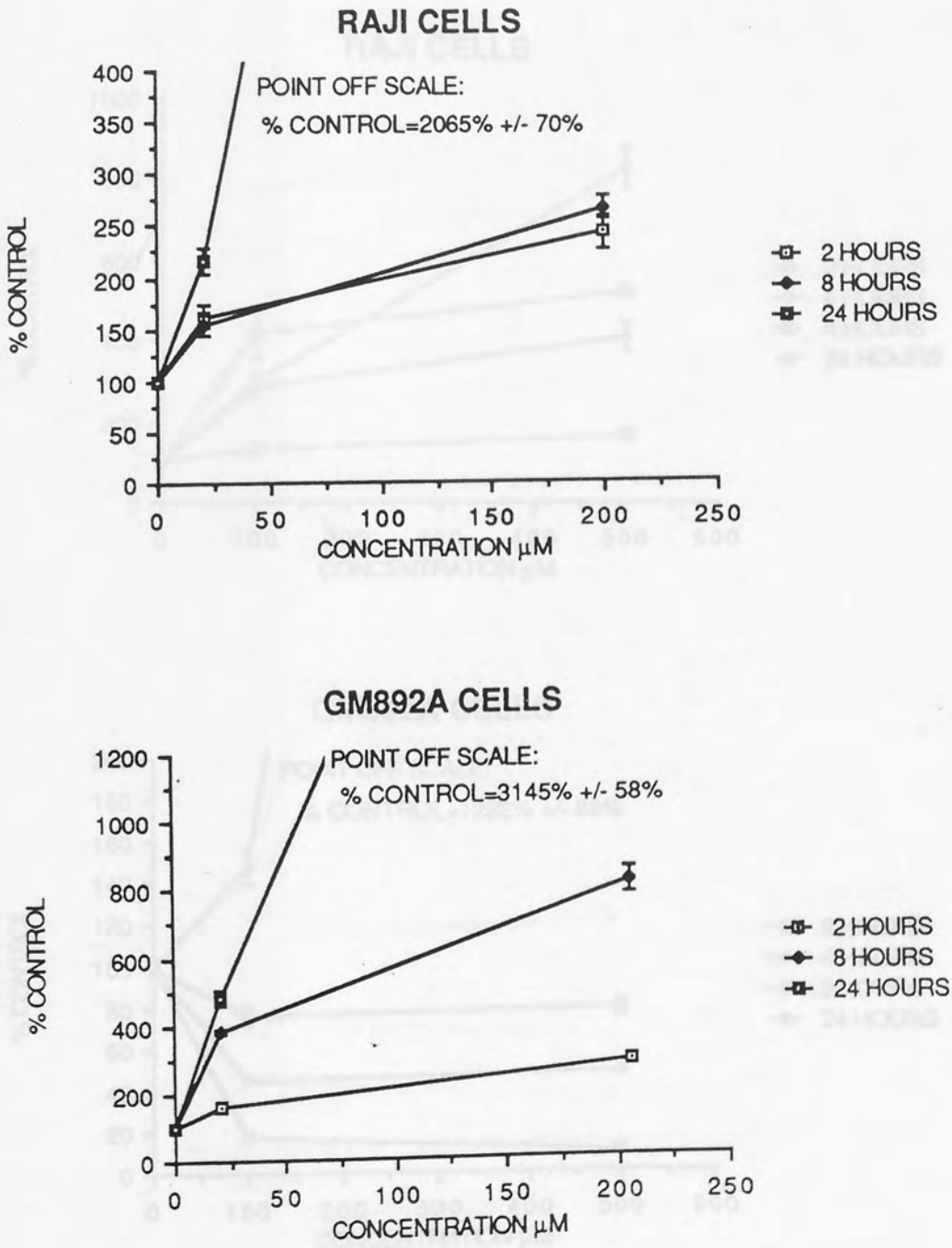


Figure 20.

Measurement of the labelled thymidine pool size in Raji and GM892A cells treated with temozolomide.

Cells at a density of $3x10^5$/ml were treated with temozolomide at the concentrations indicated in the figure. 1hr prior to the stated time-point, 1ml of the suspension was incubated with 2.5μCi of ^{3}H-thymidine at 37°C for 60min, and the pool size determined.

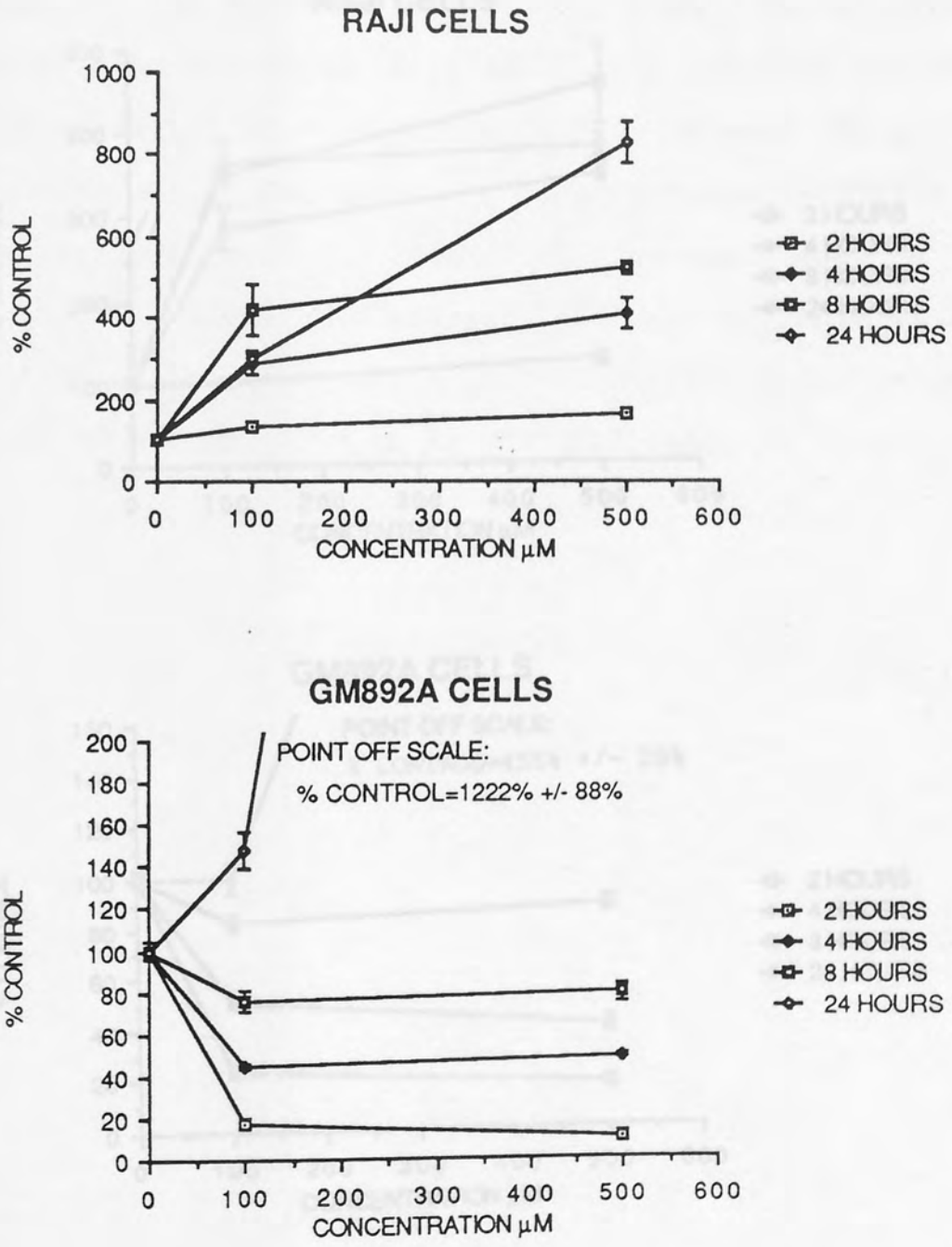
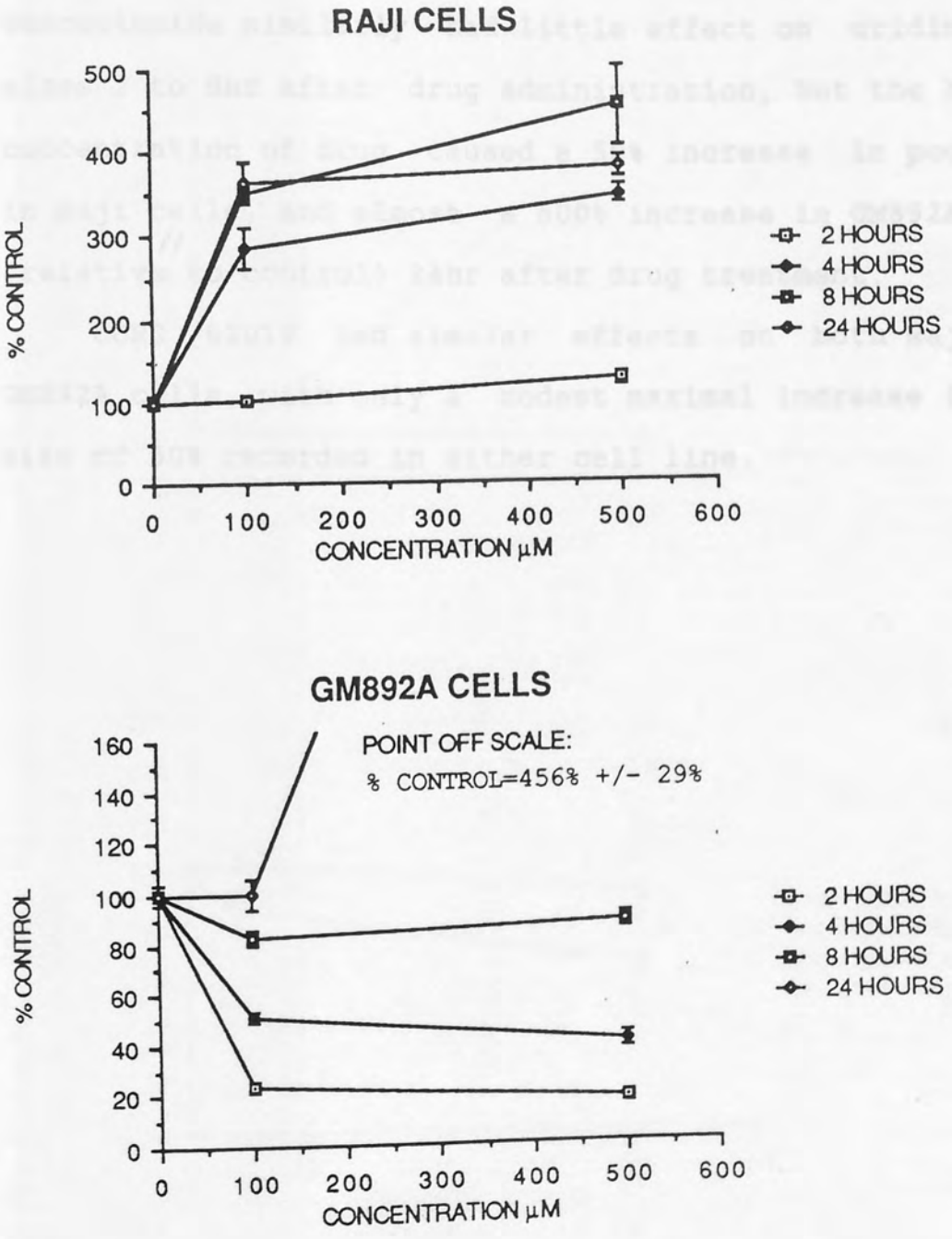


Figure 21.

Measurement of the labelled thymidine pool size in Raji and GM892A cells treated with CCRG 82019.

Cells at a density of $3x10^5$/ml were treated with CCRG 82019 at the concentrations indicated in the figure. 1hr prior to the stated time-point, 1ml of the suspension was incubated with 2.5µCi of ^{3}H-thymidine at 37°C for 60min, and the pool size determined.



4.5.2 Labelled uridine pool sizes in Raji and GM892A cells after imidazotetrazinone treatment. (Figures 22-24).

Mitozolomide had very little effect on ^{3}H-uridine pool sizes in both Raji and GM892A cells 2 to 8hr after drug treatment, but the highest concentration of the drug caused a 4- to 7-fold increase in pool size after 24hr. Temozolomide similarly had little effect on uridine pool sizes 2 to 8hr after drug administration, but the highest concentration of drug caused a 50% increase in pool size in Raji cells, and almost a 600% increase in GM892A cells (relative to control) 24hr after drug treatment.

CCRG 82019 had similar effects on both Raji and GM892A cells, with only a modest maximal increase in pool size of 50% recorded in either cell line.

Figure 22.

Measurement of the labelled uridine pool size in Raji and GM892A cells treated with mitozolomide.

Cells at a density of $3x10^5$/ml were treated with mitozolomide at the concentrations indicated in the figure. 1hr prior to the stated time-point, 1ml of the suspension was incubated with 2.5µCi of ^{3}H-uridine at 37°C for 60min, and the pool size determined.

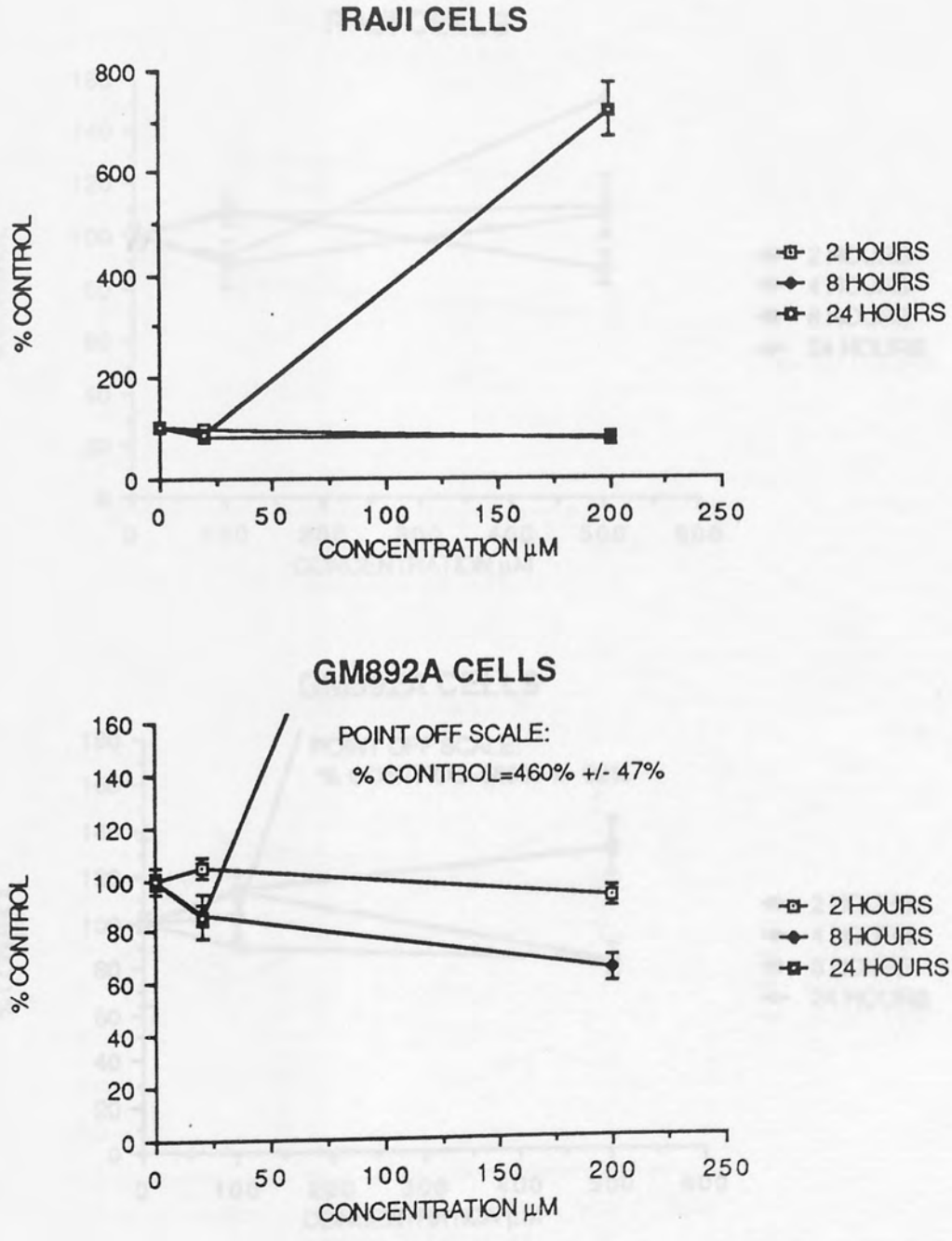
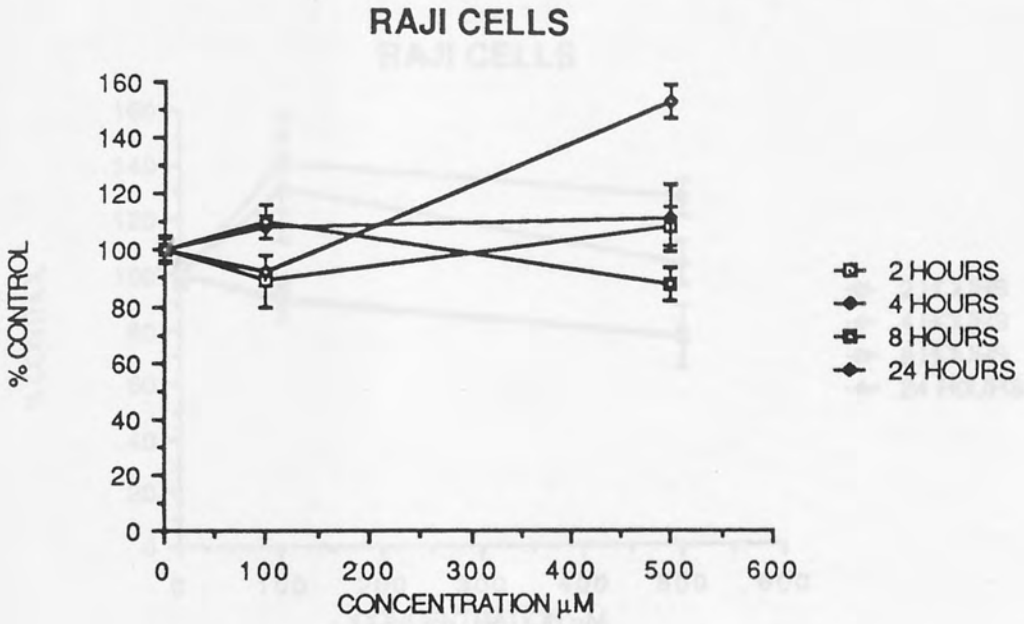


Figure 23.

Measurement of the labelled uridine pool size in Raji and GM892A cells treated with temozolomide.

Cells at a density of $3x10^5$/ml were treated with temozolomide at the concentrations indicated in the figure. 1hr prior to the stated time-point, 1ml of the suspension was incubated with 2.5µCi of ^{3}H-uridine at 37°C for 60min, and the pool size determined.



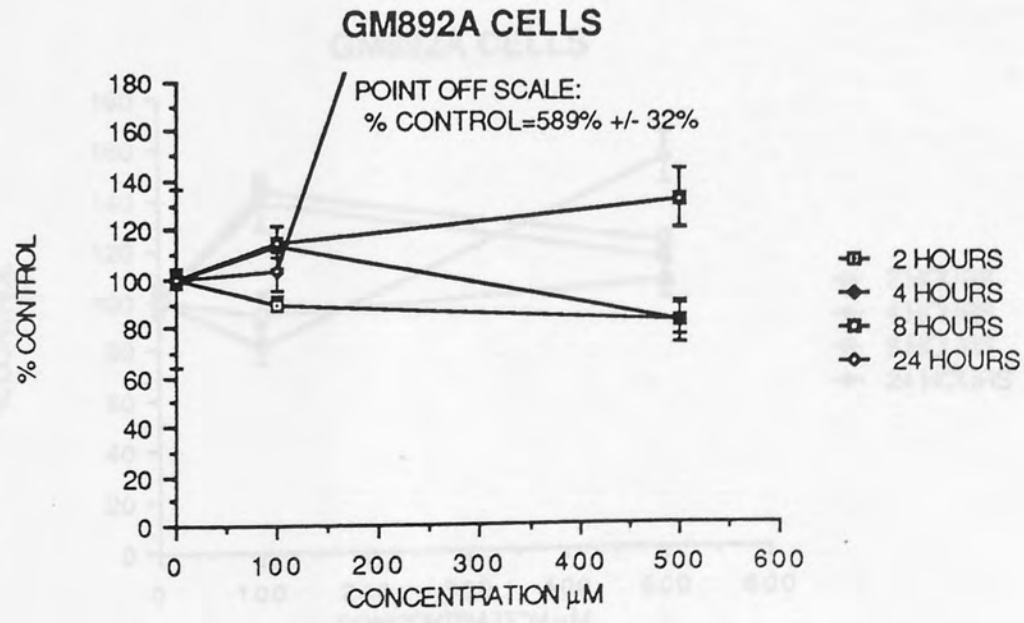
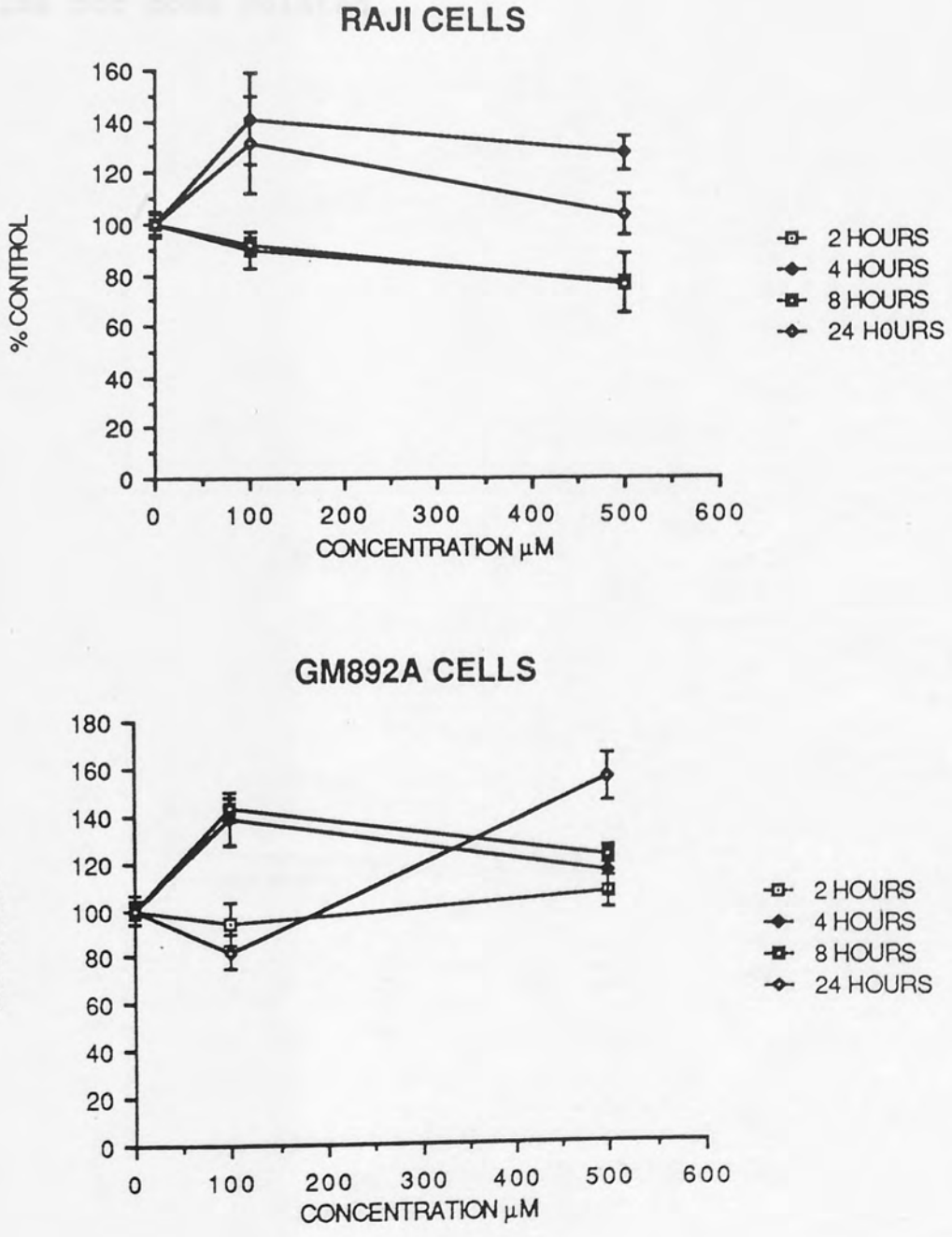


Figure 24.

Measurement of the labelled uridine pool size in Raji and GM892A cells treated with CCRG 82109.

Cells at a density of $3x10^5$/ml were treated with CCRG 82019 at the concentrations indicated in the figure. 1hr prior to the stated time-point, 1ml of the suspension was incubated with 2.5µCi of ^{3}H-uridine at 37°C for 60min, and the pool size determined.



4.5.3 Labelled leucine pool sizes in Raji and GM892A cells after imidazotetrazinone treatment. (Figures 25-27).

No pronounced effects were observed on ^{3}H-leucine pool size in either Raji or GM892A cells after treatment with any of the imidazotetrazinones. The modest fluctuations that were recorded were neither consistently time nor dose related.

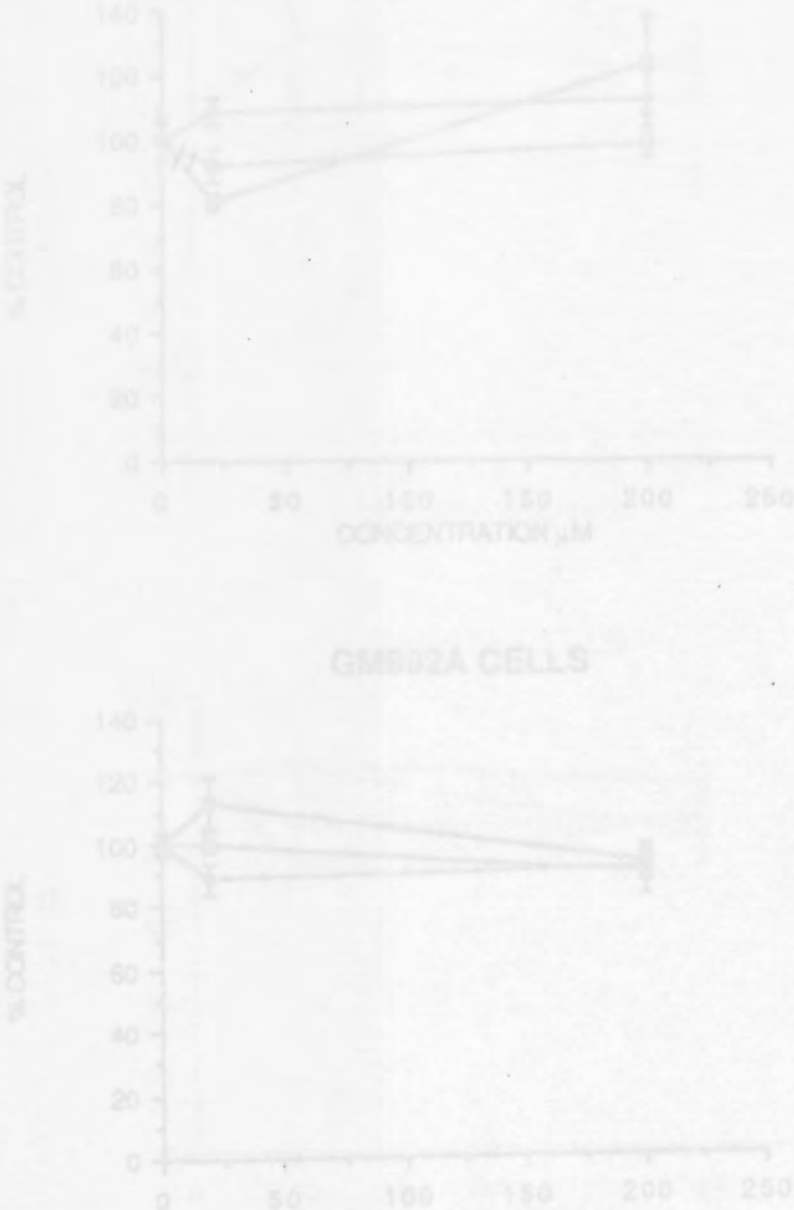


Figure 25.

Measurement of the labelled leucine pool size in Raji and GM892A cells treated with mitozolomide.

Cells at a density of $3x10^5$/ml were treated with mitozolomide at the concentrations indicated in the figure. 1hr prior to the stated time-point, 1ml of the suspension was incubated with 2.5µCi of ^{3}H-leucine at 37°C for 60min, and the pool size determined.

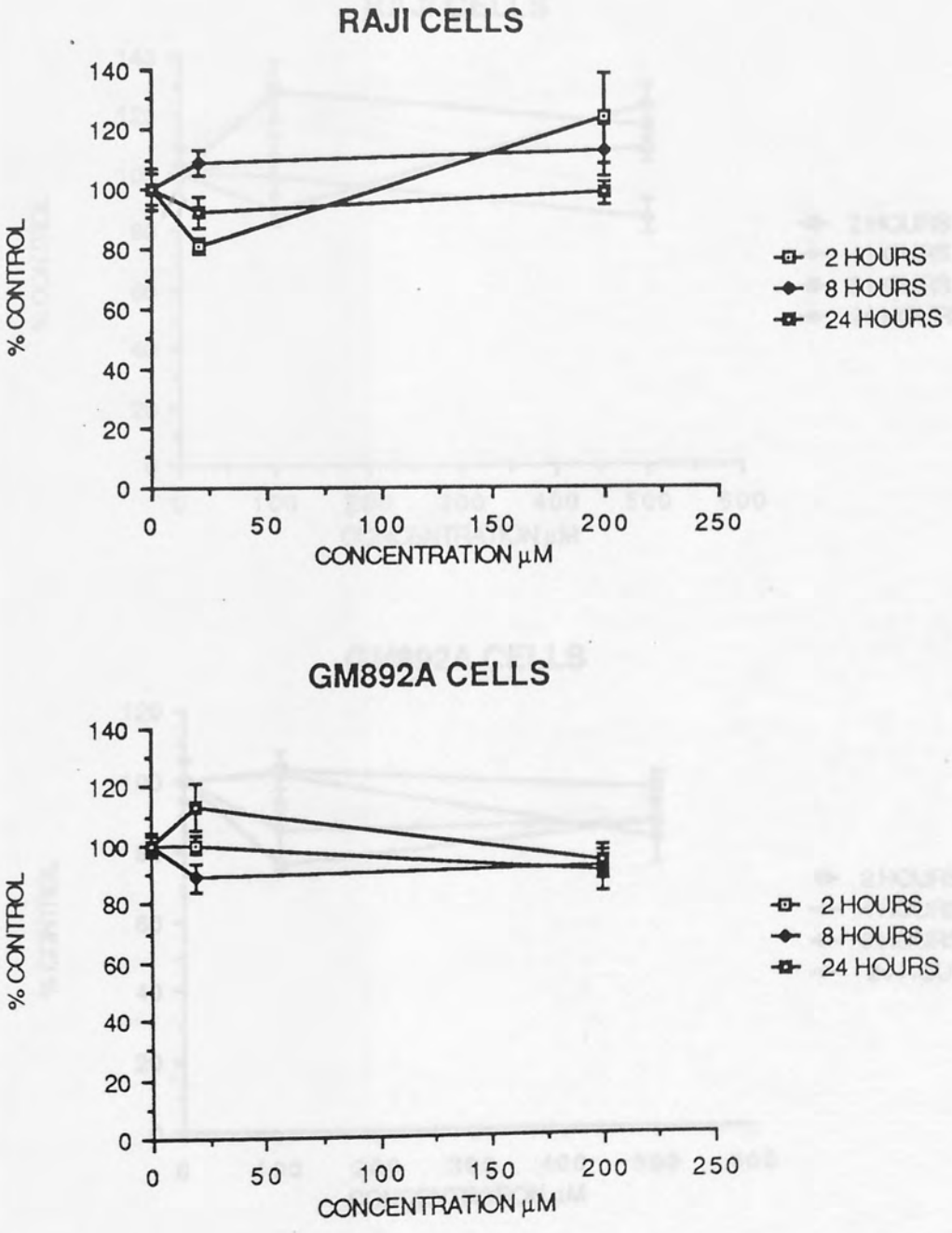


Figure 26.

Measurement of the labelled leucine pool size in Raji and GM892A cells treated with temozolomide.

Cells at a density of $3x10^5$/ml were treated with temozolomide at the concentrations indicated in the figure. 1hr prior to the stated time-point, 1ml of the suspension was incubated with 2.5µCi of ^{3}H-leucine at 37°C for 60min, and the pool size determined.

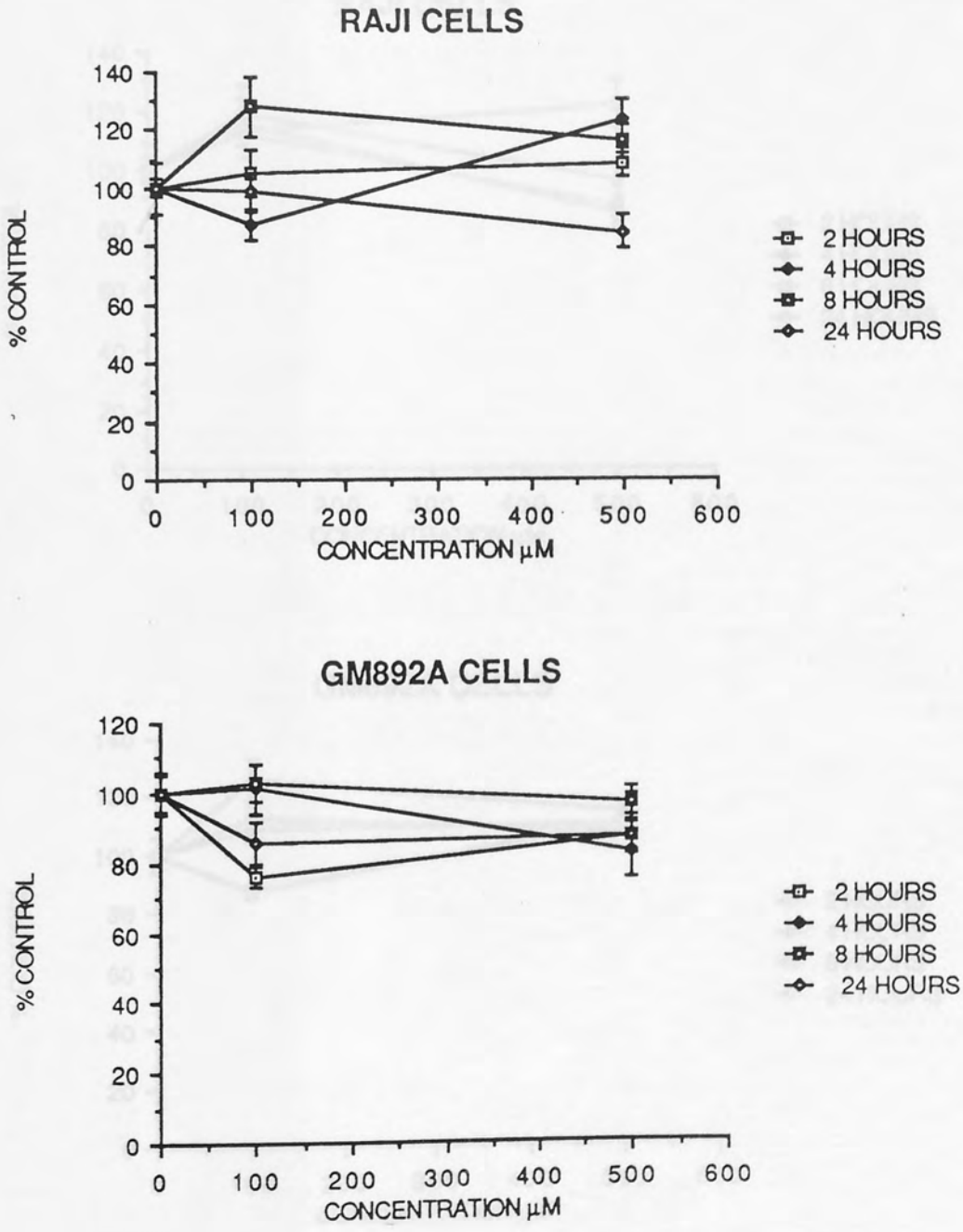
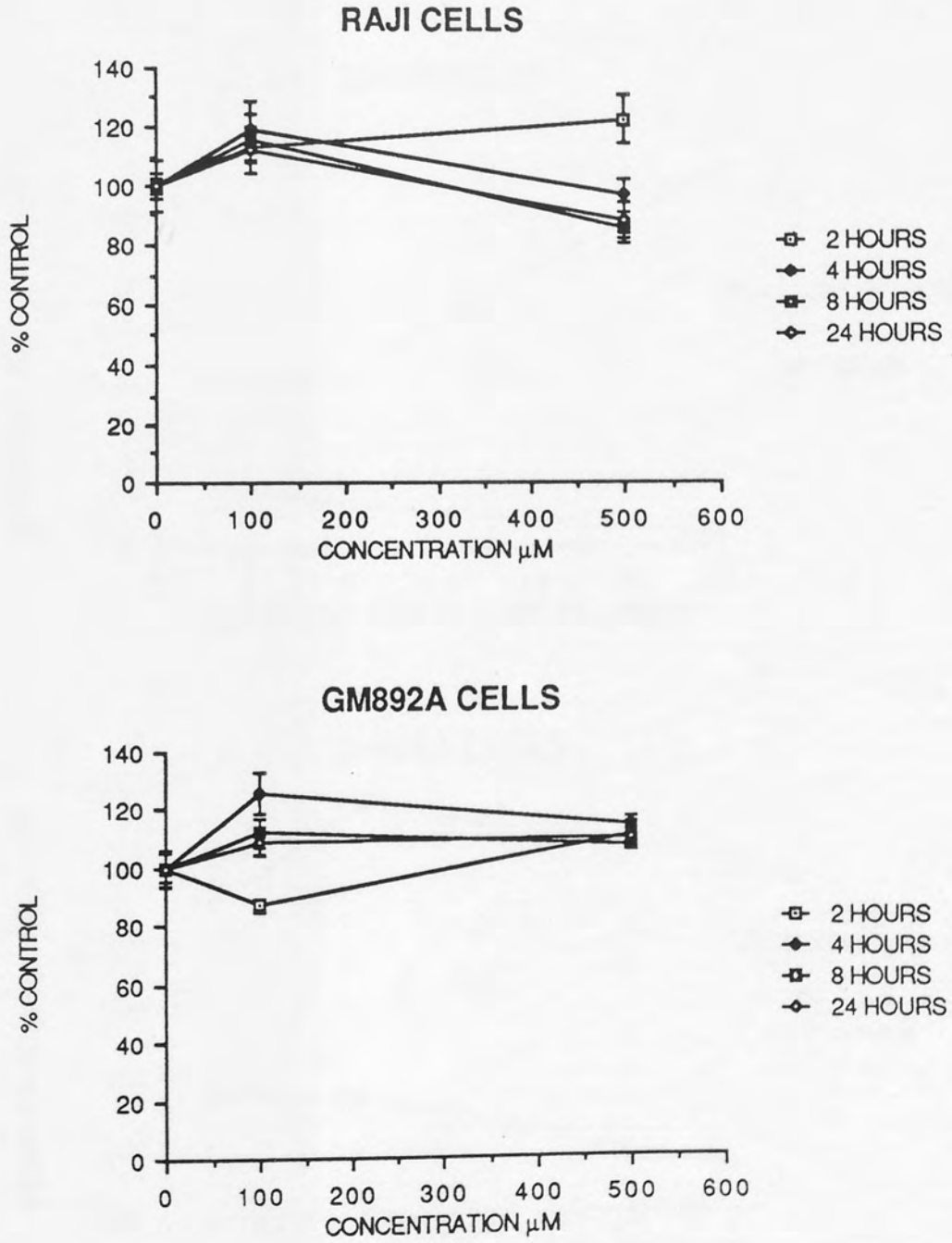


Figure 27.

Measurement of the labelled leucine pool size in Raji and GM892A cells treated with CCRG 82019.

Cells at a density of $3x10^5$/ml were treated with CCRG 82019 at the concentrations indicated in the figure. 1hr prior to the stated time-point, 1ml of the suspension was incubated with 2.5µCi of ^{3}H-leucine at 37°C for 60min, and the pool size determined.



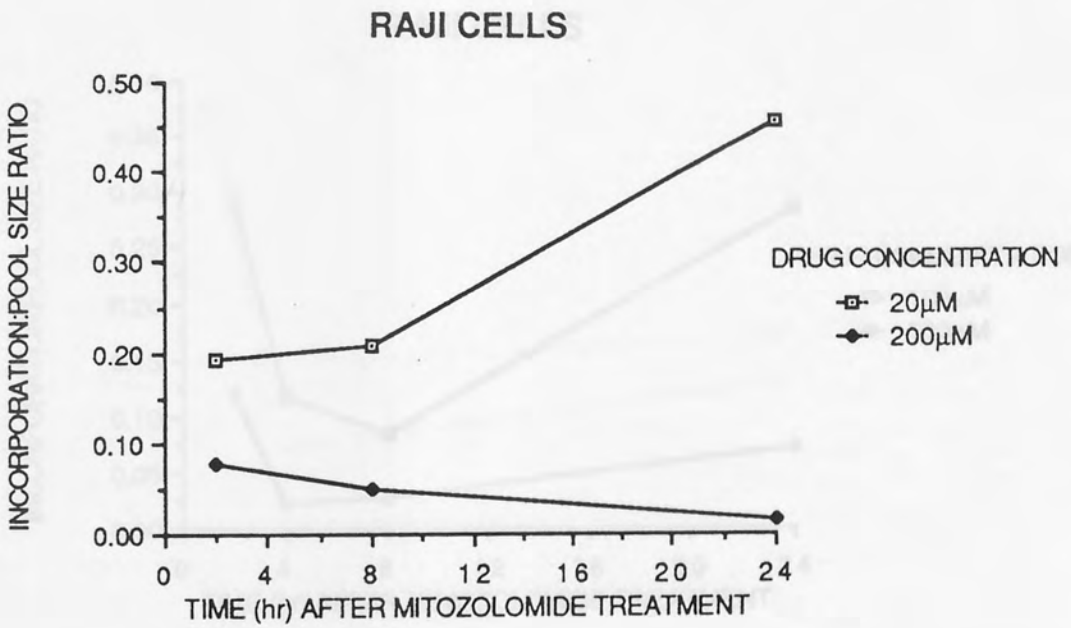
The ratios of labelled precursor incorporation into the acid-insoluble material to labelled precursor pool size in Raji and GM892A cells treated with imidazotetrazinones.

The values expressed in figures 28-36 were calculated as the ratio of labelled precursor incorporation into the acid-insoluble material of imidazotetrazinone treated cells , relative to control cells, (derived from figures 10-18), divided by the labelled precursor pool size in imidazotetrazinone treated cells, relative to control cells, (derived from figures 19-27).

Figure 28.

The ratio of labelled thymidine incorporation into acid-insoluble material to labelled thymidine pool size in Raji and GM892A cells treated with mitozolomide.

The values were calculated as the ratio of the ^{3}H-thymidine incorporated into the acid-insoluble material of treated cells (relative to control cells) versus the ^{3}H-thymidine pool size in treated cells (relative to control cells).



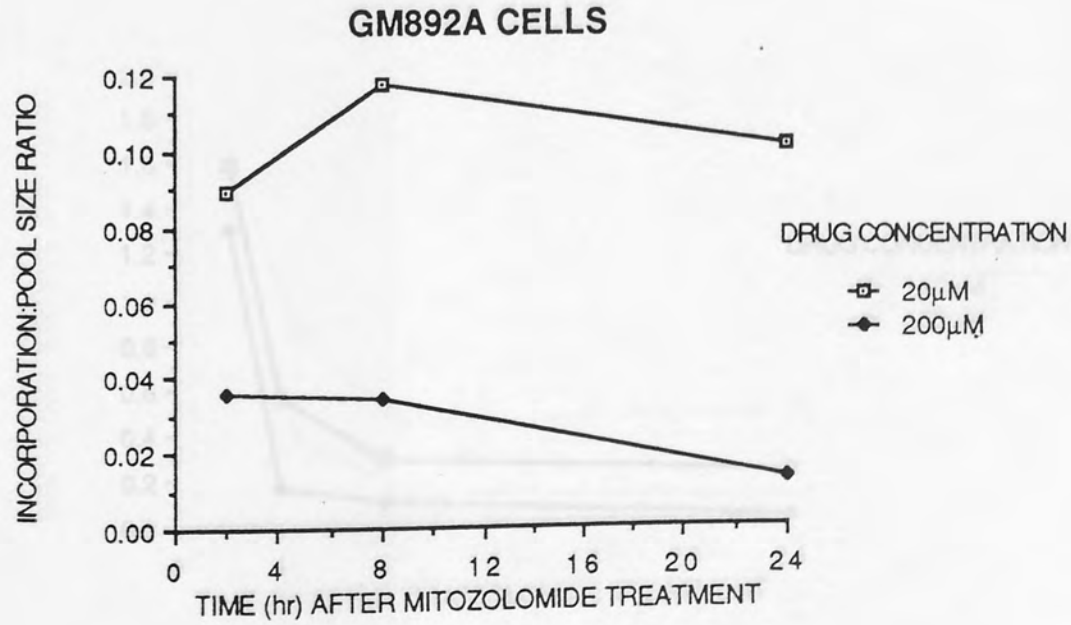
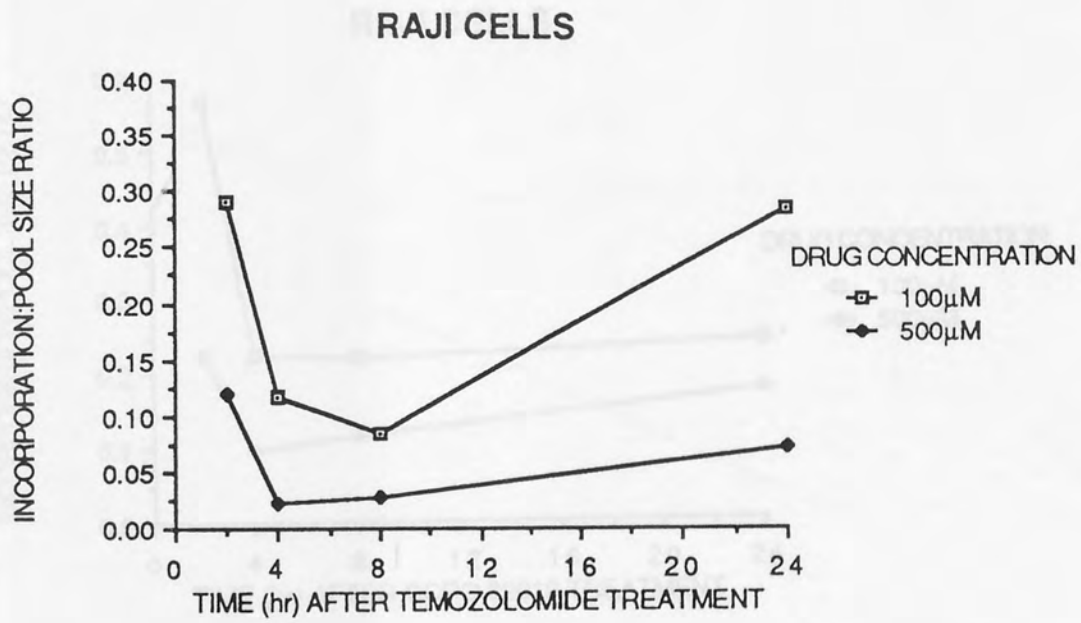


Figure 29.

The ratio of labelled thymidine incorporation into acid-insoluble material to labelled thymidine pool size in Raji and GM892A cells treated with temozolomide.

The values were calculated as the ratio of the ^{3}H-thymidine incorporated into the acid-insoluble material of treated cells (relative to control cells) versus the ^{3}H-thymidine pool size in treated cells (relative to control cells).



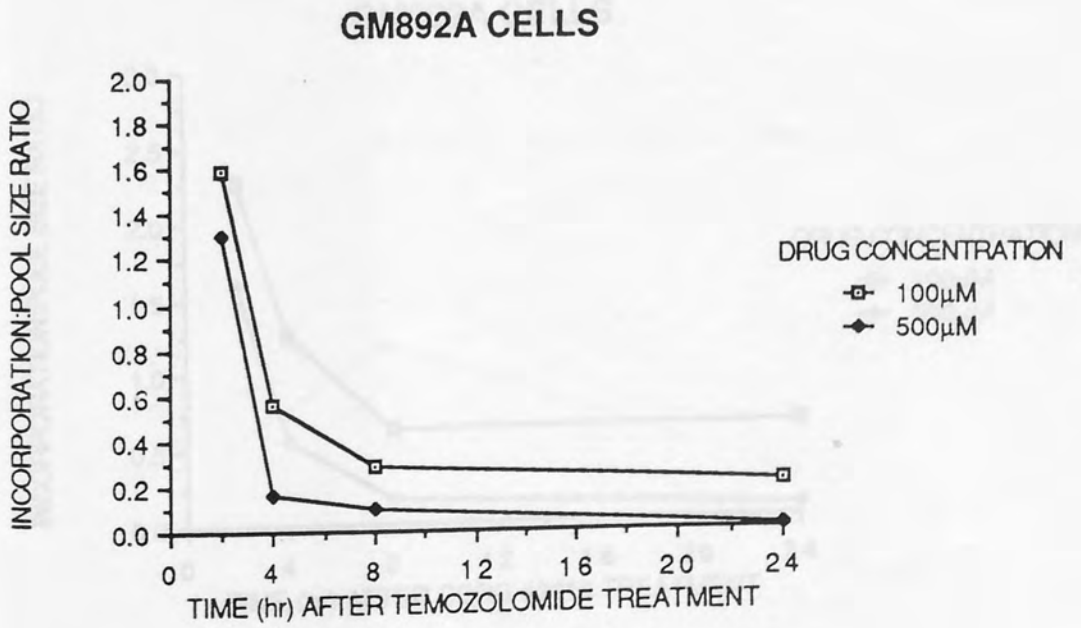


Figure 30.

The ratio of labelled thymidine incorporation into acid-insoluble material to labelled thymidine pool size in Raji and GM892A cells treated with CCRG 82019.

The values were calculated as the ratio of the ^{3}H-thymidine incorporated into the acid-insoluble material of treated cells (relative to control cells) versus the ^{3}H-thymidine pool size in treated cells (relative to control cells).

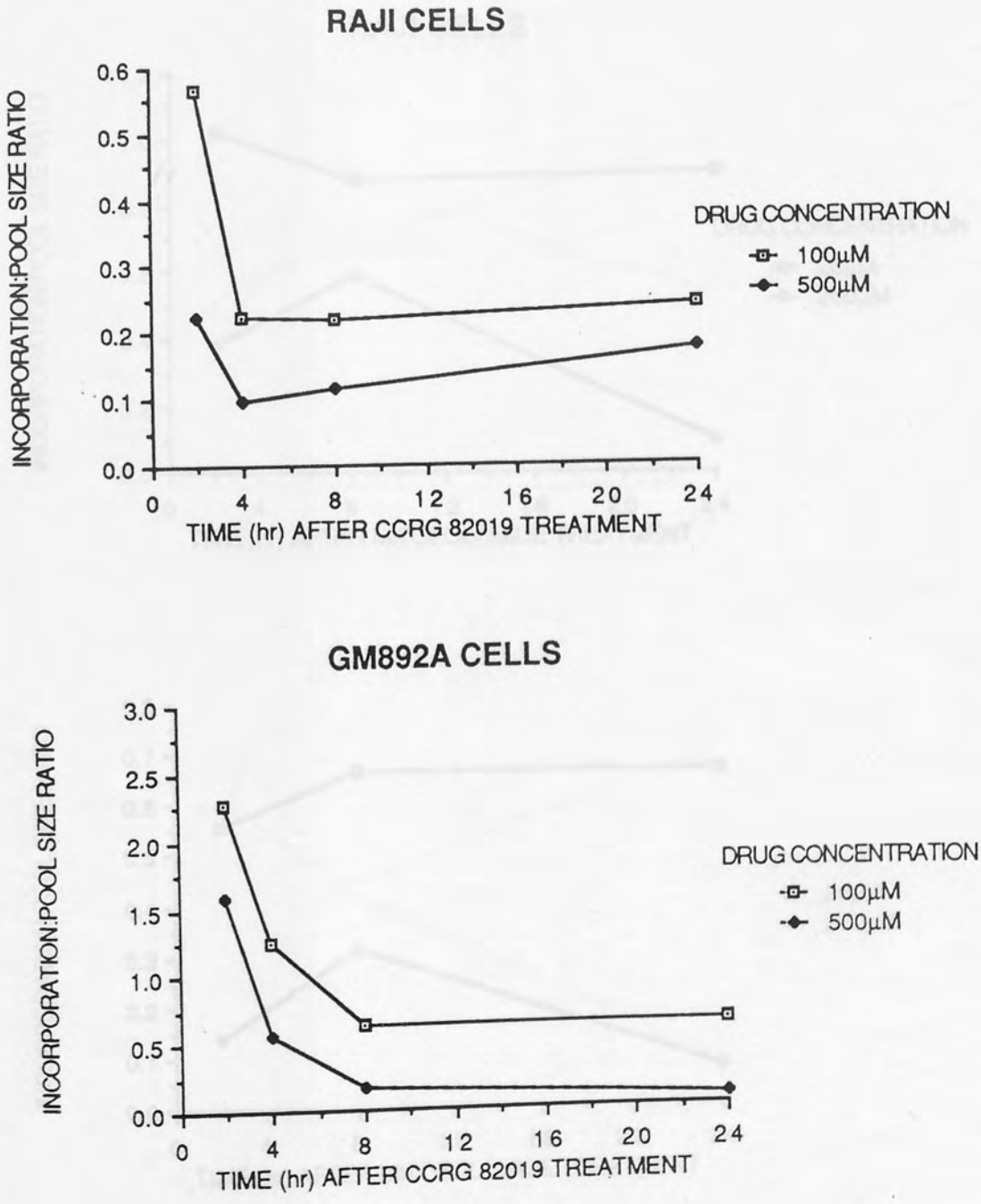
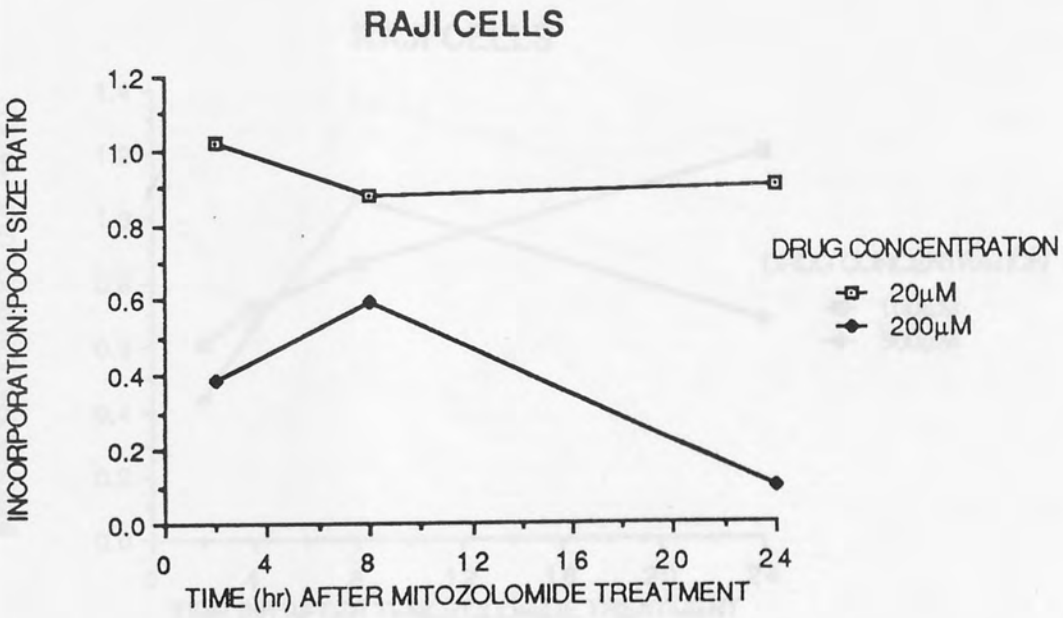


Figure 31.

The ratio of labelled uridine incorporation into acid-insoluble material to labelled uridine pool size in Raji and GM892A cells treated with mitozolomide.

The values were calculated as the ratio of the ^{3}H-uridine incorporated into the acid-insoluble material of treated cells (relative to control cells) versus the ^{3}H-uridine pool size in treated cells (relative to control cells).



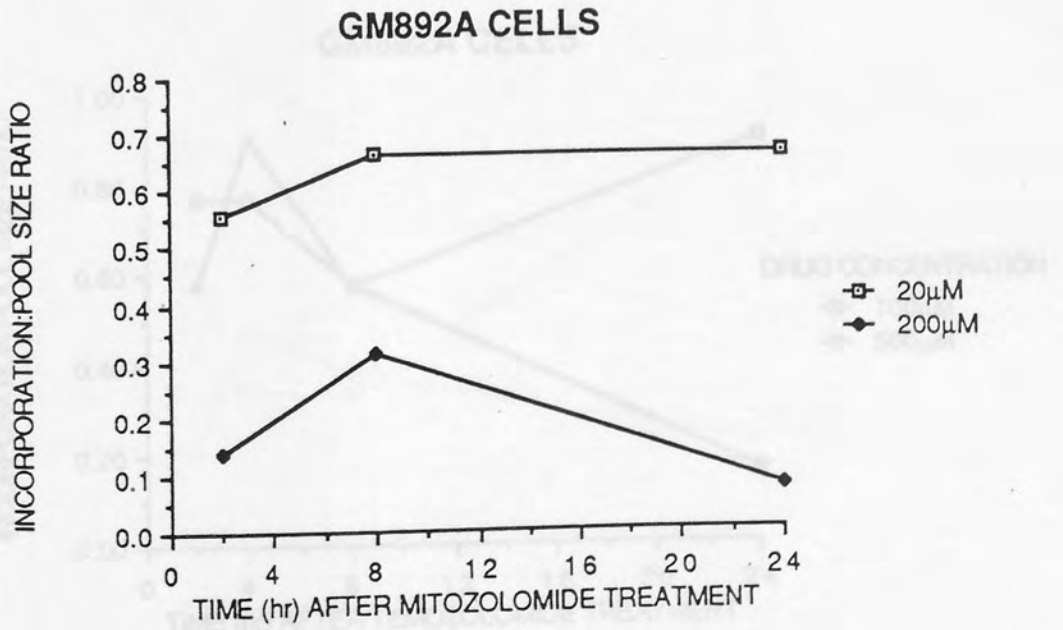
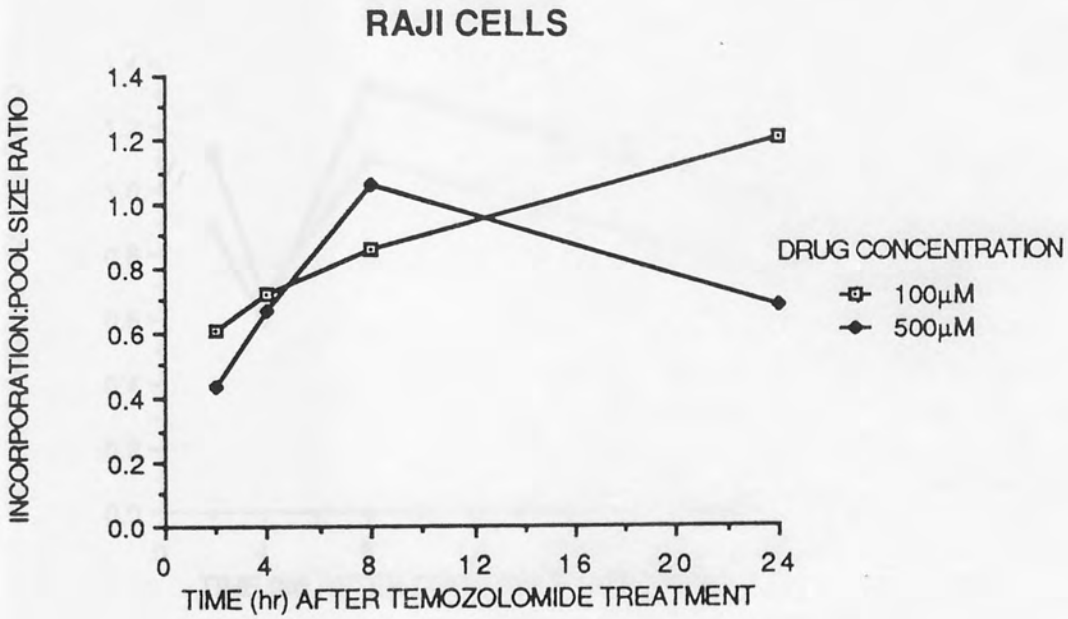


Figure 32.

The ratio of labelled uridine incorporation into acid-insoluble material to labelled uridine pool size in Raji and GM892A cells treated with temozolomide.

The values were calculated as the ratio of the ^{3}H-uridine incorporated into the acid-insoluble material of treated cells (relative to control cells) versus the ^{3}H-uridine pool size in treated cells (relative to control cells).



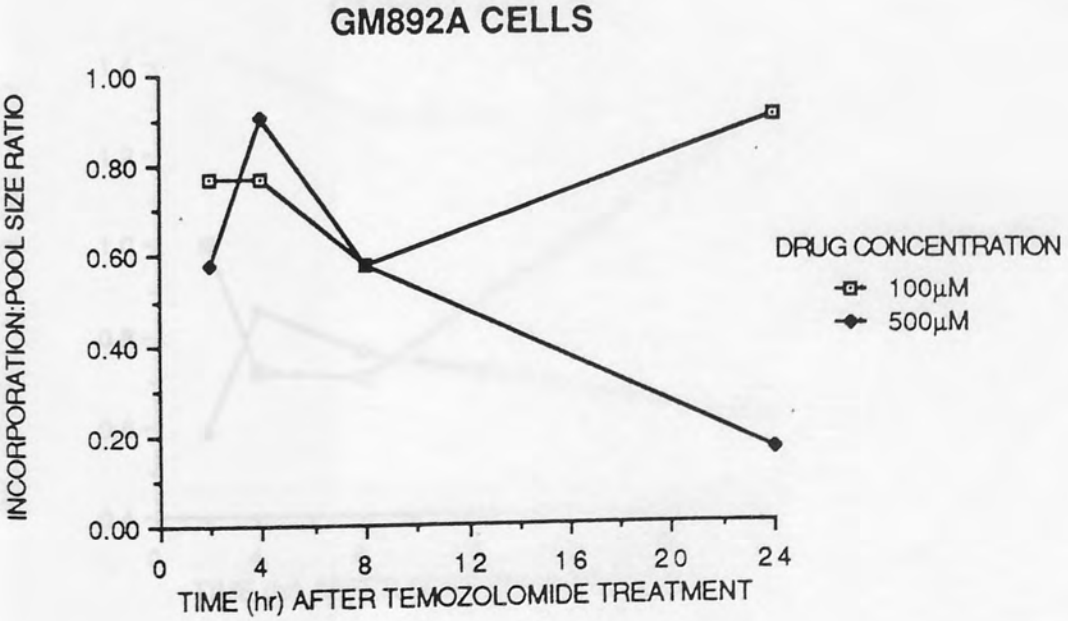
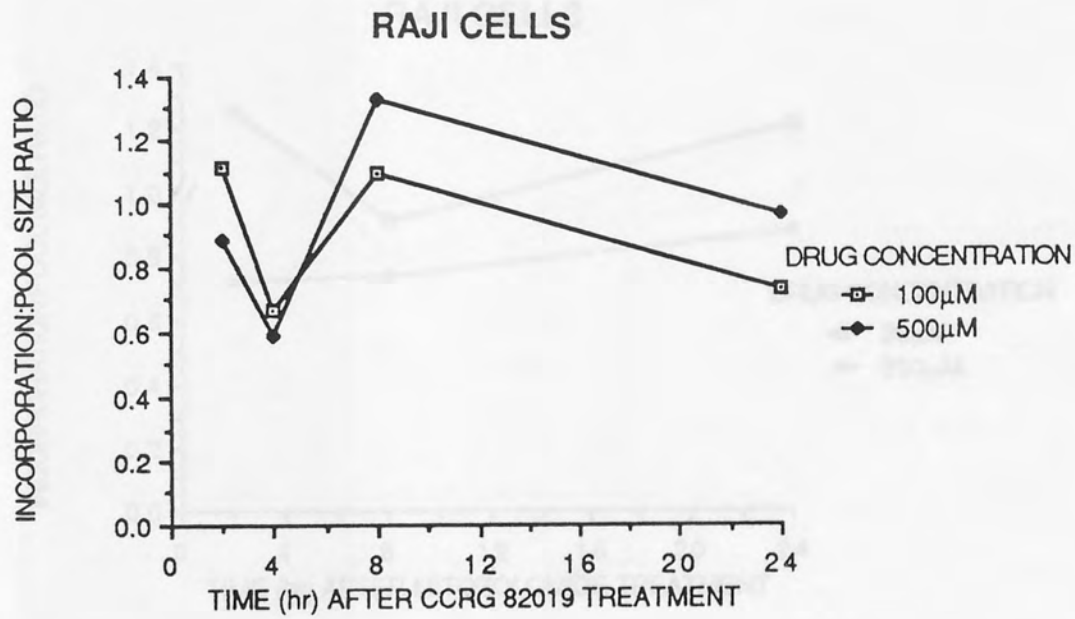


Figure 33.

The ratio of labelled uridine incorporation into acid-insoluble material to labelled uridine pool size in Raji and GM892A cells treated with CCRG 82019.

The values were calculated as the ratio of the ^{3}H-uridine incorporated into the acid-insoluble material of treated cells (relative to control cells) versus the ^{3}H-uridine pool size in treated cells (relative to control cells).



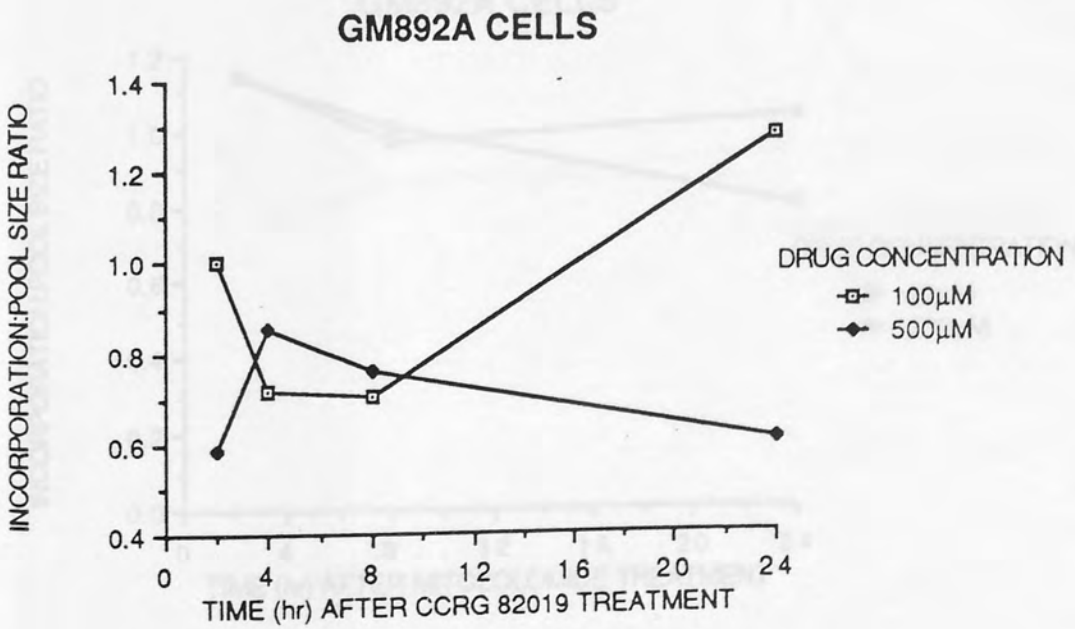


Figure 34.

The ratio of labelled leucine incorporation into acid-insoluble material to labelled leucine pool size in Raji and GM892A cells treated with mitozolomide.

The values were calculated as the ratio of the ^{3}H-leucine incorporated into the acid-insoluble material of treated cells (relative to control cells) versus the ^{3}H-leucine pool size in treated cells (relative to control cells).

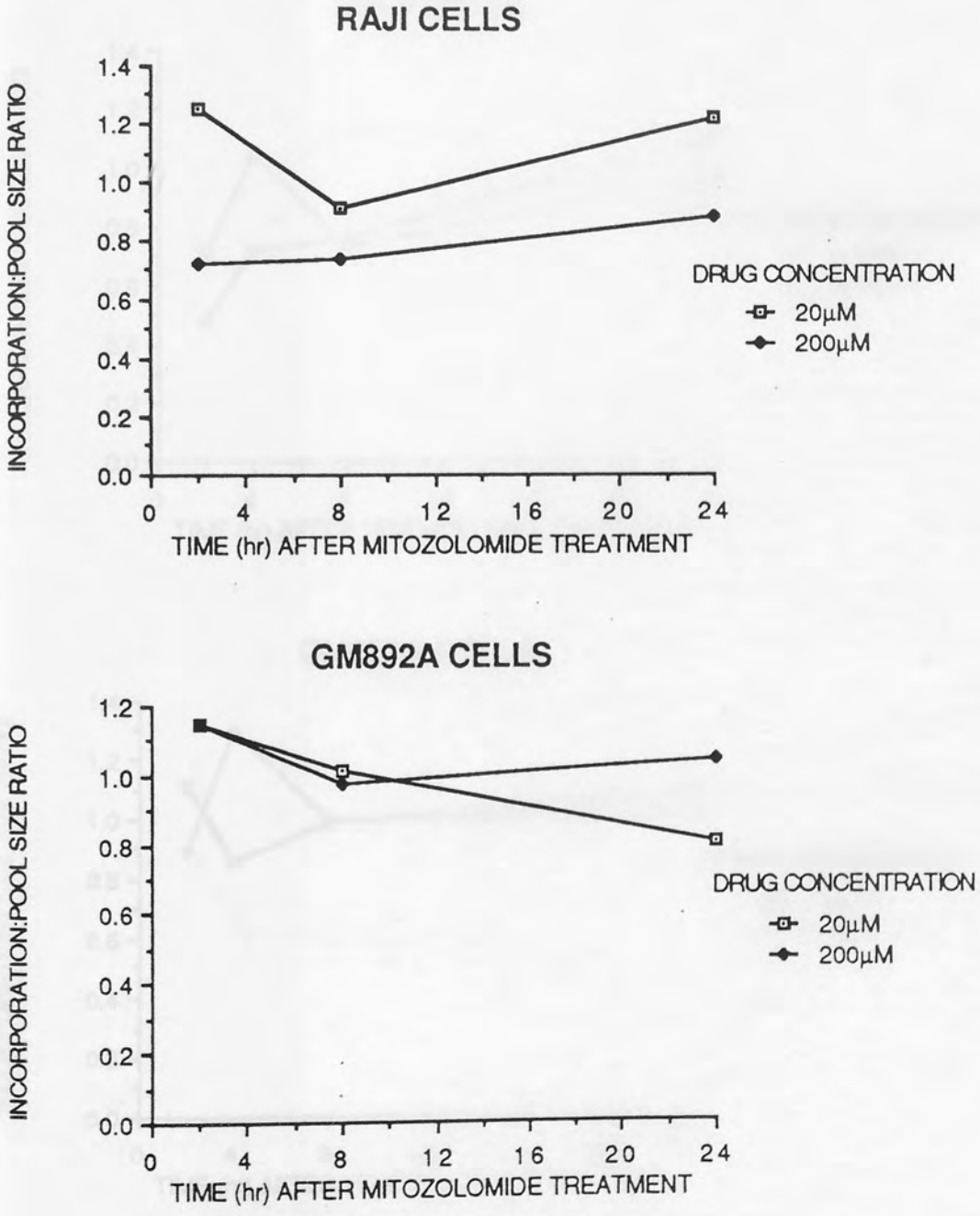
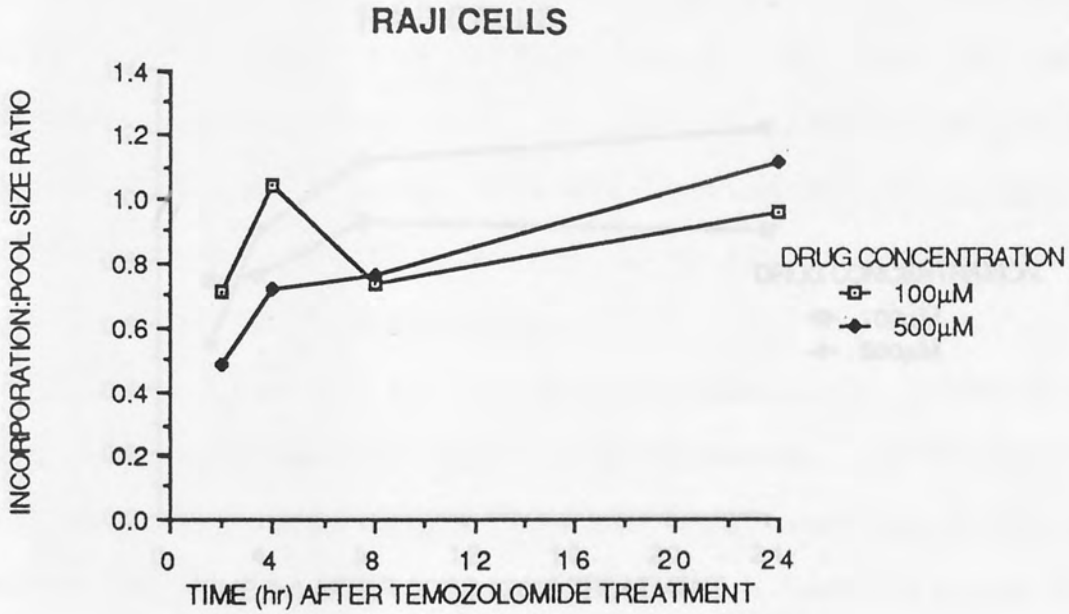


Figure 35.

The ratio of labelled leucine incorporation into acid-insoluble material to labelled leucine pool size in Raji and GM892A cells treated with temozolomide.

The values were calculated as the ratio of the ^{3}H-leucine incorporated into the acid-insoluble material of treated cells (relative to control cells) versus the ^{3}H-leucine pool size in treated cells (relative to control cells).



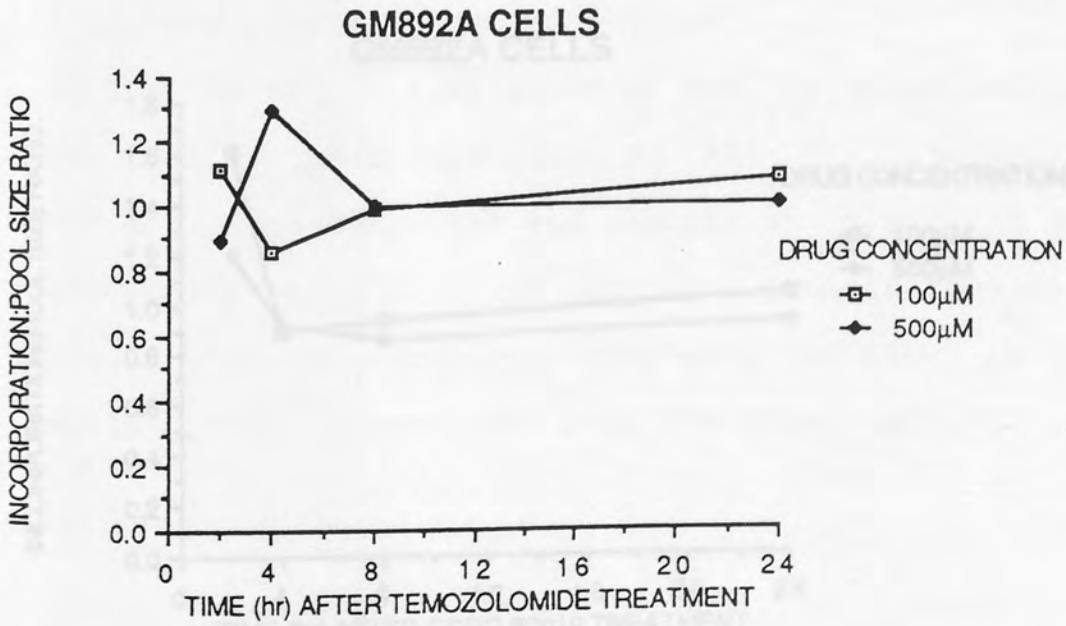
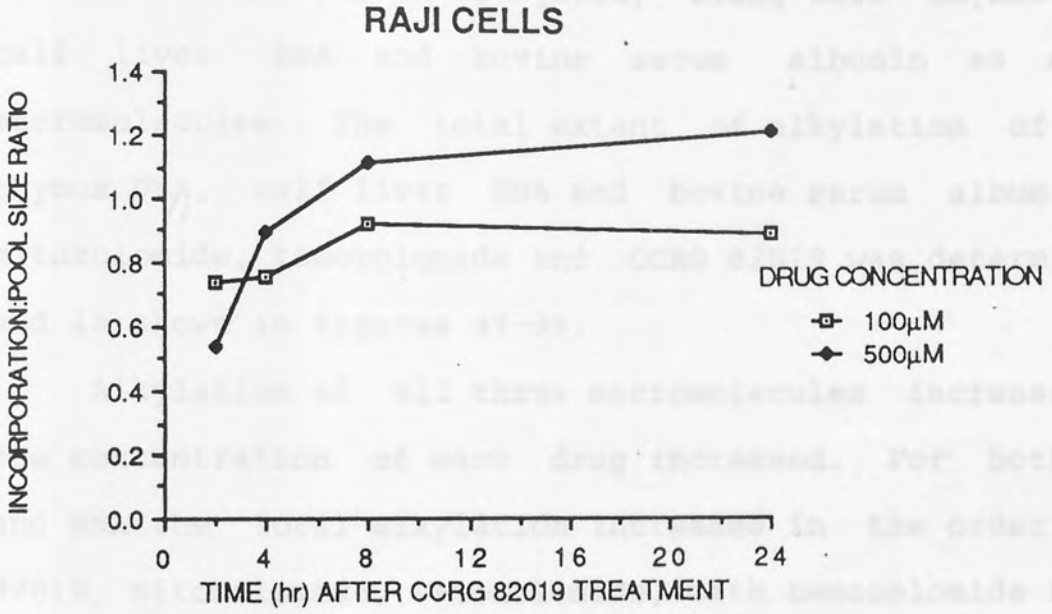
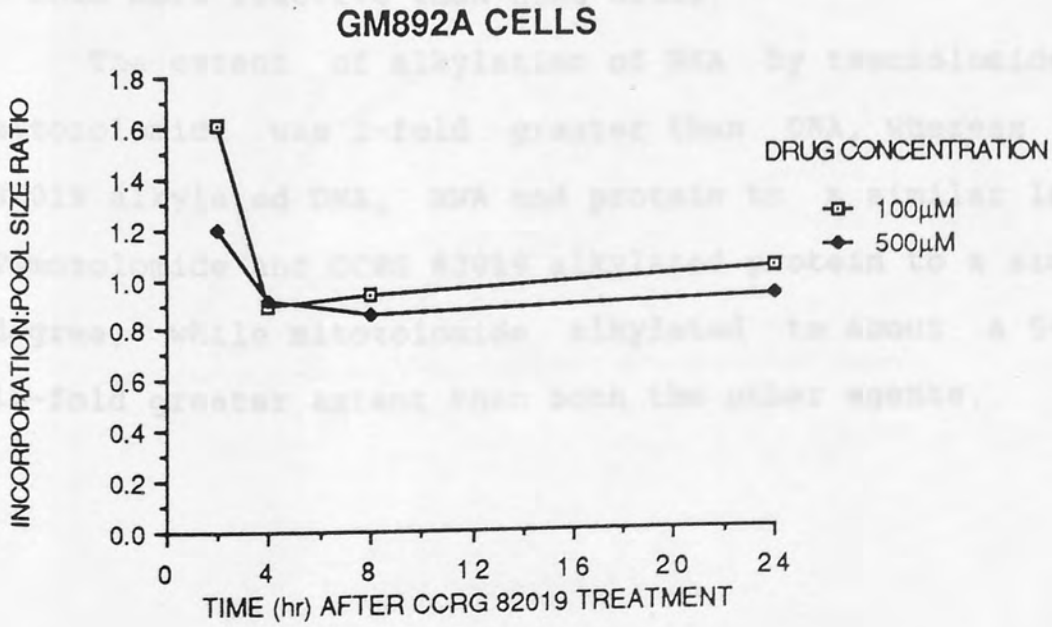


Figure 36.

The ratio of labelled leucine incorporation into acid-insoluble material to labelled leucine pool size in Raji and GM892A cells treated with CCRG 82019.

The values were calculated as the ratio of the ^{3}H-leucine incorporated into the acid-insoluble material of treated cells (relative to control cells) versus the ^{3}H-leucine pool size in treated cells (relative to control cells).





4.6 In vitro reactions of radiolabelled imidazotetrazinones with isolated macromolecules.

The cytotoxicity of the antitumour triazenes and nitrosoureas has been attributed to their extent of reaction with cellular macromolecules. For this reason the reactivity of imidazotetrazinones towards cellular macromolecules was investigated, using calf thymus DNA, calf liver RNA and bovine serum albumin as model macromolecules. The total extent of alkylation of calf thymus DNA, calf liver RNA and bovine serum albumin by mitozolomide, temozolomide and CCRG 82019 was determined, and is shown in figures 37-39.

Alkylation of all three macromolecules increased as the concentration of each drug increased. For both DNA and RNA the total alkylation increased in the order CCRG 82019, mitozolomide, temozolomide, with temozolomide being about twice as reactive as mitozolomide, and approximately 5-fold more reactive than CCRG 82019.

The extent of alkylation of RNA by temozolomide and mitozolomide was 2-fold greater than DNA, whereas CCRG 82019 alkylated DNA, RNA and protein to a similar level. Temozolomide and CCRG 82019 alkylated protein to a similar degree, while mitozolomide alkylated to about a 5- to 10-fold greater extent than both the other agents.

Figure 37.

The extent of reaction of imidazotetrazinones with calf thymus DNA.

Calf thymus DNA was incubated with drug for 2hr at 37°C, and the amount of radioactivity bound was determined.

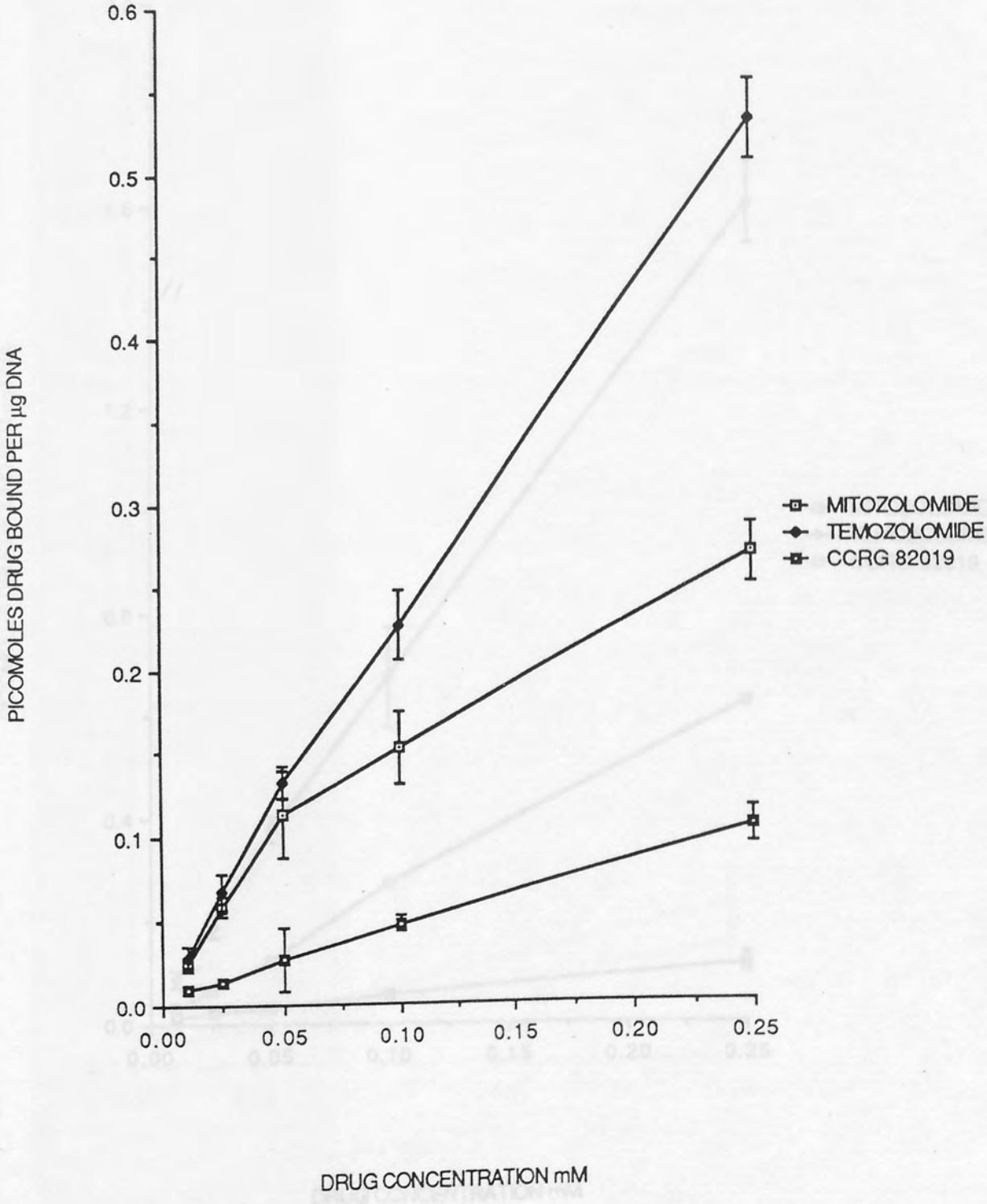


Figure 38.

<u>The extent of reaction of imidazotetrazinones with calf liver RNA.</u>

Calf liver RNA was incubated with drug for 2hr at 37°C, and the amount of radioactivity bound was determined.

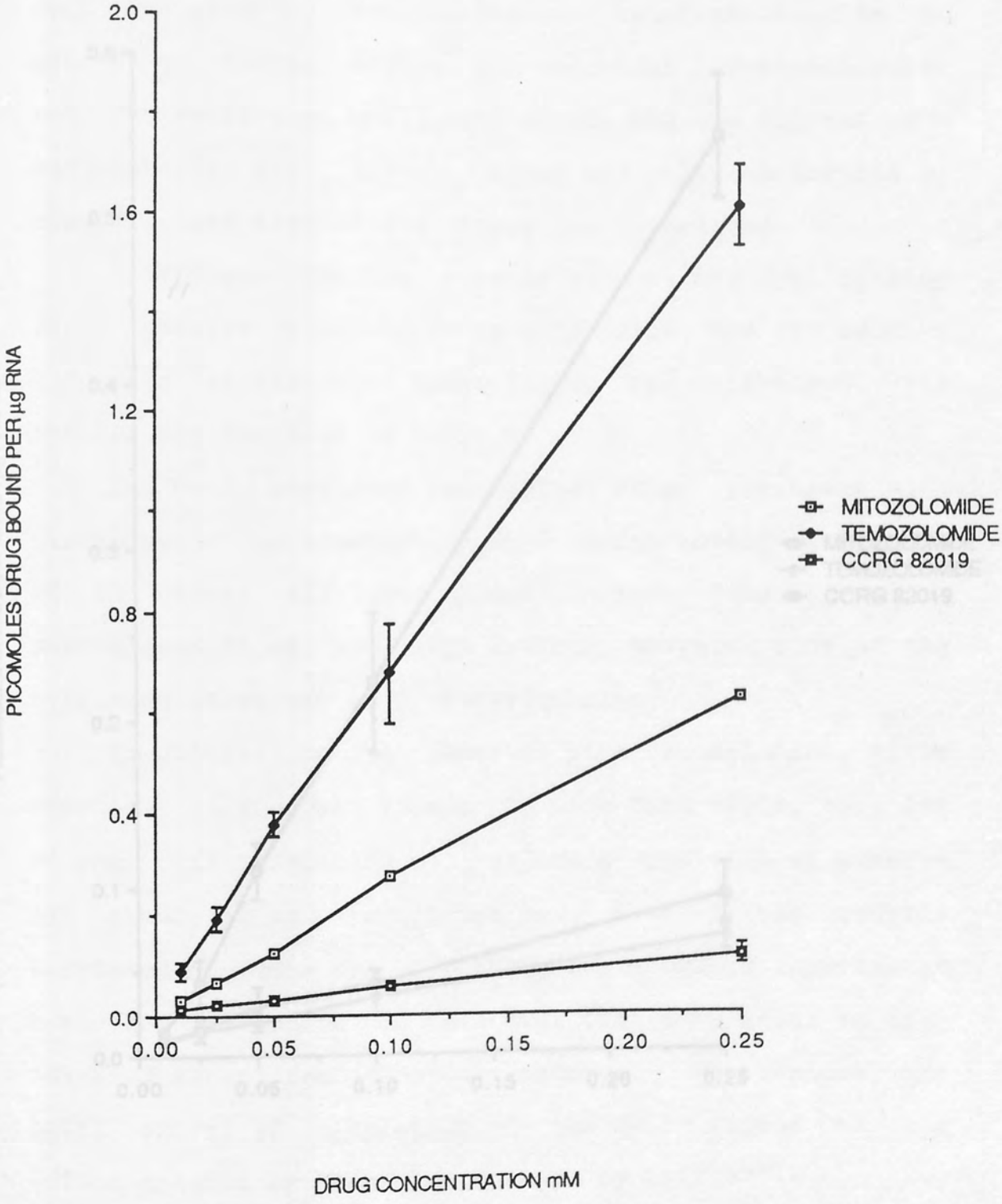
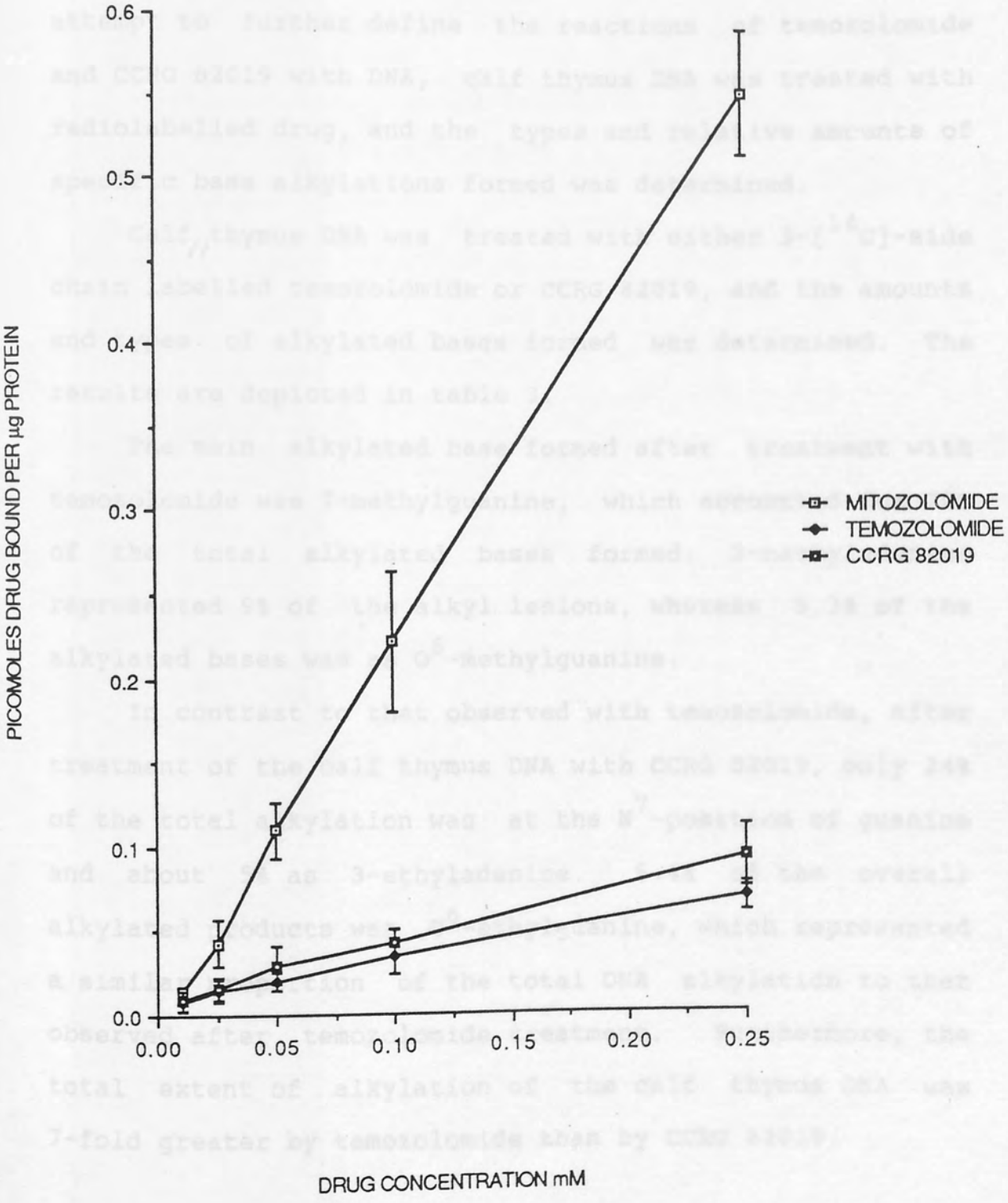


Figure 39.

<u>The extent of reaction of imidazotetrazinones with bovine serum albumin.</u>

Bovine serum albumin was incubated with drug for 2hr at 37°C, and the amount of radioactivity bound was determined.



4.7 Alkylated bases formed after treatment of calf thymus DNA with temozolomide and CCRG 82019.

The overall alkylation of DNA extracted from cells treated with imidazotetrazinones did not correlate well with the observed differences in cytotoxicity. In an attempt to further define the reactions of temozolomide and CCRG 82019 with DNA, calf thymus DNA was treated with radiolabelled drug, and the types and relative amounts of specific base alkylations formed was determined.

Calf thymus DNA was treated with either 3-[^{14}C]-side chain labelled temozolomide or CCRG 82019, and the amounts and types of alkylated bases formed was determined. The results are depicted in table 3.

The main alkylated base formed after treatment with temozolomide was 7-methylguanine, which accounted for 70% of the total alkylated bases formed: 3-methyladenine represented 9% of the alkyl lesions, whereas 5.3% of the alkylated bases was as O^6-methylguanine.

In contrast to that observed with temozolomide, after treatment of the calf thymus DNA with CCRG 82019, only 24% of the total alkylation was at the N^7-position of guanine and about 5% as 3-ethyladenine. 5.4% of the overall alkylated products was O^6-ethylguanine, which represented a similar proportion of the total DNA alkylation to that observed after temozolomide treatment. Furthermore, the total extent of alkylation of the calf thymus DNA was 7-fold greater by temozolomide than by CCRG 82019.

Attempts were made to catalogue the alkylated bases formed more fully , and to elucidate their contribution to the total alkylation. O-alkyl pyrimidines and phosphotriesters were synthesised as standards for HPLC analysis by reverse-phase chromatography, but efforts to satisfactorily separate these products proved friutless.

Table 3.

Alkylated bases formed after treatment of calf thymus DNA with temozolomide and CCRG 82019.

The values in the table represent the amount of each base, and its proportion to the total base alkylations, formed in 20mg of calf thymus DNA following treatment with 0.5mM temozolomide or CCRG 82109.

Temozolomide

Base formed	Amount formed nMoles/20mg DNA	% of total products
7-Guanine	11.59	70.3
O^6-Guanine	0.87	5.3
3-Adenine	1.52	9.2
Total extent of alkylation.	16.49	

CCRG 82019

Base formed	Amount formed nMoles/20mg DNA	% of total products
7-Guanine	0.58	24.0
O^6-Guanine	0.13	5.4
3-Adenine	0.12	4.9
Total extent of alkylation.	2.43	

4.8 Extent of reaction of radiolabelled imidazotetrazinones with macromolecules in intact cells.

The amount of radioactivity associated with macromolecules in Raji and GM892A cells at various times after treatment with 3-[^{14}C]-side chain labelled imidazotetrazinones is shown in figures 40-42. Possible incorporation of the radiolabel via the 1-carbon pool was suppressed by the addition of 20mM sodium formate to the medium 30min prior to drug addition.

In both cell lines the radioactivity remaining bound to DNA, RNA and protein after mitozolomide treatment exceeded that of temozolomide and CCRG 82019 by a factor of between 4- and 7-fold. The extent of alkylation of DNA by mitozolomide was higher than that of RNA or protein, and the DNA was alkylated almost twice as much in GM892A cells as in Raji cells.

The overall alkylation of DNA in Raji cells by temozolomide and CCRG 82019 was almost identical over the 24hr period. In GM892A cells the overall alkylation of DNA by temozolomide was greater than that of CCRG 82019 only after 24hr. In contrast to Raji cells, the overall alkylation of DNA in GM892A cells by temozolomide continued to rise slightly over the 24hr period. Alkylation of RNA was slightly greater by temozolomide than by CCRG 82019, but was of a similar order of magnitude in both cell lines, and similar to the extent of alkylation of DNA.

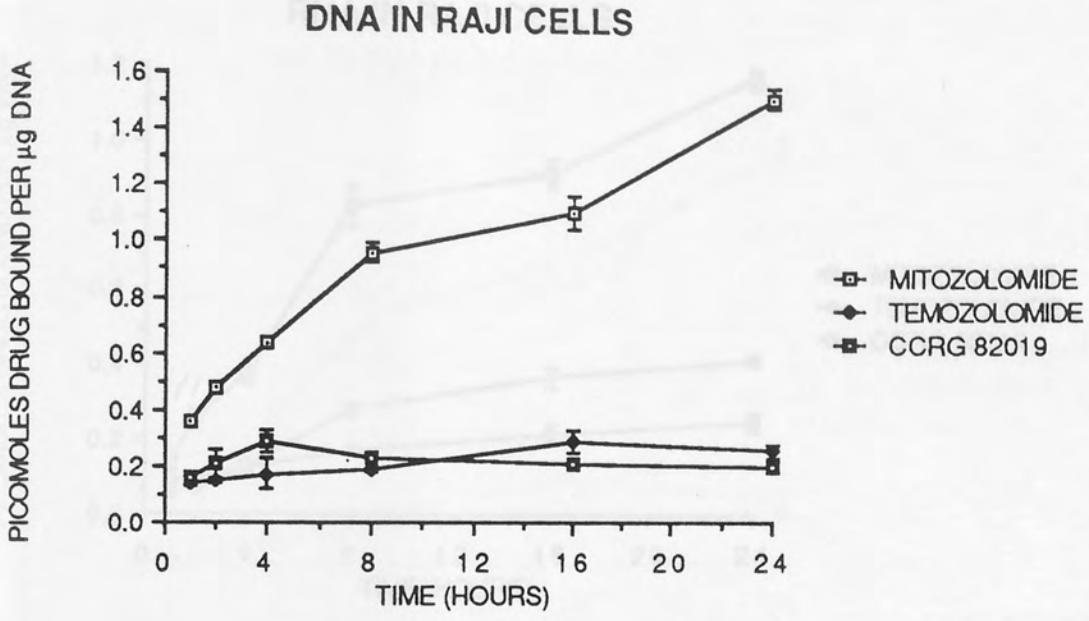
The level of protein alkylation by all three agents was about 10- to 20-fold less than that of DNA or RNA. Temozolomide and CCRG 82019 alkylated proteins to a similar order of magnitude, whereas mitozolomide alkylated to a 10-fold greater extent. The level of protein alkylation by each agent was similar in both cell lines.

The extent of reaction of imidazotetrazinones with macromolecules in intact cells.

$8x10^6$ cells were treated with sodium formate to give a final concentration of 20mM, 30min prior to the addition of 0.1mM (final concentration) of 3-^{14}C side-chain labelled imidazotetrazinones. At various time intervals, a portion of the cell suspension was removed, and the radioactivity associated with the cellular DNA, RNA and protein determined.

Figure 40.

The radioactivity associated with the DNA extracted from Raji and GM892A cells treated with imidazotetrazinones.



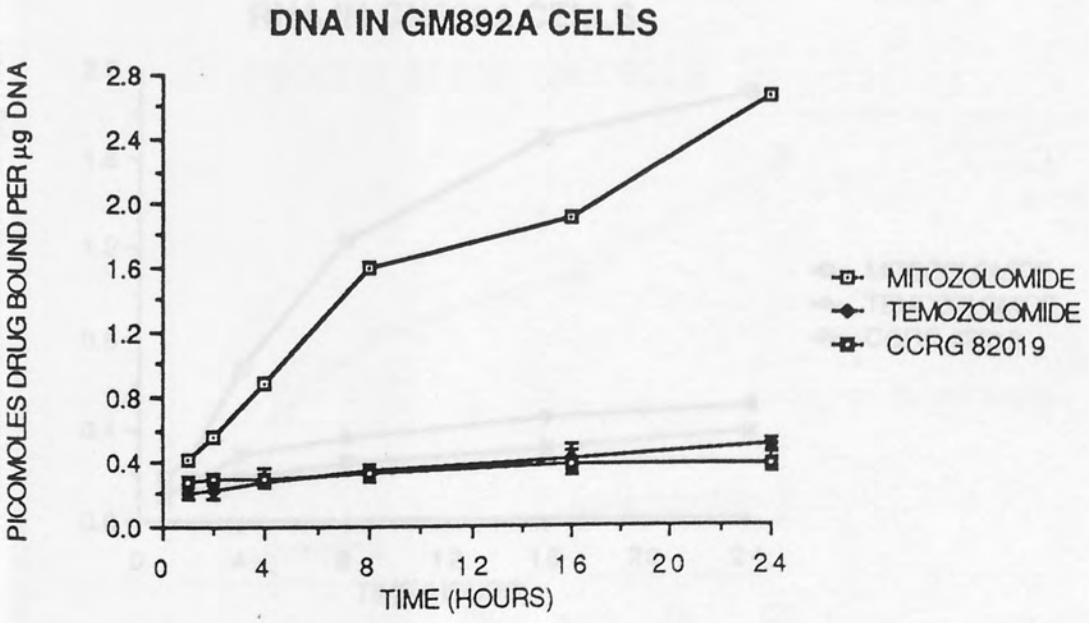
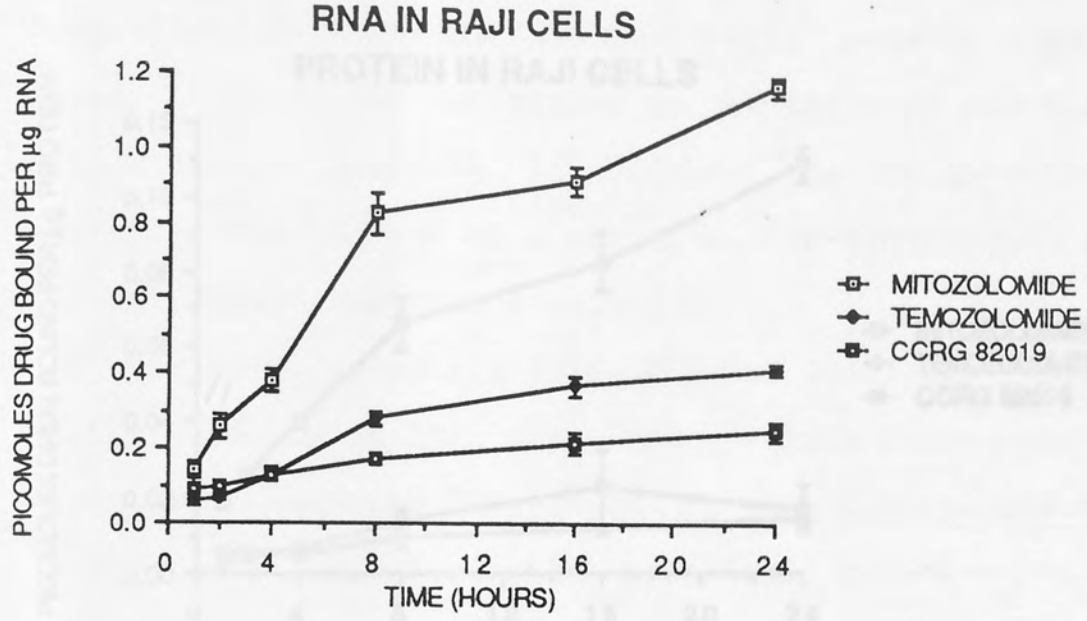


Figure 41.

The radioactivity associated with the RNA extracted from Raji and GM892A cells treated with imidazotetrazinones.



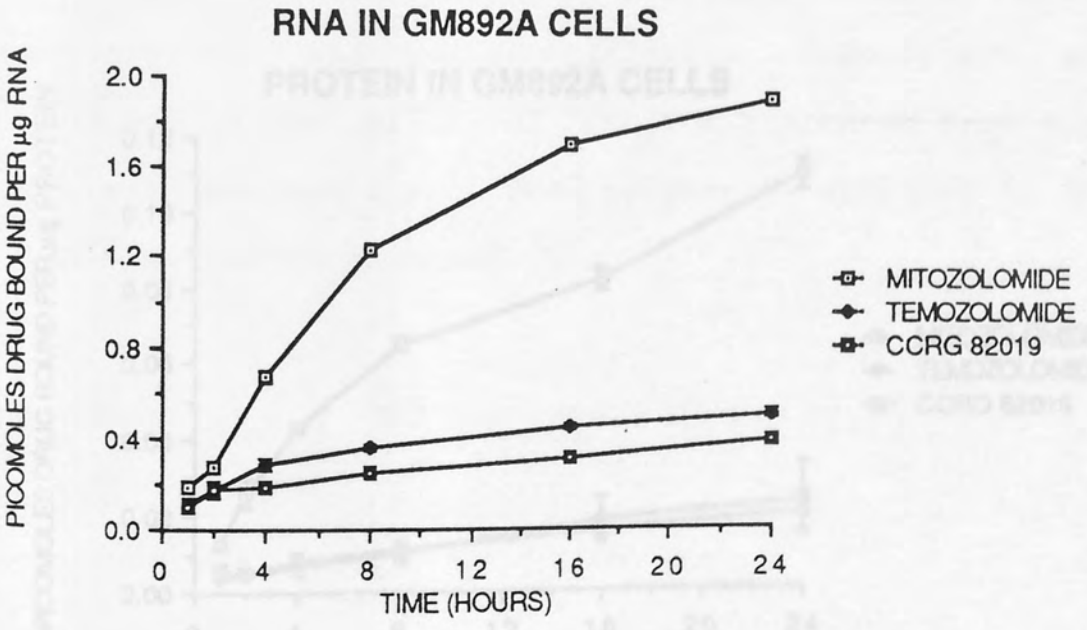
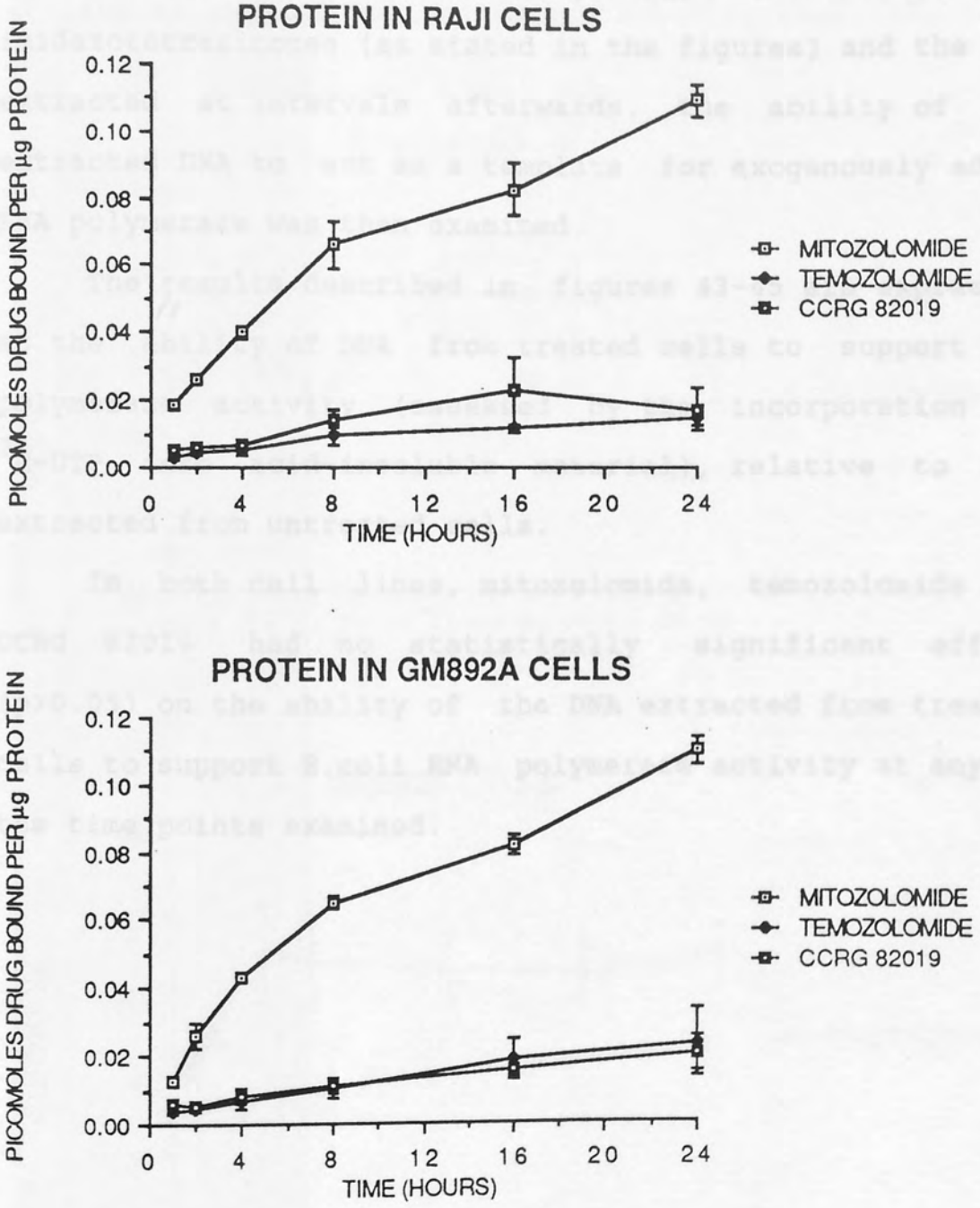


Figure 42.

The radioactivity associated with the protein extracted from Raji and GM892A cells treated with imidazotetrazinones.



4.9 The ability of DNA extracted from Raji and GM892A cells treated with imidazotetrazinones to support RNA polymerase activity.

In an attempt to define more closely the effects of drug alkylation on DNA function, cells were treated with imidazotetrazinones (as stated in the figures) and the DNA extracted at intervals afterwards. The ability of the extracted DNA to act as a template for exogenously added RNA polymerase was then examined.

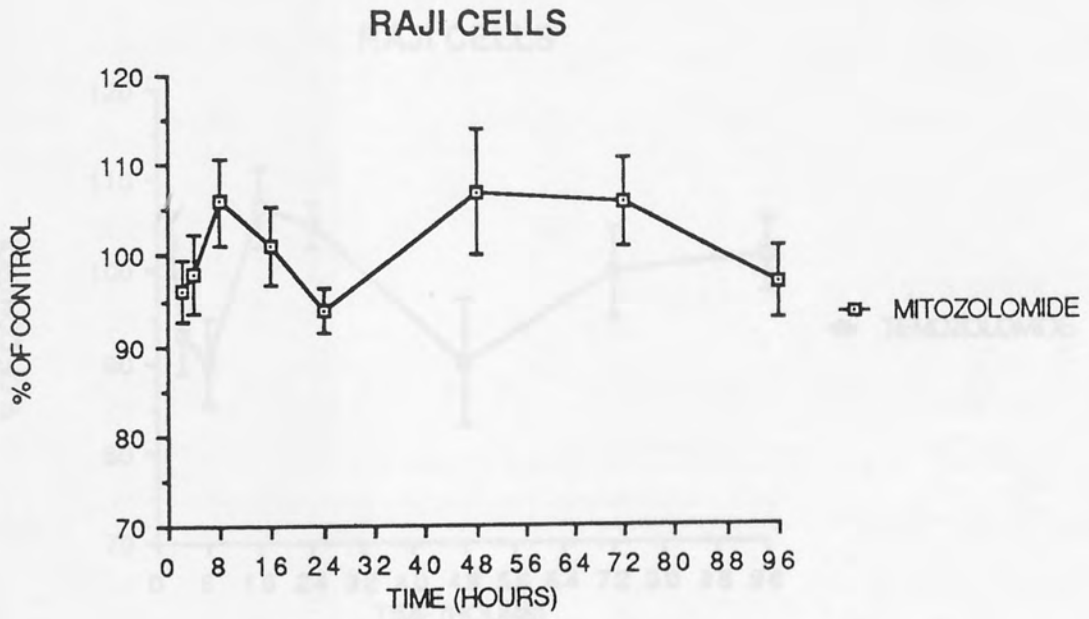
The results described in figures 43-45 are expressed as the ability of DNA from treated cells to support RNA polymerase activity (assessed by the incorporation of ^{3}H-UTP into acid-insoluble material), relative to DNA extracted from untreated cells.

In both cell lines, mitozolomide, temozolomide and CCRG 82019 had no statistically significant effect ($p>0.05$) on the ability of the DNA extracted from treated cells to support E.coli RNA polymerase activity at any of the time points examined.

Figure 43.

The ability of DNA extracted from mitozolomide-treated Raji and GM892A cells to support RNA polymerase activity.

Raji cells were treated with mitozolomide at a concentration of 20μM, and GM892A cells were treated with mitozolomide at a concentration of 2μM. At various time intervals the DNA was extracted from $2x10^7$ cells, and its ability to support RNA polymerase activity measured.



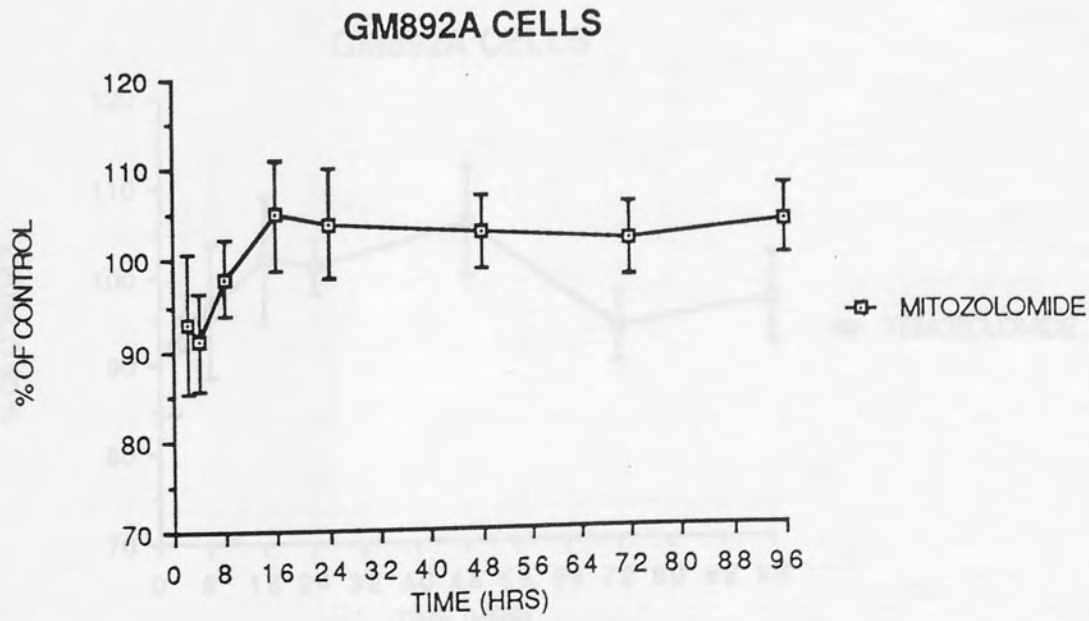
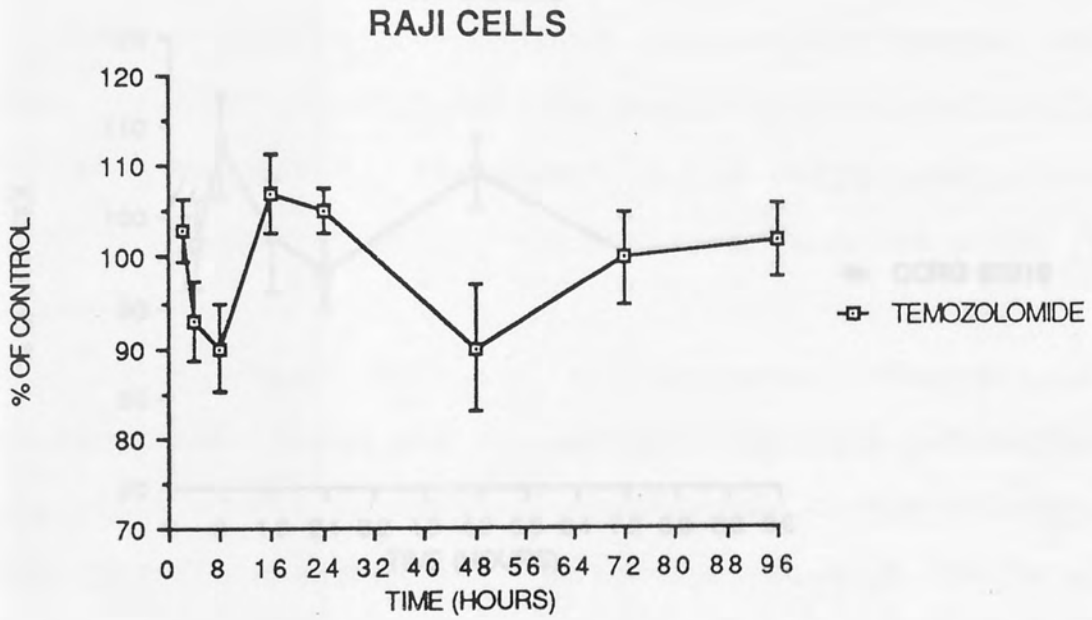


Figure 44.

The ability of DNA extracted from temozolomide-treated Raji and GM892A cells to support RNA polymerase activity.

Raji cells were treated with temozolomide at a concentration of 206μM, and GM892A cells were treated with temozolomide at a concentration of 10μM. At various time intervals the DNA was extracted from $2x10^7$ cells, and its ability to support RNA polymerase activity measured.



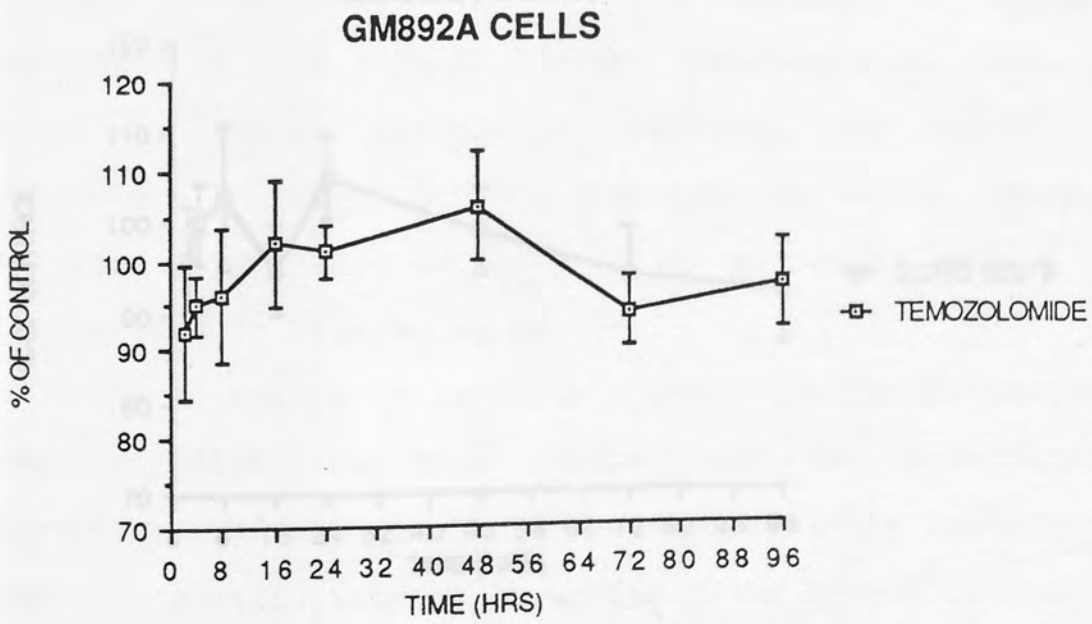
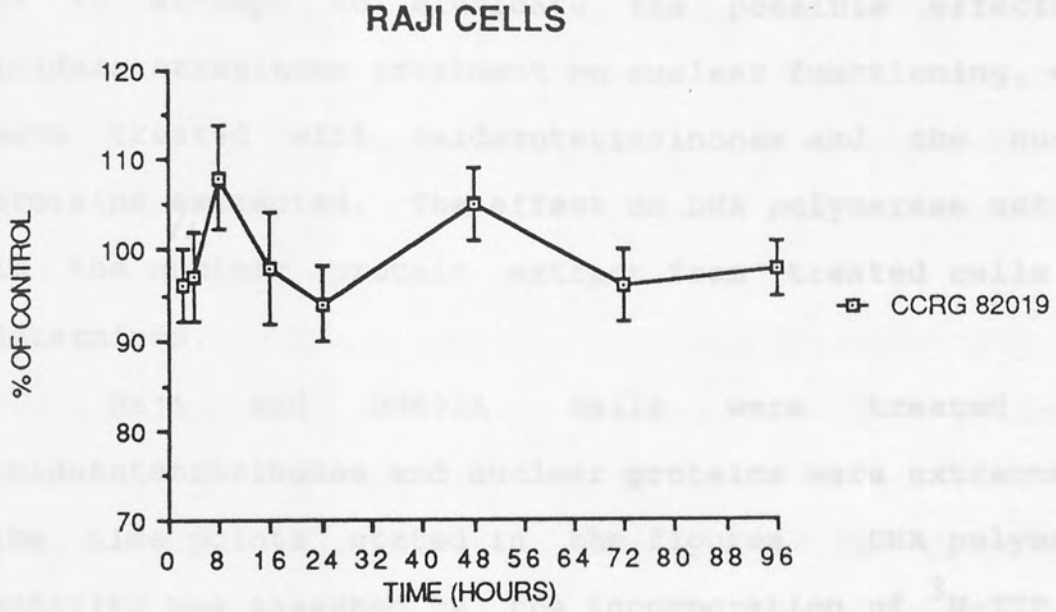
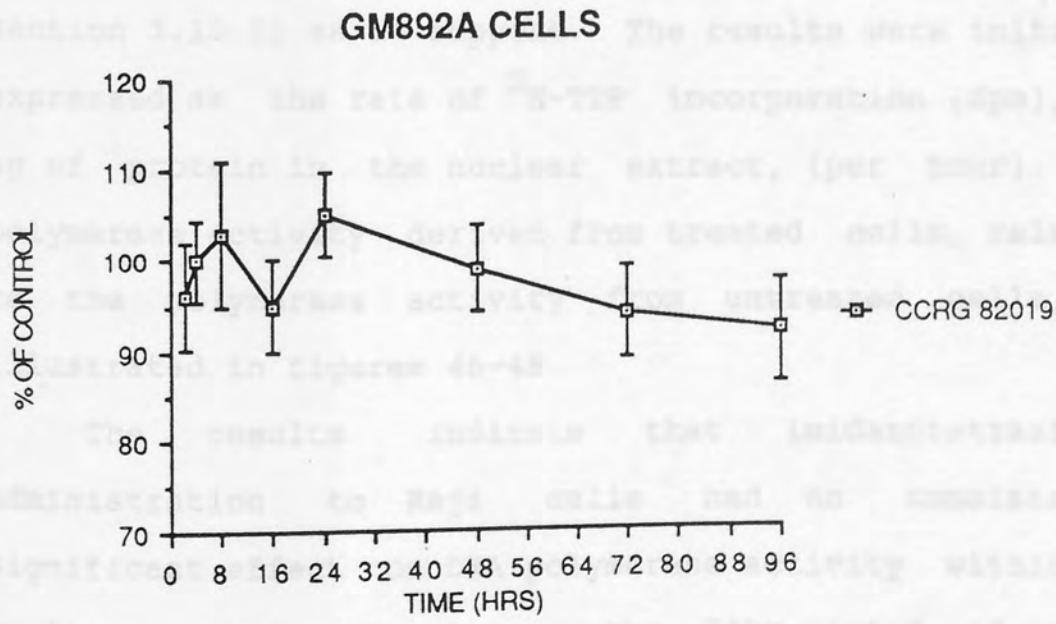


Figure 45.

The ability of DNA extracted from CCRG 82019-treated Raji and GM892A cells to support RNA polymerase activity.

Raji cells were treated with CCRG 82019 at a concentration of 361μM, and GM892A cells were treated with CCRG 82019 at a concentration of 245μM. At various time intervals the DNA was extracted from $2x10^7$ cells, and its ability to support RNA polymerase activity measured.





4.10 DNA polymerase activity in nuclear protein extracts from Raji and GM892A cells treated with imidazotetrazinones.

As previously shown (section 4.9), DNA extracted from cells treated with imidazotetrazinones was unaffected in its ability to support E. coli RNA polymerase activity. In an attempt to elucidate the possible effects of imidazotetrazinone treatment on nuclear functioning, cells were treated with imidazotetrazinones and the nuclear proteins extracted. The effect on DNA polymerase activity in the nuclear protein extract from treated cells was determined.

Raji and GM892A cells were treated with imidazotetrazinones and nuclear proteins were extracted at the time points stated in the figures. DNA polymerase activity was assessed by the incorporation of ^{3}H-TTP into acid insoluble material, using "activated" DNA (see section 3.10.2) as a support. The results were initially expressed as the rate of ^{3}H-TTP incorporation (dpm), per µg of protein in the nuclear extract, (per hour). DNA polymerase activity derived from treated cells, relative to the polymerase activity from untreated cells, is illustrated in figures 46-48.

The results indicate that imidazotetrazinone administration to Raji cells had no consistently significant effect on DNA polymerase activity within the nuclear protein extract over the 24hr period of study.

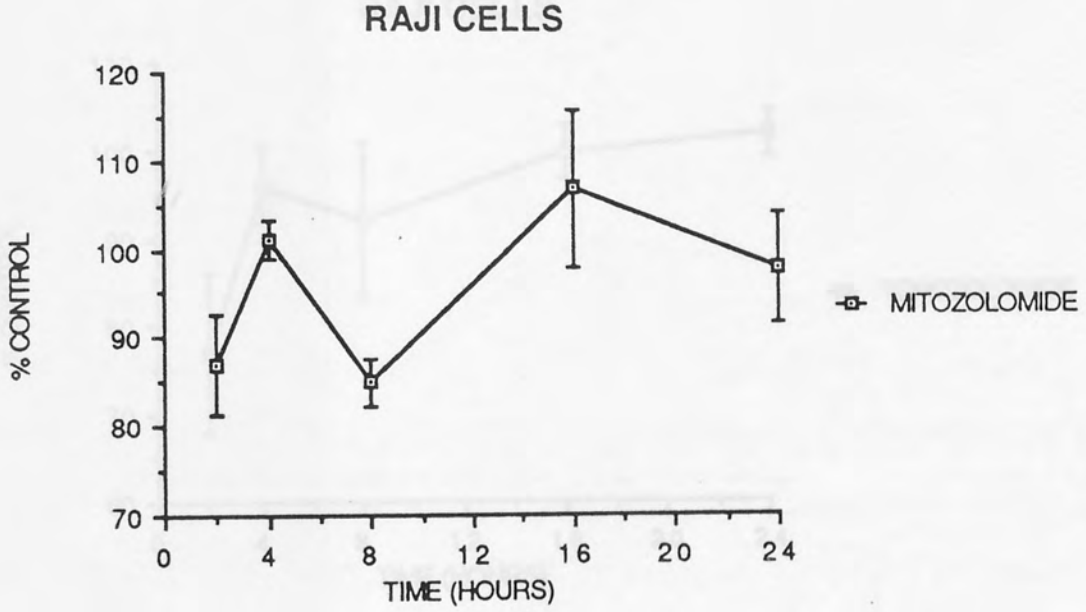
However in GM892A cells, whereas mitozolomide had no consistently significant effect on polymerase activity, temozolomide did appear to cause a slight, though significant ($p<0.05$) decrease in activity 16 to 24hr after drug treatment. Furthermore CCRG 82019 had a marked, significant effect ($p<0.025$) over the whole 24hr period, with a maximal 40% decrease in DNA polymerase activity.

To ascertain whether the observed effects on DNA polymerase activity were due to a direct interaction between drug and enzyme, nuclear proteins were extracted from untreated Raji and GM892A cells and incubated with the same concentrations of drugs as used to treat the cells from which the extract had been derived. The drug-treated extract was then assayed for DNA polymerase activity in the same manner as used for extracts from treated cells. It was found that none of the three agents examined had a direct effect on the DNA polymerase in the nuclear protein extract.

Figure 46.

DNA polymerase activity extracted from mitozolomide-treated Raji and GM892A cells.

Raji cells were treated with mitozolomide at a concentration of 20μM, and GM892A cells were treated with mitozolomide at a concentration of 2μM. At various time intervals nuclear proteins were extracted from $2x10^7$ cells, and the DNA polymerase activity present measured.



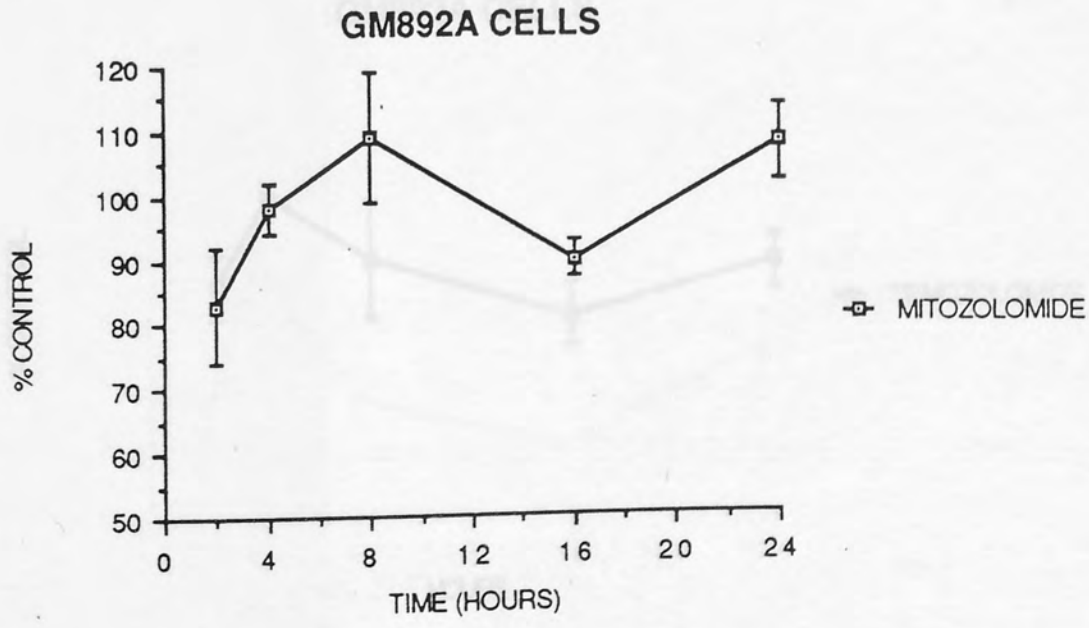
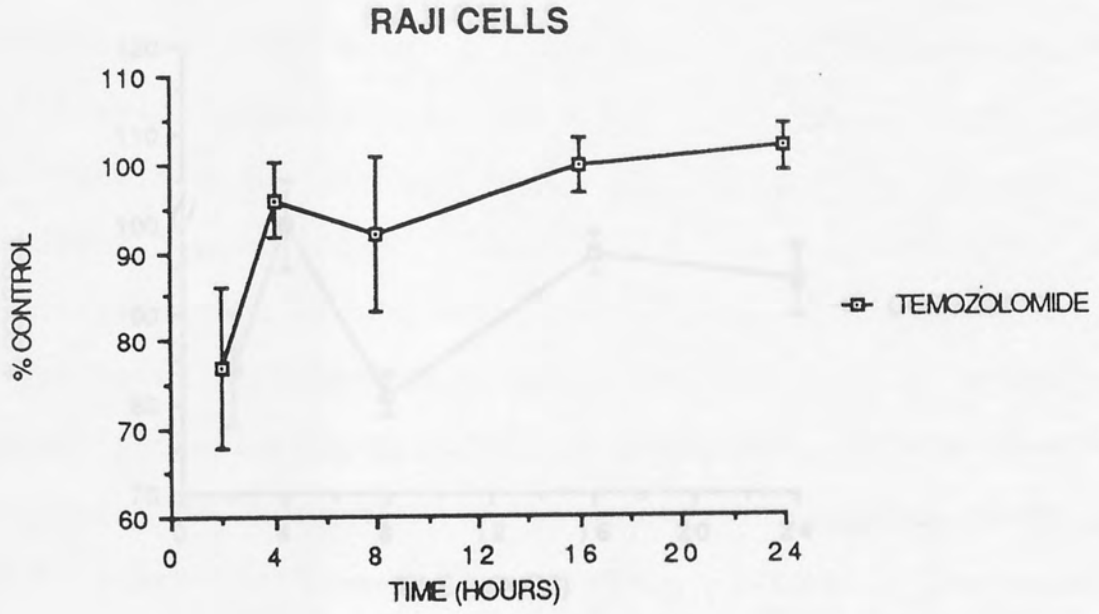


Figure 47.

DNA polymerase activity extracted from temozolomide-treated Raji and GM892A cells.

Raji cells were treated with temozolomide at a concentration of 206μM, and GM892A cells were treated with temozolomide at a concentration of 10μM. At various time intervals nuclear proteins were extracted from $2x10^7$ cells, and the DNA polymerase activity present measured.



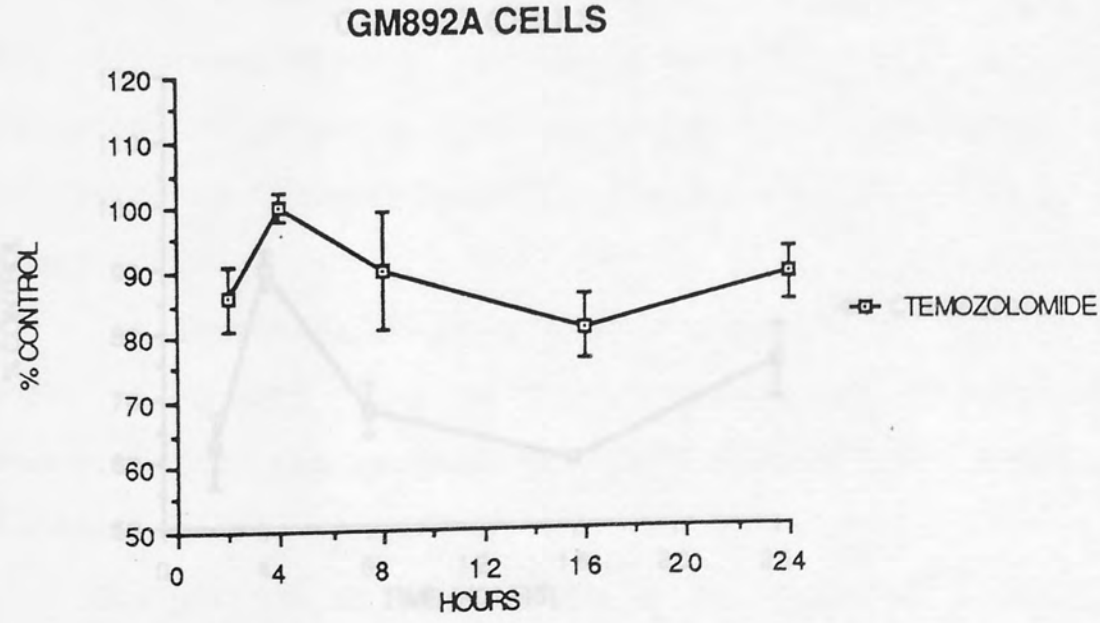
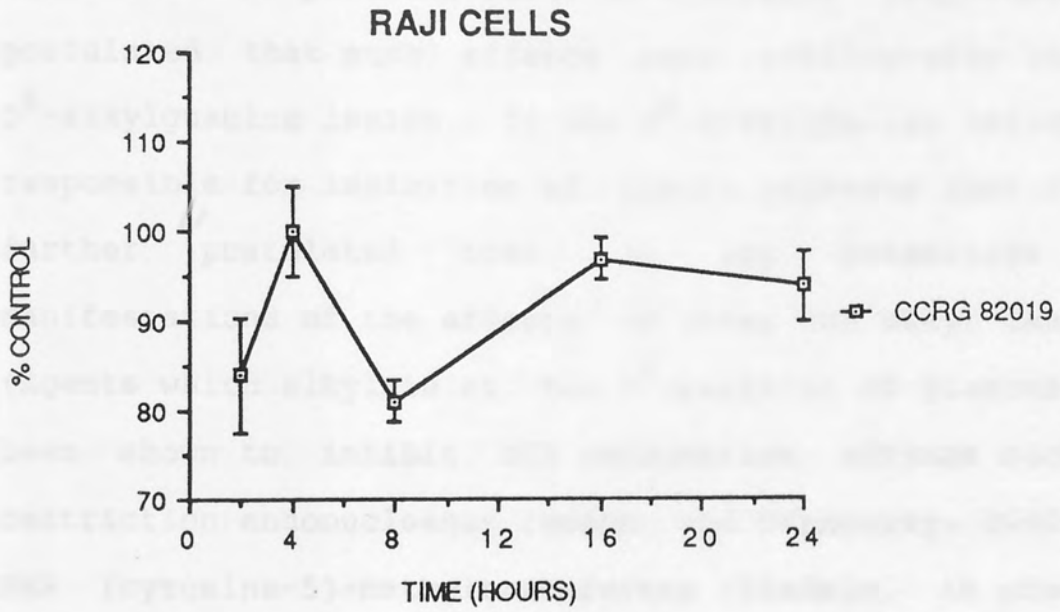
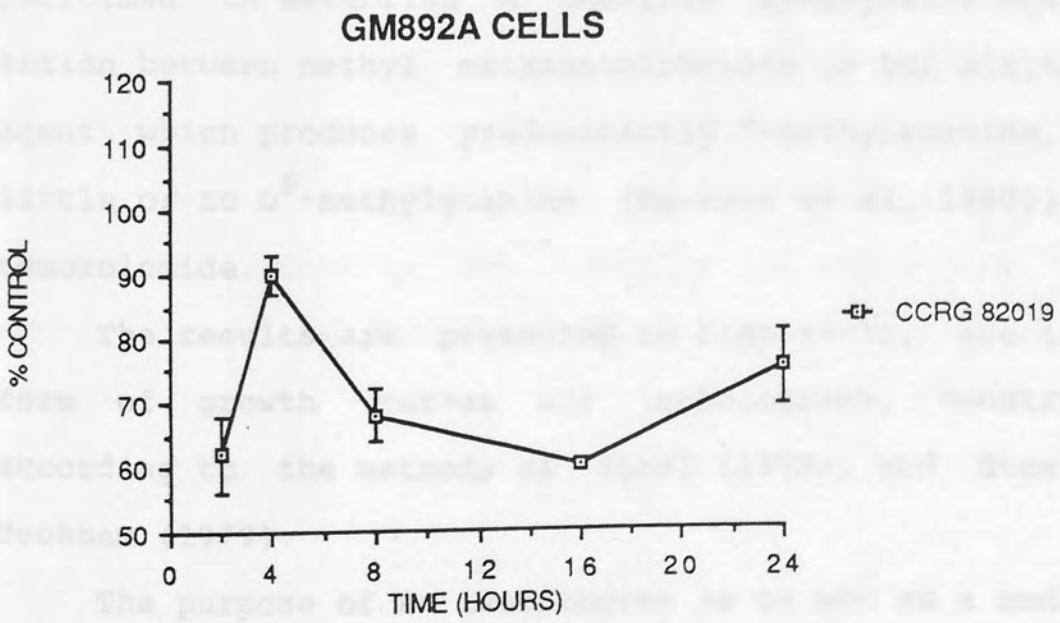


Figure 48.

DNA polymerase activity extracted from CCRG 82019-treated Raji and GM892A cells.

Raji cells were treated with CCRG 82019 at a concentration of 361μM, and GM892A cells were treated with CCRG 82019 at a concentration of 245μM. At various time intervals, nuclear proteins were extracted from $2x10^7$ cells, and the DNA polymerase activity present measured.





4.11 Investigation of a possible synergistic mode of action between temozolomide and methyl methanesulphonate.

Earlier work in this study (section 4.10) demonstrated that treatment of GM892A cells with temozolomide and CCRG 82019 inhibited the DNA polymerase activity extracted from the cells. It was thought that other nuclear proteins could be similarly inhibited, and postulated that such effects were attributable to the O^6-alkylguanine lesion. If the O^6-alkylguanine lesion was responsible for inhibition of repair proteins then it was further postulated that it may potentiate the manifestations of the effects of other DNA alkyl lesions. (Agents which alkylate at the O^6-position of guanine have been shown to inhibit DNA recognition enzymes such as restriction endonucleases (Boehm and Drahovsky, 1980) and DNA (cytosine-5)-methyltransferase (Tisdale, in press)). To further investigate this possibility experiments were performed to establish a possible synergistic mode of action between methyl methanesulphonate (a DNA alkylating agent which produces predominantly 7-methylguanine, but little or no O^6-methylguanine (Beranek et al, 1980)), and temozolomide.

The results are presented in figs 49-51, are in the form of growth curves and isobolograms, constructed according to the methods of Steel (1979), and Steel and Peckham (1979).

The purpose of an isobologram is to act as a model to

predict whether two modes of therapy act to a lesser, equal or greater extent than would be expected by the concomitant administration of the two therapies independently. An isobologram is constructed by initially establishing the effects of each therapy (in this case two drug treatments, methyl methanesulphonate and temozolomide) singly. The data is then analysed to predict the concentrations of each drug that would be anticipated to cause a given effect (in this case a stated inhibition of growth i.e. 20, 30 or 50%).

In this experiment isobolograms were constructed, and cells were treated with various concentration combinations of each drug, and the effect on growth inhibition observed. Combinations of the drug concentrations which achieved the stated growth inhibition were plotted on the relevant isobologram (the combinations are described as experimental data in the figures) to ascertain whether they acted sub-additively, additively or supra-additively.

In each of the figures shown the experimental data lie in the area of sub-additivity, but relatively close to the envelope of additivity, probably indicating that both agents act independently. The proximity of the experimental data to the envelope of additivity would suggest that they do not negatively interact with one another.

Figure 49.

Experiments to establish a possible synergistic mode of action between temozolomide and methyl methanesulphonate.

Raji and GM892A cells were treated with a range of concentrations of either temozolomide or methyl methanesulphonate, and the effects on cell population growth determined.

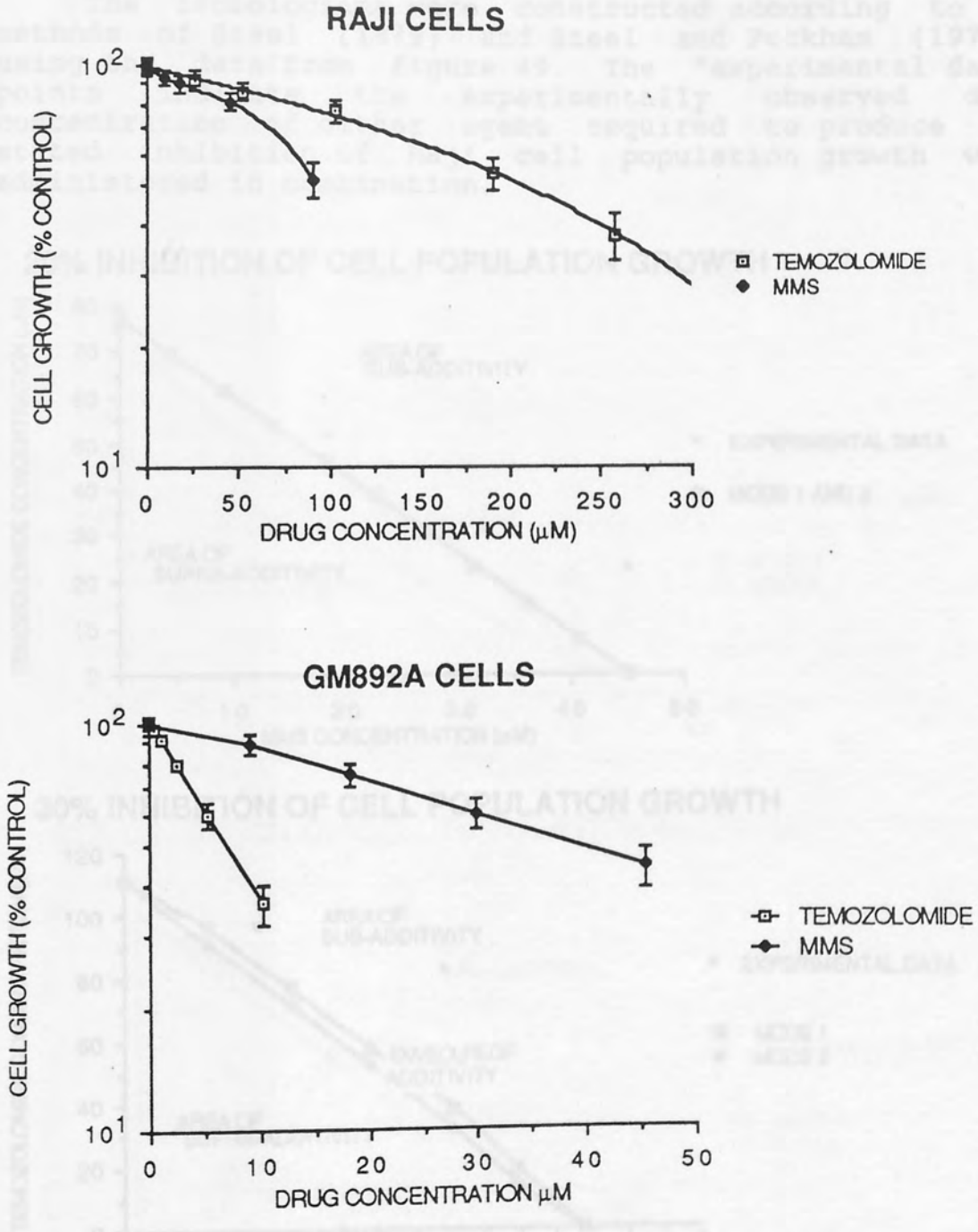


Figure 50.

The construction of isobolograms to predict the concentrations of temozolomide and methyl methane-sulphonate required to cause either a 20% or 30% inhibition of Raji cell population growth when administered in combination.

The isobolograms were constructed according to the methods of Steel (1979) and Steel and Peckham (1979), using the data from figure 49. The "experimental data" points indicate the experimentally observed drug concentration of either agent required to produce the stated inhibition of Raji cell population growth when administered in combination.

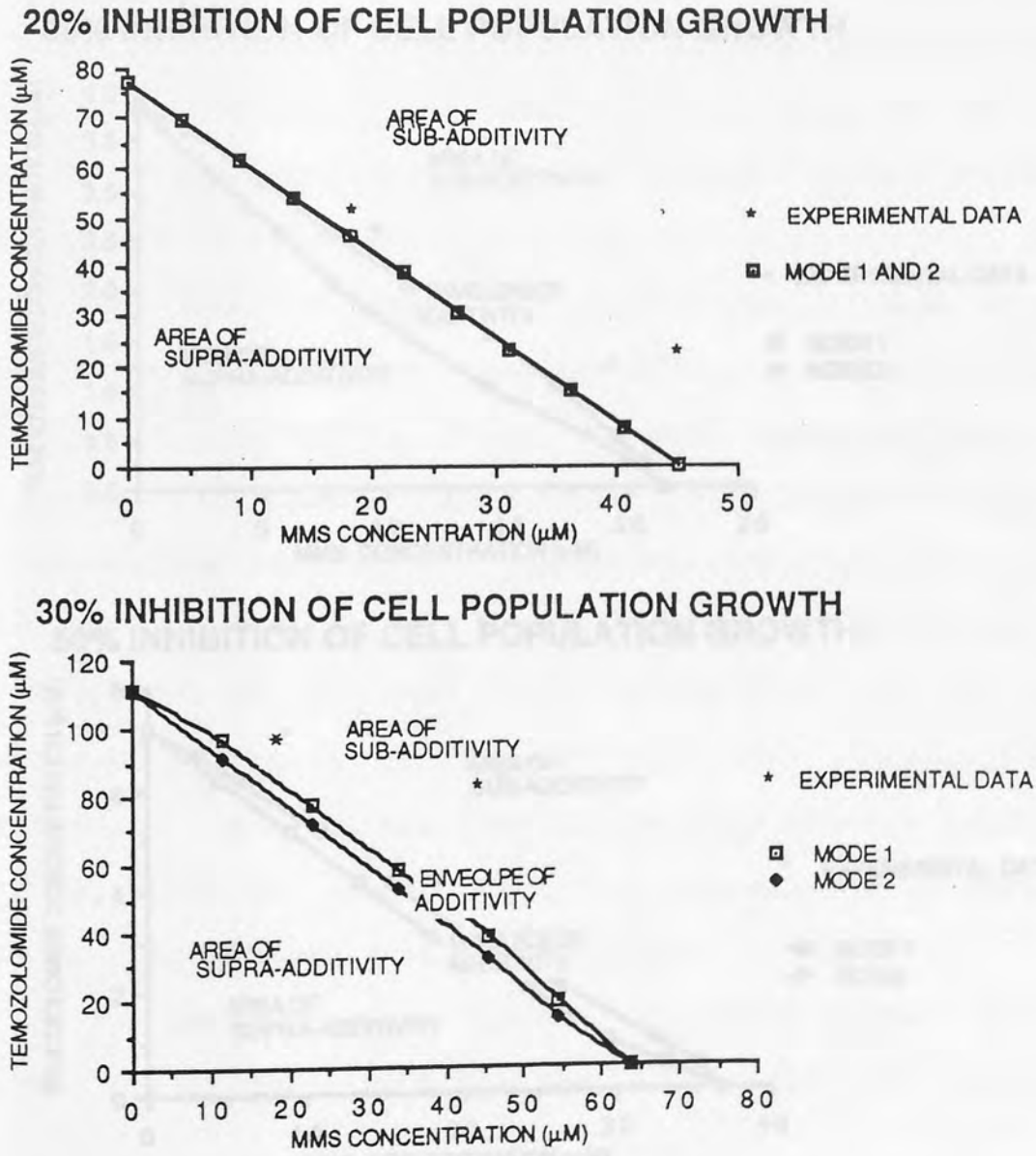
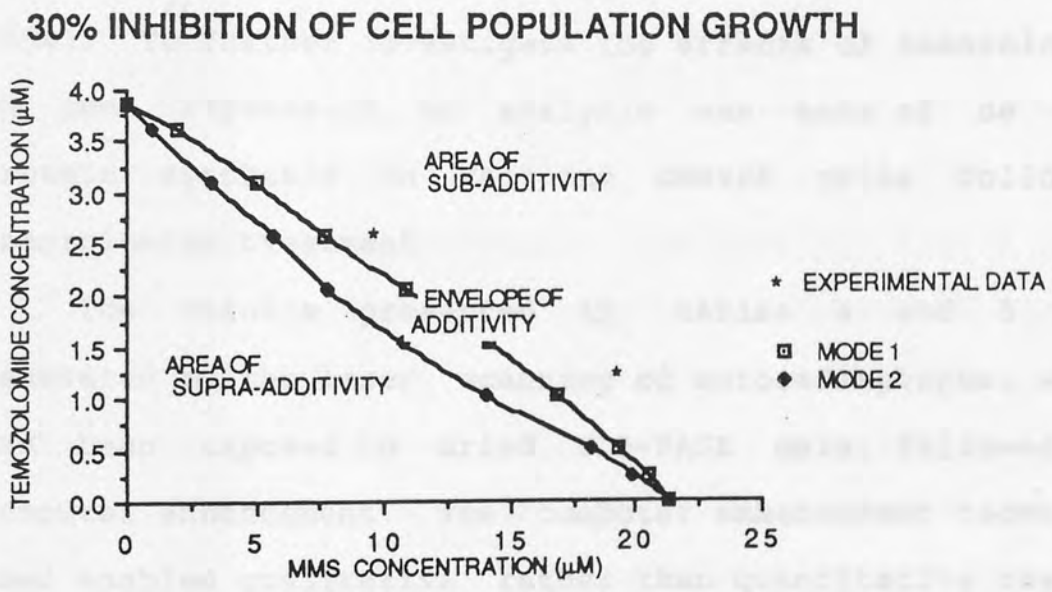
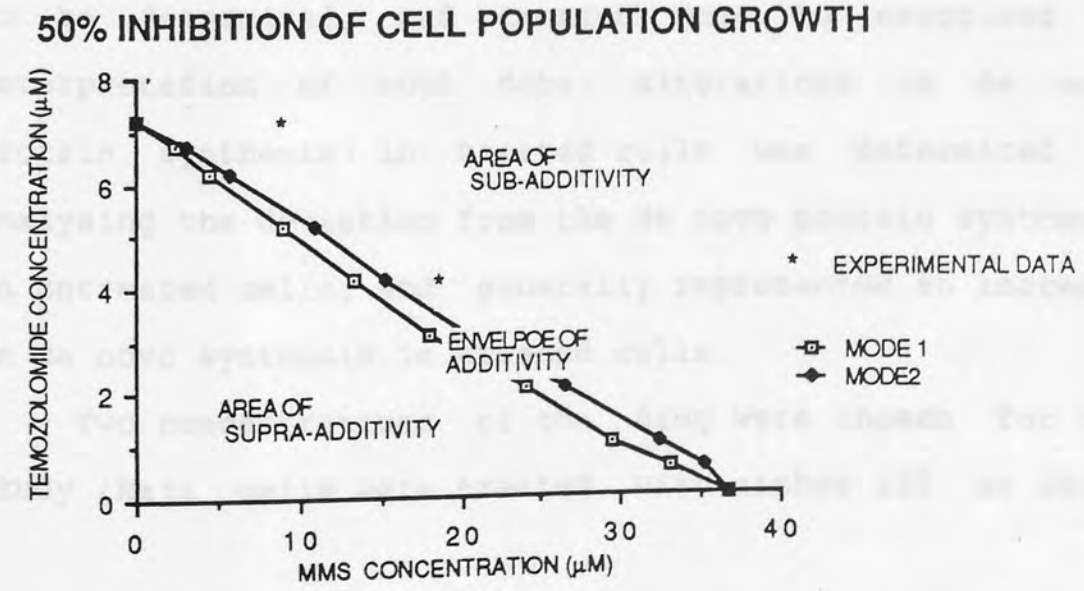


Figure 51.

The construction of isobolograms to predict the concentrations of temozolomide and methyl methanesulphonate required to cause either a 30% or 50% inhibition of GM892A cell population growth when administered in combination.

The isobolograms were constructed according to the methods of Steel (1979) and Steel and Peckham (1979), using the data from figure 49. The "experimental data" points indicate the experimentally observed drug concentration of either agent required to produce the stated inhibition of GM892A cell population growth when administered in combination.





4.12 Analysis by SDS-PAGE of de novo protein synthesis in Raji and GM892A cells treated with temozolomide.

It has been recently demonstrated that temozolomide, but not CCRG 82019, induced both differentiation and hypomethylation in the K562 human erythroleukaemia cell line (Tisdale, 1985, Tisdale, 1986), and was also shown to cause hypomethylation in Raji and GM892A cells. DNA methylation is thought to play an important role in the governing of gene function and expression, as well as differentiation (Razin and Riggs, 1980, Razin and Cedar, 1984). To further investigate the effects of temozolomide on gene expression, an analysis was made of de novo protein synthesis in Raji and GM892A cells following temozolomide treatment.

The results presented in tables 4 and 5 were generated by the laser scanning of autoradiographs, which had been exposed to dried SDS-PAGE gels, followed by computer enhancement. The computer enhancement technique used enabled qualitative rather than quantitative results to be determined, and caution must be exercised in interpretation of such data. Alterations in de novo protein synthesis in treated cells was determined by analysing the deviation from the de novo protein synthesis in untreated cells, and generally represented an increase in de novo synthesis in treated cells.

Two concentrations of the drug were chosen for the study (Raji cells were treated with either 125 or 250μM

temozolomide and GM892A cells with either 50 or 100μM temozolomide), in an attempt to determine possible dose-related changes in de novo protein synthesis. The data presented is a summary of two experiments.

Five bands (representing single proteins or mixtures of proteins) have apparently been altered in Raji cells treated with temozolomide in both experiments, though not identically. All the changes are of proteins of relatively low molecular weight (between about 35 and 54 kD), and in most instances occur between 72 and 96hr after drug treatment.

In contrast, four changes were observed in the treated GM892A cells which correlated well between the two experiments, and these generally occurred between 24 and 72hr after drug administration. Of particular note is an 80kD band which appeared to be markedly increased for the period 24 and 120hr after drug treatment. Furthermore the protein changes appeared to be of relatively larger proteins than that seen in Raji cells. The techniques used did not allow for a further, finer determination of differential effects of temozolomide on de novo protein synthesis in the two cell lines utilised for the study.

Table 4.

Apparent changes in de novo protein synthesis in Raji cells treated with temozolomide.

Raji cells were treated with temozolomide at a concentration of either 125uM or 250uM, and de novo protein synthesis assessed. The table indicates the approximate molecular weights of proteins which had apparently altered, and the time(s) after drug addition at which the changes had been observed.

Experiment a.

m.w. of proteins changed (kD)	Time (hrs) when changes observed.
35	72/96
36.5	120
43	72/96/120
47	72/96
54	96/120

Experiment b.

m.w. of proteins changed (kD)	Time (hrs) when changes observed.
35	72/96
36.5	72/96/120
43	24/72/96
≈ 45/47	24/72/96
50.5	96
54	96
56.5	96

Table 5.

Apparent changes in de novo protein synthesis in GM892A cells treated with temozolomide.

GM892A cells were treated with temozolomide at a concentration of either 50uM or 100uM, and de novo protein synthesis assessed. The table indicates the approximate molecular weights of proteins which had apparently altered, and the time(s) after drug addition at which the changes had been observed.

Experiment a.			Experiment b.	
m.w. of proteins changed (kD)	Time (hrs) when changes observed.		m.w. of proteins changed (kD)	Time (hrs) when changes observed.
			45/47	48/96/120
			≈ 50.5/53	24/48
			60	24/48
76/78	96/120		80	24/48/72/96/120
92	72		96	24/48
120	72		118	24
127	72		126	24/48
			135	96
			169	24
			188	24

4.13 Analysis by two-dimensional electrophoresis of de novo protein synthesis in Raji and GM892A cells treated with temozolomide.

The technique of SDS-PAGE yields limited information concerning de novo protein synthesis, and in particular is unable to distinguish whether a single protein or a group of proteins of similar molecular weight have been affected. For this reason two-dimensional electrophoresis was performed in an attempt to more specifically identify individual protein changes by distinguishing between proteins by both isoelectric focussing and molecular weight.

A greater resolution between proteins was obtained by two-dimensional electrophoresis, but the effects on de novo synthesis could not be satisfactorily determined. This was mainly due to the insufficient specific activity of the ^{35}S-methionine label in the newly synthesised proteins. This may be possibly remedied by incubating the cells in a medium containing a greater specific activity of ^{35}S-methionine label.

4.14 The effects of temozolomide and CCRG 82019 on the Raji and GM892A cell cycle.

The effects of both temozolomide and CCRG 82019 on the cell cycle progression of the Raji and GM892A cell lines was examined by means of flow cytometric analysis to elucidate more clearly the point in the cell cycle at which these agents act, and to determine any differences.

Flow cytometry provides a rapid method of assessing the effects of drugs on the cell cycle. The results are presented as histograms of cellular DNA content (figures 52-57), which generally reflects the proportion of the cell population at a given stage of the cell cycle, and is measured by analysing the amount of the fluorochrome, propidium iodide, bound to the DNA of each individual cell in the population.

The concentration of drugs chosen for this study were that which caused an 80% inhibition of cell population growth, in order to enhance, and facilitate the observation of the effects on the cell cycle (ie Raji cells were treated with either 2.06mM temozolomide or 2.88mM CCRG 82019, and GM892A cells were treated with either 103µM temozolomide or 1.92mM CCRG 82019). Control cells were treated with the concentration of DMSO which represented the volume of the vehicle administered to either cell line in which the temozolomide was dissolved, in order to determine whether the DMSO was responsible for any of the observed effects.

Neither temozolomide nor CCRG 82019 were observed to affect the cell cycle of either cell line until 24hr after drug treatment, at which point, both drugs were beginning to cause an apparent block of GM892A cells in late S/G_2M phase, with a diminution of cells in G_1 phase. By 48hr after treatment with either drug, there was an even greater decrease in the proportion of cells in G_1 phase, with much of the population being in S/G_2M phase, and with a marked increase in the amount of fluorescent debris.

Towards Raji cells, the effects of drug treatment are less apparent. This was due to the fact that DMSO alone caused a blockage of cells in G_1 phase, which became apparent 24-48hr after administration, thus making it difficult to separate the individual effects of either drug or vehicle. Following temozolomide treatment, however, there did appear to be a build up of cells in S/G_2M phase 24hr after treatment, and after 48hr there was both a diminution of the G_1 population with a a concomitant increase in cell debris which had retained fluorescence. CCRG 82019 similarly had little effect on the Raji cell cycle until 24hr after administration, at which point there was an increase in the S/G_2M population, which furthered increased after 48hr, again with an increase in flourescent debris.

Thus the observations made of the effects of drug treatment on the cell cycle of both the Raji and GM892A cell lines would appear to be a combination of the effects of the drug and that of the vehicle in which it was

dissolved. DMSO alone does not appear to markedly perturb the GM892A cell cycle, as compared to its effects on the Raji cell cycle, but this is simply explained by the fact that a much smaller volume of the vehicle was added to the GM892A control cells, as a much less was required to dissolve the temozolomide administered to the drug treated cells. Thus the observed effects of drug/vehicle treatment on the GM892A cell cycle was a more accurate reflection of the effects of drug alone.

Figure 52.

Flow cytometric analysis of the effects of temozolomide and CCRG 82019 on the Raji cell cycle 12h after drug treatment.

Raji cells were treated with either temozolomide at a concentration of 2.06mM (plot B) or CCRG 82019 at a concentration of 2.88mM (plot C), and to the control cells was added 20µl/ml DMSO (plot A). 12h after drug or vehicle addition, cells were fixed, stained and analysed by flow cytometry.

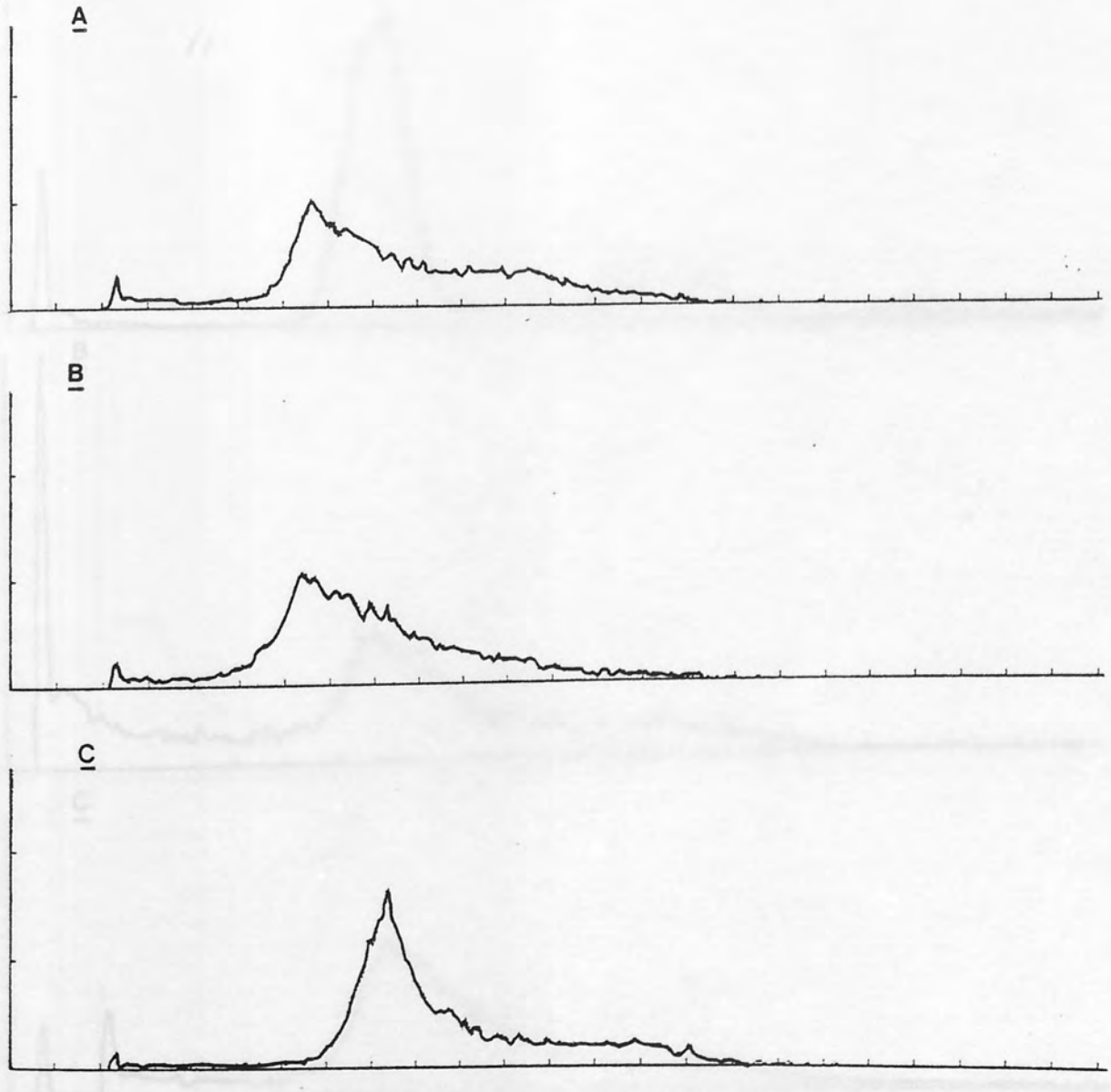


Figure 53.

Flow cytometric analysis of the effects of temozolomide and CCRG 82019 on the Raji cell cycle 24h after drug treatment.

Raji cells were treated with either temozolomide at a concentration of 2.06mM (plot B) or CCRG 82019 at a concentration of 2.88mM (plot C), and to the control cells was added 20µl/ml DMSO (plot A). 24h after drug or vehicle addition, cells were fixed, stained and analysed by flow cytometry.

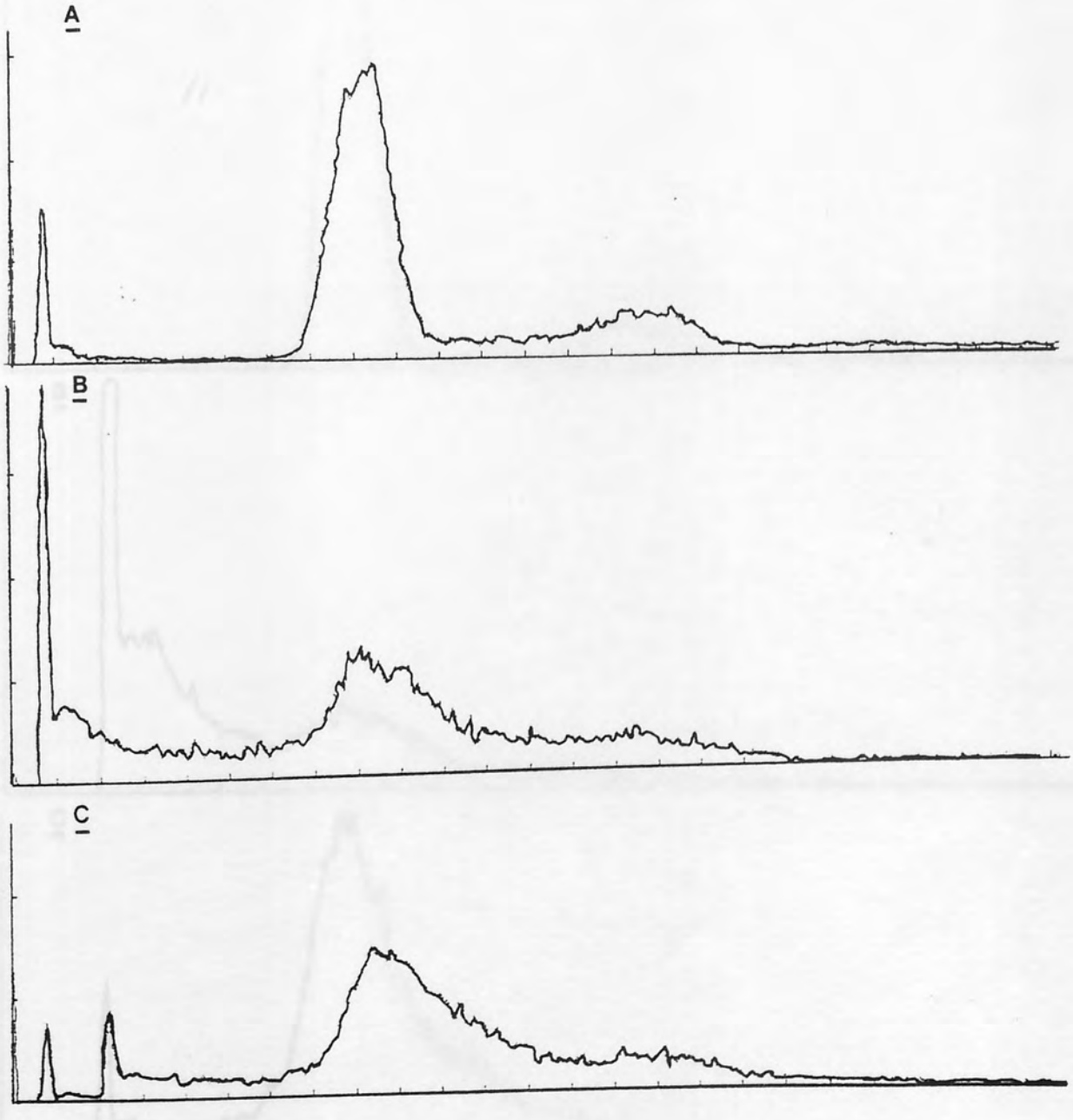


Figure 54.

Flow cytometric analysis of the effects of temozolomide and CCRG 82019 on the Raji cell cycle 48h after drug treatment.

Raji cells were treated with either temozolomide at a concentration of 2.06mM (plot B) or CCRG 82019 at a concentration of 2.88mM (plot C), and to the control cells was added 20µl/ml DMSO (plot A). 48h after drug or vehicle addition, cells were fixed, stained and analysed by flow cytometry.

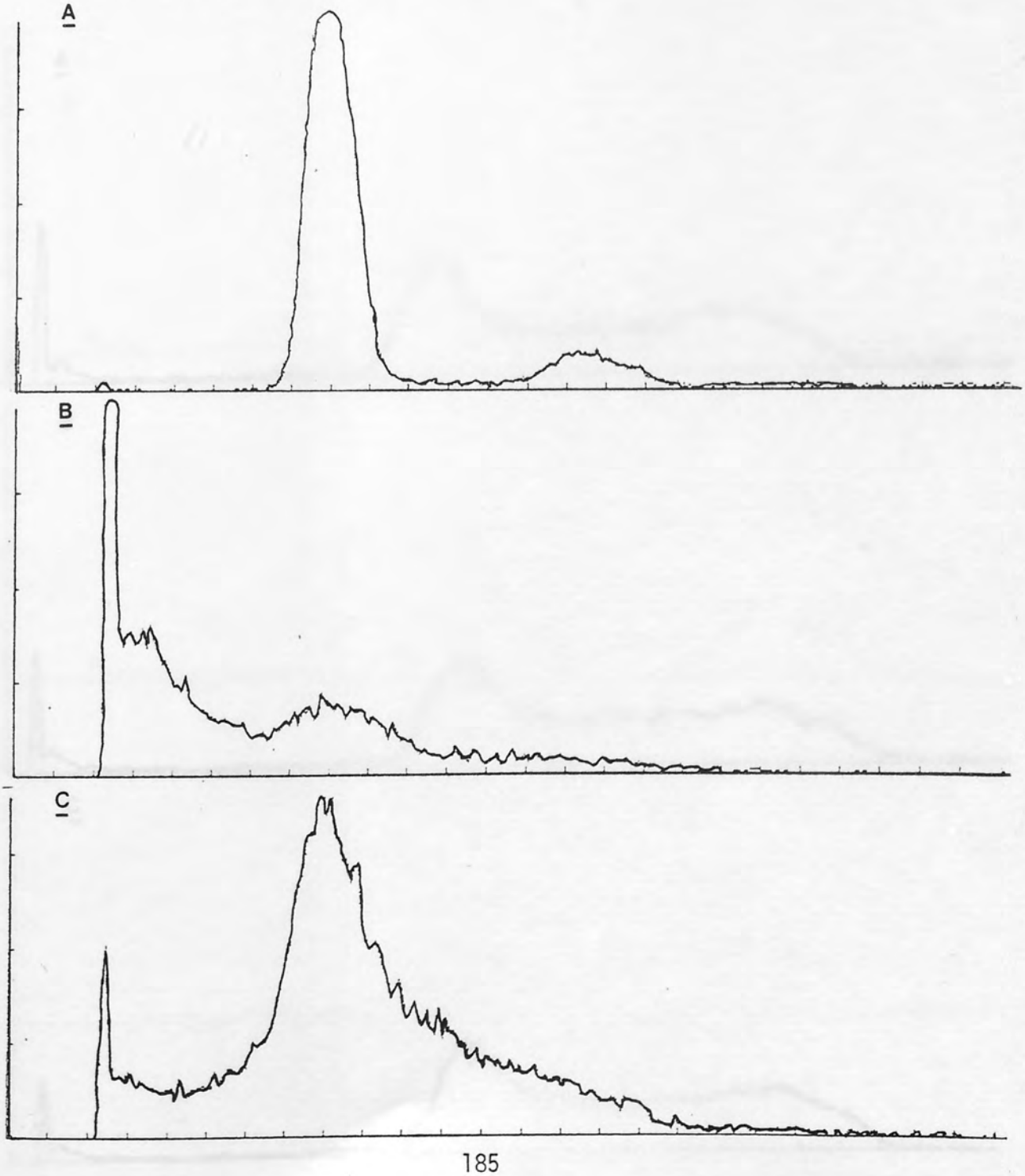


Figure 55.

Flow cytometric analysis of the effects of temozolomide and CCRG 82019 on the GM892A cell cycle 12h after drug treatment.

GM892A cells were treated with either temozolomide at a concentration of 103µM (plot B) or CCRG 82019 at a concentration of 1.92mM (plot C), and to the control cells was added 1µl/ml DMSO (plot A). 12h after drug or vehicle addition, cells were fixed, stained and analysed by flow cytometry.

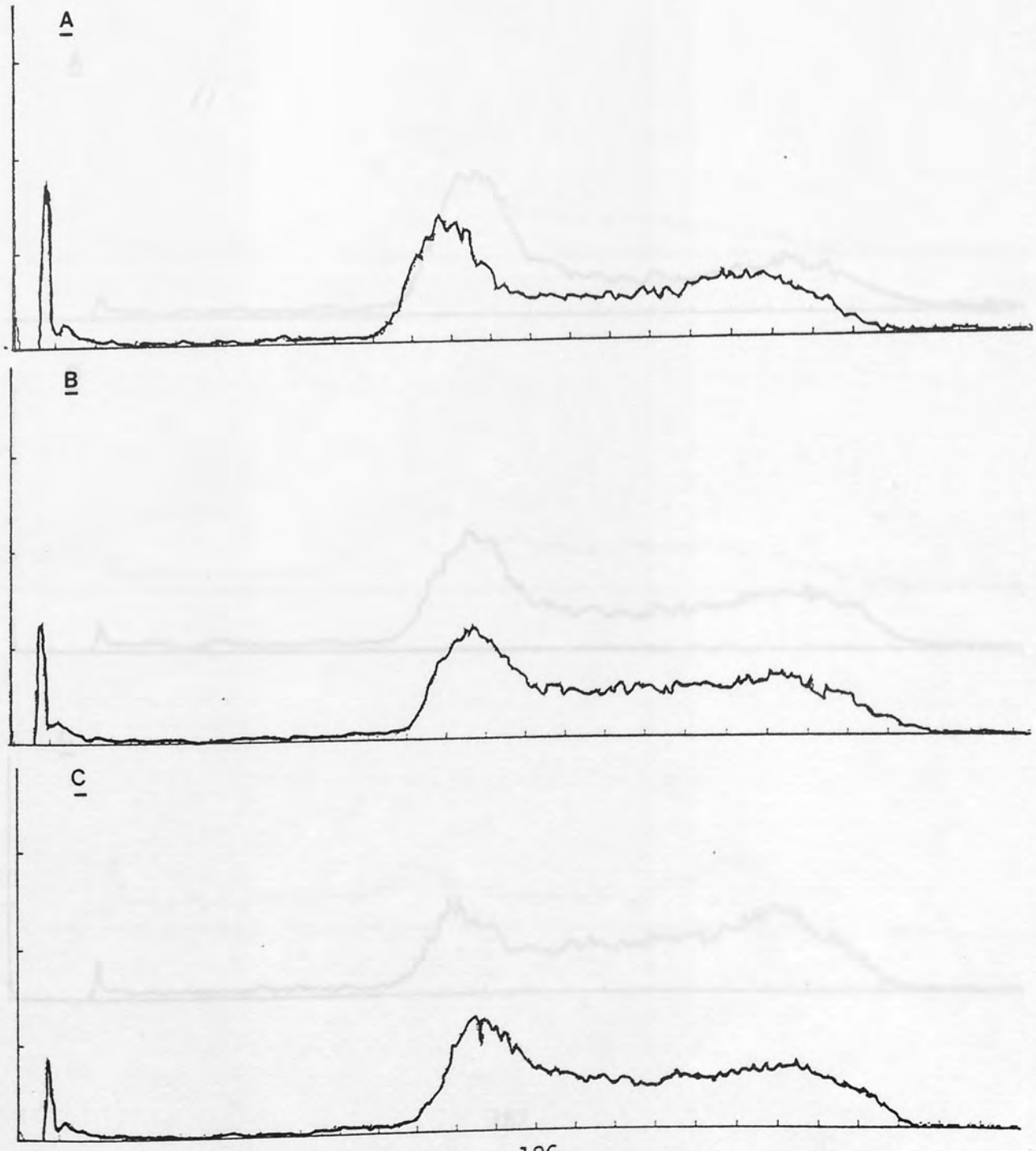


Figure 56.

Flow cytometric analysis of the effects of temozolomide and CCRG 82019 on the GM892A cell cycle 24h after drug treatment.

GM892A cells were treated with either temozolomide at a concentration of 103μM (plot B) or CCRG 82019 at a concentration of 1.92mM (plot C), and to the control cells was added 1μl/ml DMSO (plot A). 24h after drug or vehicle addition, cells were fixed, stained and analysed by flow cytometry.

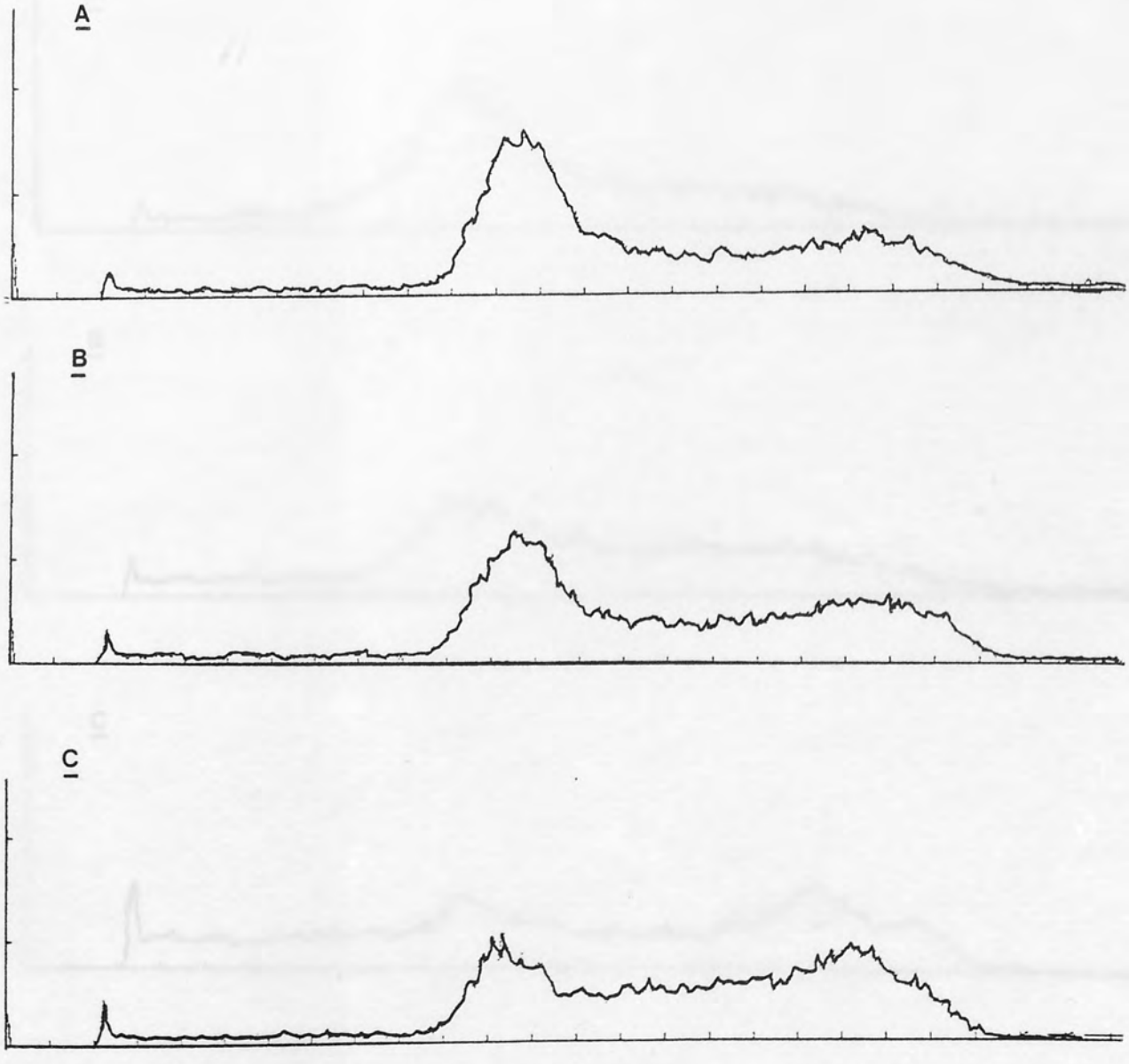
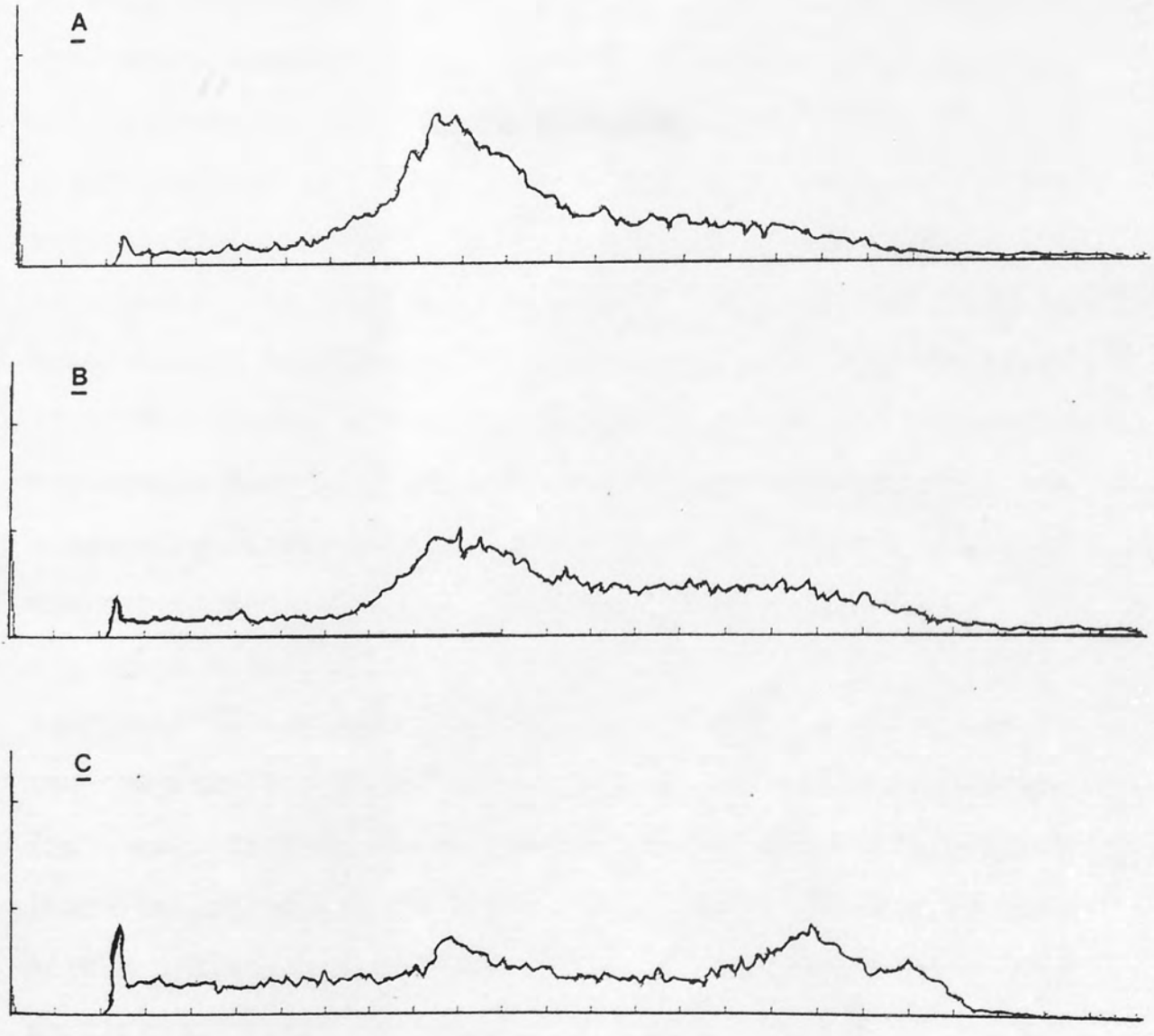


Figure 57.

Flow cytometric analysis of the effects of temozolomide and CCRG 82019 on the GM892A cell cycle 48h after drug treatment.

GM892A cells were treated with either temozolomide at a concentration of 103µM (plot B) or CCRG 82019 at a concentration of 1.92mM (plot C), and to the control cells was added 1µl/ml DMSO (plot A). 48h after drug or vehicle addition, cells were fixed, stained and analysed by flow cytometry.



5. DISCUSSION.

The imidazotetrazinones are a new group of antitumour agents which have demonstrated good activity against a range of murine tumours and human xenografts. (Stevens et al, 1984, Hickman et al, 1985, Fødstad et al, 1985). Chemical (Stevens et al, 1984) and biological studies (Horgan and Tisdale, 1984) suggest that these agents ring open under alkaline conditions to yield acyclic triazenes.

The work in this thesis was prompted by the observation that the chloroethyl and methyl imidazotetrazinone analogues are active as potential antitumour agents, while the ethyl and higher homologues are inactive in both in vivo (Stevens et al, 1984) and in vitro studies (Tisdale, 1987). This is similar to the structure activity relationship observed for the antitumour triazenes (Gibson, 1986). The cytotoxicity of mitozolomide has been attributed to its ability to cross-link DNA (Gibson, 1984b), whereas its methyl and ethyl analogues, temozolomide and CCRG 82019 respectively, are chemically incapable of forming such cross-links, in analogy to the triazenes (Gibson, 1984b).

Both mitozolomide and temozolomide exhibit a marked differential toxicity towards cells which are deficient in the repair protein O^6-alkylguanine-DNA alkyltransferase (Mer- cell lines) than to cells proficient in the protein (Mer+ cells), while for CCRG 82019 the difference is less marked (Tisdale, 1987). The correlation between the ability of cells to repair O^6-alkylguanine lesions and cytotoxicity has also been observed with both the

triazenes (Gibson et al, 1984a) and nitrosoureas (Scudiero et al, 1984). This suggests that alkylation at the O^6-position of guanine may be the primary reaction responsible for cytotoxicity, but it is not clear why the O^6-methylguanine should be a cytotoxic lesion, and the O^6-ethylguanine not.

For the purposes of this study two cell lines were chosen with differing sensitivities to the imidazotetrazinones: The Raji cell line which has been previously shown to be proficient in O^6-alkylguanine alkyltransferase, (O^6-AGAT) and the GM892A cell line which has been shown to be deficient (Harris et al, 1983, Tisdale, 1987).

Attempts were made to elucidate the differential effects of mitozolomide, temozolomide and CCRG 82019 on the two cell lines, and the ability of all three drugs to inhibit Raji and GM892A cell growth was investigated. It was found that GM892A cells were 8- and 25-fold more sensitive to mitozolomide and temozolomide, respectively, than were Raji cells. However CCRG 82019 demonstrated little differential toxicity between the two cell lines, and in GM892A cells was 6-fold less potent than temozolomide and 30-fold less than mitozolomide.

These results concur with those recently described by Tisdale (1987) who investigated the effects of all three agents on several cell lines which varied in their constitutive levels of O^6-AGAT. It was found that the toxicity of mitozolomide and temozolomide correlated well

with the level of O^6-AGAT in the cell: Cells with a low level of O^6-AGAT (such as GM892A) were found to be sensitive, while those with high levels were found to be relatively resistant. However the toxicity of CCRG 82019 was not found to correlate with the cellular levels of O^6-AGAT, as was also the case for the bifunctional alkylating agent chlorambucil.

O^6-AGAT may be depleted by incubation of cells with free O^6-alkylguanine base (thought to be a weak substrate for the protein) (Dolan et al, 1985) and this has been shown to sensitise Mer+ cells to cell killing by CNU (Dolan et al, 1985b, Dolan et al, 1986) and MNNG (Yarosh et al,1986). The Mer+ cells used in Tisdale's study (1987) were also sensitised to the cytotoxic effects of mitozolomide and temozolomide following depletion of O^6-AGAT (by incubation of cells with free O^6-alkylguanine base), but were not sensitised to the effects of CCRG 82019. The sensitisation of cells to mitozolomide and temozolomide by depletion of O^6-AGAT would implicate the O^6-guanine position to be a prime target for the cytotoxic action of these agents, but questions the role of this site in the cytotoxicity of CCRG 82019.

The role of O^6-AGAT in modifying the cytotoxic effects of mitozolomide and temozolomide, but not CCRG 82019, is further implicated by the shape of the growth inhibition curves. A shoulder on a curve may be thought to imply the necessity for depletion of a repair enzyme prior to maximum elicition of cytotoxicity. Against the

Raji cell line, all three agents exhibit a shoulder to their growth inhibition curves, whereas only CCRG 82019 has such a dose-response towards the GM892A cell line. This may indicate that the cytotoxic lesion(s) produced by CCRG 82019 is different from that produced by either mitozolomide or temozolomide.

As discussed previously (section 4.2), observations made on cell growth after drug treatment does not necessarily reveal whether a drug has had a cytotoxic effect or has merely caused a delay in cell growth. Attempts to assess cell viability by other methods proved unsuccessful for a variety of reasons.

The ability of cells to grow and form colonies in a soft-agar medium generally avoids the constraint of growth delay effects. Even though a variety of changes were made to the original agar-growth medium, neither cell line would grow to form colonies. This is perhaps not surprising, as there have been no reports in the literature of either cell line being cloned. Raji cells grow in agar-free medium as clumps of cells, but such groups tend to be fragile, and rapidly segregate if disturbed, and thus could not be considered as a colony forming assay.

The ability of cells to exclude trypan blue is frequently used and cited as a measure of cell viability. The assay is based on the premise that only the cell membrane of viable cells is competent to exclude the dye. However, an estimation of viability may be artificially

high if the drug treatment results in a cytostatic effect. Furthermore, changes in the ability of the membrane to exclude the dye may take place some time after the loss of viability, or non-viable cells may rapidly disintegrate, which would also affect the estimate of viability. Preliminary experiments were performed to ascertain the usefulness of trypan blue exclusion in the assessment of cell viability. As reported earlier, apparent cell viability remained high after imidazotetrazinone treatment, and did not appear to be either time or dose related, and was not considered further.

Rhodamine 123, a cationic fluorescent dye, has been shown to be selectively accumulated by mitochondria in living cells (Johnson et al, 1980). The specific uptake of the dye into mitochondria depends upon the maintenance of a high mitochondrial membrane potential (Johnson et al, 1981). Furthermore the loss of rhodamine 123 uptake was found to correlate with the loss of clonogenic ability, and occurred prior to observed changes in cell membrane permeability (as indicated by trypan blue staining) (Bernal et al 1982).

Attempts were made to assess viability by using this technique, but it proved impossible to distinguish between viable and non-viable cells with the facilities available. However, it is thought that monitoring of the dye uptake by flow-cytometric analysis would have greatly enhanced the chances of success with this assay.

The differential effects of a group of drugs in a

given cell line, or the effect of one drug in a variety of cell lines may be the result of differences in the rate of drug uptake. Horgan and Tisdale (1985) have previously shown that uptake of mitozolomide into TLX5 murine lymphoma cells was rapid, with equilibrium being reached within one minute at 37^{o}C, but took 10min to reach at 4^{o}C, and the cell:medium concentration remained constant at approximately 1.3 for 60min. The uptake of mitozolomide, temozolomide and CCRG 82019 into both Raji and GM892A cells at 4^{o}C was examined in an attempt to assess more accurately whether differences in cytotoxicity related to differences in drug penetration into cells.

Equilibrium was reached, for all three agents, some 4 to 8min after drug addition, and the cell:medium distribution remained constant for at least 30min, at approximately 1.3 in Raji cells and 1.8 in GM892A cells. This was in agreement with the results described by Horgan and Tisdale (1985). The only observed discrepancy between the uptake of drugs was the inexplicably lower equilibrium concentration attained by temozolomide in GM892A cells, as compared to mitozolomide and CCRG 82019. However such a difference was not sufficient to explain the observed differential toxicities of each drug.

The principal site for the cytotoxic effects of the imidazotetrazinones is thought to be nuclear DNA. In an attempt to define the mode of action of these agents more closely, observations were made of the incorporation of radiolabelled thymidine, uridine and leucine into cellular

acid-insoluble material. However the incorporation of precursors into cellular macromolecules may be influenced by drug-mediated effects not directly related to the effect on nucleic acids. Changes in the observed level of incorporation may be due to changes in the concentration of free precursor within the cell (ie the pool size) (Russell and Partick, 1980, Bianchi et al, 1983). Furthermore conclusions drawn from pool measurements may be complicated by the considerable variation of dNTP pool sizes throughout the cell cycle (Reichard, 1987).

The incorporation of ^{3}H-thymidine into the acid insoluble material of both Raji and GM892A cells was found to be inhibited, in a dose-dependent manner, maximally 2hr after treatment with each imidazotetrazinone. With respect to the drug concentration required to cause inhibition, mitozolomide was the most potent agent, and CCRG 82019 the least potent. Furthermore there was a recovery towards control levels of thymidine incorporation in Raji cells 24hr after drug treatment, the recovery being most pronounced in the CCRG 82019 and temozolomide treated cells, and least in the the mitozolomide treated cells. In comparison recovery towards control levels of ^{3}H-thymidine incorporation was much less pronounced in the GM892A cells.

The observed decrease in thymidine incorporation in Raji cells could not be accounted for by changes in pool size, which increased in a time and dose-dependent manner. Mitozolomide was apparently the most effective drug in

eliciting pool size increases, with CCRG 82019 the least effective.

In GM892A cells mitozolomide caused an increase in ^{3}H-thymidine pool size in a time and dose-dependent fashion, similar to that observed in Raji cells. However, the observed decrease of ^{3}H-thymidine incorporation into the acid-insoluble material of GM892A cells over the first 4hr after treatment with either temozolomide or CCRG 82019, could partly be explained by an observed concomitant decrease in the ^{3}H-thymidine pool size. By 8hr after drug treatment there was a virtual return to control level of pool size, and after 24hr the highest concentration of temozolomide and CCRG 82019 caused, respectively, a 12-fold and 4.5-fold increase in pool size, whereas there was virtually no recovery towards control levels of incorporation in GM892A cells treated with either drug.

Mitozolomide caused a marked inhibition of ^{3}H-uridine incorporation into the acid-insoluble material of GM892A cells that mirrored the inhibition of ^{3}H-thymidine incorporation, but which could not be accounted for by changes in the pool size. Inhibition of ^{3}H-uridine incorporation into Raji cells after mitozolomide treatment was less marked than that of ^{3}H-thymidine incorporation, and less than that observed in GM892A cells. Whereas temozolomide caused a similar depression of ^{3}H-uridine incorporation into the acid-insoluble material of both cell lines, which again could not be accounted for by

changes in pool sizes, CCRG 82019 had virtually no effect on either the pool size or level of ^{3}H-uridine incorporation in either cell line.

None of the imidazotetrazinones used in this study had a marked effect on either the pool size or level of incorporation of ^{3}H-leucine into the acid-insoluble material in either cell line.

These results would appear to be in direct contrast to those observed by Horgan and Tisdale (1984), where mitozolomide was reported not to have any effect on the levels of ^{3}H-thymidine or ^{3}H-uridine incorporated into cellular acid-insoluble material 2hr after treatment, but with an inhibition which gradually appeared over a 24hr period, with a concomitant decrease in pool size. The results in this study would appear to be more closely allied to the effects of BCNU on incorporation of precursors recorded by Horgan and Tisdale. BCNU was found to cause a marked inhibition of both ^{3}H-thymidine and ^{3}H-uridine incorporation after 2hr (as also observed by Connors and Hare, 1975), with no recovery 24hr after drug treatment.

The explanation for such a discrepancy is not immediately apparent: The method used for assessing incorporation and pool sizes was essentially the same. However whereas in this study two human-derived cell lines were used, Horgan and Tisdale used the TLX5(S) murine lymphoma. Furthermore for some time points TLX5(S) cells freshly obtained from mice were utilised, while for other

time points cells maintained in vitro were used, and these differences may be the source of the observed discrepancies.

Interpretation of these results is made more difficult due to the observed changes in radiolabelled precursor pool size. Whereas an apparent decrease in the incorporation of a precursor into acid-insoluble material may be explained by a concomitant decrease in pool size, an increase in in the radiolabelled precursor pool size would increase the specific activity of the pool, which in turn may cause an actual inhibition of precursor incorporation to be observed as a return to or an increase above the control levels of incorporation. Thus the observed recovery of ^{3}H-thymidine incorporation toward control levels 24hr after treatment may have been caused by increases in the specific activity of the pool size, and may not reflect a true recovery of incorporation.

The cause of the observed inhibition of ^{3}H-thymidine and ^{3}H-uridine incorporation and changes in pool sizes after drug treatment is speculative. It is unlikely to be due to an indiscriminate interaction with the cell membrane affecting precursor uptake, as there was no apparent effect on leucine pool sizes after drug treatment. It is also unlikely to be be due to a drug-derived carbamoylating species, as exogenously added 2-chloroethyl isocyanate was not found to affect either ^{3}H-thymidine or ^{3}H-uridine incorporation into the acid-insoluble material of TLX5(S) cells (Horgan and Tisdale,

1984), though a carbamoylating species generated intracellularly may be responsible.

Alterations in ^{3}H-thymidine and ^{3}H-uridine pool sizes may reflect a disruption in the processes of nucleotide anabolism which may be responsible for the observed increases in the uptake of radiolabelled precursors (Bianchi et al, 1983), or it may reflect alterations in the activities of the membrane kinases responsible for the uptake of precursors.

The rapid inhibition of ^{3}H-thymidine and ^{3}H-uridine incorporation, and later recovery, may indeed reflect an inhibition of DNA and RNA synthesis caused by direct DNA damage with subsequent repair. Of note is that ^{3}H-thymidine incorporation recovers towards control levels 24hr after drug treatment in the Raji, O^{6}-alkylguanine repair proficient cell line, whereas there is little increase in levels of ^{3}H-thymidine incorporation in the GM892A, O^{6}-alkylguanine repair deficient cell line over the 24hr period.

To try and clarify the effect of changes in the precursor pool size on the observed levels of precursor incorporation, the ratios of incorporation to pool size (the I/P ratio) were calculated and are shown in figures 28-36 (pages 138-147).

In Raji cells the relatively low I/P ratio for ^{3}H-thymidine was mainly due to large increases in the pool size, whilst the level of incorporation remained depressed. Consequently the I/P ratio of ^{3}H-thymidine was

decreased after treatment with all three drugs in a dose-dependent fashion, with mitozolomide causing the greatest decrease, and CCRG 82019 the least. In GM892A cells mitozolomide caused a similar effect on the I/P ratio of ^{3}H-thymidine as seen in the Raji cells. However, temozolomide and CCRG 82019 caused an initial concomitant decrease in ^{3}H-thymidine incorporation and pool size, but whilst the level of incorporation remained static, there was a marked increase in the ^{3}H-thymidine pool size.

The effects of each drug on the ^{3}H-uridine I/P ratio in Raji cells was much less dramatic than the effects on the ^{3}H-thymidine I/P ratio. The effects of 20μM mitozolomide on the level of incorporation of ^{3}H-uridine was mirrored by changes in the pool size (thus the I/P ratio remained close to unity). The higher concentration of mitozolomide caused the I/P ratio to decrease due to a large increase in the pool size. Temozolomide and CCRG 82019 initially caused a decrease in ^{3}H-uridine incorporation, which recovered with time, but the I/P ratio returned to near unity with time as there was a much less marked effect on the pool size. In GM892A cells mitozolomide caused a dose-dependent decrease in the ^{3}H-uridine I/P ratio, which was constant over a 24hr period and arose mainly from the maintenance of a depression in the levels of ^{3}H-uridine incorporation. The ^{3}H-uridine ratio after temozolomide and CCRG 82019 treatment remained much closer to unity over the 24hr, and the overall effects were independent of the drug

concentration. The marked decrease in the ^{3}H-uridine I/P ratio seen in the GM892A cells treated with temozolomide was mainly due to a marked increase in the uridine pool size.

The effects of mitozolomide, temozolomide and CCRG 82019 on the ^{3}H-leucine I/P ratio were marginal, with the ratio remaining close to unity over the 24hr period of study, as none of the agents markedly affected either the level of incorporation of ^{3}H-leucine or its pool size.

Alterations in the precursor pool size may affect the observed level of radiolabelled precursor incorporation by increasing the specific activity of the radiolabelled pool. Observations made of the ratio of precursor incorporation to pool size in this study unfortunately do not entirely clarify whether the observed changes in precursor incorporation are real or artefactual.

In an attempt to define the reactivity of the imidazotetrazinones towards macromolecules more closely, radiolabelled drugs were incubated with isolated macromolecules. It was found that the extent of reaction of mitozolomide, temozolomide and CCRG 82019 with calf thymus DNA, calf liver RNA and bovine serum albumin increased in a concentration dependent manner. Of the three drugs investigated, temozolomide was the most reactive towards both DNA and RNA, and CCRG 82019 the least reactive, whereas mitozolomide was more reactive towards protein than were either temozolomide or CCRG 82019. This is perhaps a somewhat surprising result, given that

mitozolomide was found to be the most toxic agent towards tissue culture cells. Furthermore, temozolomide and mitozolomide were found to be 2- to 3-fold more reactive towards calf liver RNA than calf thymus DNA.

An investigation was made of the specific base alkylations formed after treatment of calf thymus DNA with temozolomide and CCRG 82019. It was found that the greatest proportion of the adducts formed after temozolomide treatment (70%) was 7-methylguanine, while 7-ethylguanine represented only 24% of the bases formed after CCRG 82019 treatment. The amount of 3- alkyladenine formed, as a percentage of the total bases, after treatment with either drug was similar, with 9% 3-methyladenine formed and 5% 3-ethyladenine formed. The relative proportion of O^6-alkylguanine formed, after either temozolomide or CCRG 82019 treatment, was almost identical, with O^6-methylguanine and O^6-ethylguanine representing just over 5% of the total bases formed. However at the equimolar concentrations used to treat the calf thymus DNA, temozolomide alkylated to a 7-fold greater extent than CCRG 82019.

These results are in close accord with those reported by other workers (for example, Sun and Singer, 1975, Beranek et al, 1980, Lawley and Warren, 1981, Warren, 1984), who have measured the bases formed in isolated DNA treated with either radiolabelled MNU or ENU. It is thought that in line with the results described by these authors, the largest proportion of of the specific bases

formed after treatment of calf thymus DNA with CCRG 82019 will be as ethylphosphotriesters, and may be predicted to account for about 50-60% of the total bases. The amount of phosphotriesters formed after treatment of isolated DNA with MNU is generally reported to represent about 12% of the total bases formed, and it would seem reasonable to assume that such a figure would represent the amount of phosphotriesters formed after treatment of calf thymus DNA with temozolomide.

The cellular reactivity of mitozolomide, temozolomide and CCRG 82019 towards macromolecules in Raji and GM892A cells was somewhat different from that with isolated macromolecules. More alkyl groups remained bound to the DNA, RNA and protein of both Raji and GM892A cells after mitozolomide treatment than after either temozolomide or CCRG 82019 treatment. The number of alkyl groups remaining bound to DNA and proteins were similar for both temozolomide and CCRG 82019. This may in part explain the superior cytotoxicity of mitozolomide over the other two agents, but does not explain the superior toxicity of temozolomide over CCRG 82019.

The number of alkyl groups remaining bound to the DNA in GM892A cells after treatment with each of the three drugs were greater than those remaining bound to the DNA in Raji cells. This may partially correlate with the superior ability of Raji cells to remove O^6-alkylguanine from DNA, although the adduct represents only a small proportion of the lesions formed.

There are a number of factors which may explain the observed apparent differential alkylation of isolated macromolecules, and macromolecules in cells.

If the specific bases formed in cellular DNA after treatment with temozolomide or CCRG 82019 are of a similar proportion to the total alkylation to those found after treatment of calf thymus DNA with these agents, then this may further explain the observed discrepancies between the the total extent of alkylation of isolated and cellular DNA, and the total extents of DNA alkylation observed in Raji and GM892A cells.

The extent of alkylation observed in cells will be a function of both the rate of alkylation and the rate of repair. The only reported difference in the repair capacities of the two cell lines used is in their respective capacities to repair O^6-alkylguanine lesions. The observation that the number of alkyl groups remaining bound to the DNA of GM892A cells was greater than those remaining bound to the DNA of Raji cells for all three imidazotetrazinones will presumably have been in part influenced by such a discrepancy in O^6-alkylguanine repair capabilities. However if the observations made of the bases formed with isolated DNA can be extrapolated to the case of the cellular DNA, the amount formed as a proportion of the total alkylation by either agent should be similar, and as such would not fully explain the differences between the total extent of alkylation, and the number of alkyl groups remaining bound to cellular

DNA. It may, however, explain the difference between the number of alkyl groups remaining bound to the DNA of the two cell lines.

If the predominant lesion caused by temozolomide in cellular DNA is 7-methylguanine (in analogy to the formation of bases in isolated DNA), then this may be depleted from cellular DNA by both spontaneous depurination and excision by the action of a glycosylase; 3-methyladenine would similarly be depleted from cellular DNA (Medcalf and Lawley, 1981).

However the predicted predominant lesion produced by CCRG 82019 in cellular DNA is ethylphosphotriesters. It is thought that the phosphotriester lesion is not subjected to repair in mammalian cells (Saffhill et al, 1985)d19, and thus may be expected to be as persistent in cellular DNA as in isolated DNA.

Thus, whereas after temozolomide treatment of cells, the predominant lesions expected to have been formed are subjected to constant loss from the DNA, the majority of lesions formed after CCRG 82019 treatment persist. This may be the major cause of the anomalies observed between the number of alkyl groups remaining bound to calf thymus DNA and cellular DNA after drug treatment.

There are a number of other factors which may contribute to cause such differences. For example, it has been reported that the half-life of mitozolomide considerably shortens with an increase in pH (Goddard, 1985) and thus variations in the medium pH and also local

intracellular pH may account in part for the observed differences between the alkylation of macromolecules in vitro and those in cells.

Marushige and Marushige (1983a) have reported that the extent of alkylation of DNA in the form of chromatin by MNU was about 50% less than free DNA, and alkylation of free DNA by ENU was about 4- to 5-fold greater than that of chromatin DNA. MNU was found to alkylate both free and chromatin DNA to about a 15-fold greater extent than ENU. The effects of nuclear proteins may thus also account in part for the observed differences between the alkylation of isolated and cellular DNA by imidazotetrazinones.

It has also been shown previously that the ability of O^6-AGAT to repair alkyl lesions depends on the type of lesion formed, with methyl adducts being repaired much faster than ethyl or higher adducts (Yarosh, 1985), and this may also influence the total numbers of adducts remaining bound to DNA in cells. Any one, or combinations of these factors may therefore be responsible for the discrepancies observed between the extent of alkylation of macromolecules and the numbers of alkyl groups remaining bound to macromolecules in cells.

To further investigate the effects of drug treatment on DNA function, the ability of DNA from imidazotetrazinone treated cells to support E. coli RNA polymerase activity was examined. It was however found that mitozolomide, temozolomide and CCRG 82019 did not appear to affect the ability of the treated DNA to act as

a template for the RNA polymerase.

Tisdale (1986) has previously shown that the ability of calf thymus DNA to support RNA polymerase activity was reduced by pre-treatment with temozolomide, and to a lesser extent CCRG 82019. This was in agreement with the observations made by Marushige and Marushige (1983b), that the ablility of MNU and ENU treated calf thymus DNA to support E. coli RNA polymerase activity was inhibited, with MNU having the more pronounced effect, based on the effects of equimolar concentrations of drugs.

The observations made in this study, however, are not necessarily in conflict with those of Tisdale (1986) or Marushige and Marushige (1983b). Both studies treated the calf thymus DNA with mM concentrations of drugs (1mM and greater), whereas the maximum concentration used in this study for any of the drugs was 360μM. Furthermore, as discussed earlier, the total extent of alkylation of calf thymus DNA does not necessarily reflect the amount of alkyl groups remaining bound to DNA in cells.

It has also been shown that the incubation conditions used for the RNA polymerase assay may affect the observed degree of inhibition. For example, Marushige and Marushige (1983b) showed that E. coli RNA polymerase was inhibited to a greater extent in their assay conditions than was wheat germ RNA polymerase under the same conditions. The fact that no significant inhibition of polymerase activity was observed in this study may reflect that the enzyme used in the assay is relatively

insensitive to the DNA alkylation caused by the action of the imidazotetrazinones, but this would seem unlikely, and no attempts were made to further optimise the assay conditions.

In a further attempt to elucidate the possible effects of the imidazotetrazinones on nuclear functioning, the nuclear proteins were extracted from treated cells, and the DNA polymerase therein was assayed.

None of the imidazotetrazinones had a consistently significant effect on DNA polymerase activity extracted from Raji cells over the 24hr period of study. However in GM892A cells, whereas mitozolomide had no consistently significant effect on the polymerase activity, temozolomide was found to cause a slight, but significant inhibition of activity 16 to 24hr after treatment of the cells with drug. Moreover, CCRG 82019 caused a markedly significant inhibition of the DNA polymerase activity extracted from GM892A cells over the 24hr period examined.

Baril et al (1975) had previously described the inhibition of DNA polymerase II in a cell-free system by nitrosoureas and isocyanates, and noted that pre-treatment of the "activated" DNA support with drug had no effect on polymerase activity. The concentration of drugs used to elicit this inhibition was 1mM, and it was speculated that the observed inhibition was mediated by carbamoylation of the enzyme via an isocyanate species.

In this study pre-incubation of drugs with the nuclear protein extract was found not to affect the DNA

polymerase activity. The lack of direct inhibition of the enzyme may be explained in part by the fact that much lower drug concentrations were used, and that mitozolomide, at least, has been shown to be unlikely to produce an isocyanate moiety or to carbamoylate (Horgan and Tisdale, 1984). Connors and Hare (1975) have previously reported that the direct incubation of a crude cell extract of DNA polymerase with BCNU had no effect on enzyme activity.

The observation that inhibition of the DNA polymerase was observed in the GM892A cell line, and not in the Raji, and that the apparent efficacy for inhibition of enzyme activity increases in the order mitozolomide, temozolomide, CCRG 82019, in contrast to their potencies for cell growth inhibition, may be of significance.

The lesion 3-alkyladenine has been shown to act as an efficient block to DNA polymerases (Lawley and Warren, 1976, McCarthy et al, 1984), whereas the O^6-methylguanine lesion does not (Abbott and Saffhill, 1979, Loechler et al, 1984), with the polymerase interpreting the lesion to specify the incorporation of a pyrimidine (either cytosine or thymidine) into the newly synthesised strand. 7-methylguanine is apparently an innocuous lesion which does not promote miscoding. However, spontaneous transformation of the base into an imidazole ring-opened form, a formamido-pyrimidine, is known to act as a powerful block of DNA chain elongation (Boiteux and Laval, 1983).

The ability, however, of DNA extracted from either Raji and GM892A cells treated with imidazotetrazinones, to support RNA polymerase activity was not observed to have been affected, but there was a decrease in DNA polymerase activity extracted from GM892A cells treated with either temozolomide or CCRG 82019. The decrease in polymerase activity could not be attributed to a direct drug inhibition of the enzyme, as incubation of a nuclear protein extract from treated cells with any of the drugs had no apparent effect. The observed decrease in DNA polymerase activity may have been due to a decrease in de novo synthesis of the enzyme, but this was not investigated.

It has been recently demonstrated that nuclear proteins form a more stable non-covalent complex with DNA pre-treated with CCRG 82019 than with DNA pre-treated with temozolomide (Tisdale, in press). He also demonstrated that relative to the number of alkyl groups bound to DNA, that CCRG 82019 treated calf thymus DNA was a more effective inhibitor of methylation of M. lysodiktious DNA by eukaryotic DNA methylase than was temozolomide treated calf thymus DNA. This leads to the intriguing possiblity that the observed decrease in DNA polymerase activity was caused by an increased affinity of the enzyme for the alkylated DNA, making it more difficult to extract.

It has been shown that temozolomide treatment of cells causes a greater degree of hypomethylation in GM892A cells than in Raji cells (Tisdale, in press). It has also

been demonstrated that MNU (Boehm and Drahovsky, 1981a) and MNNG (Boehm and Drahovsky, 1981b) cause hypomethylation of newly synthesised DNA in Raji cells in a dose dependent manner. Both compounds can directly inhibit the DNA (cytosine-5)-methyltransferase enzyme, but the concentration of drug required is much higher than obtained *in vivo* (Chan *et al*, 1983).

One possibility is that specific alkylated bases may be responsible for the inhibition of methylase activity. Pfohl-Leszkowicz *et al* suggested that the lesion responsible was neither 7-methylguanine nor 3-methyladenine, but either O^6-methylguanine or methylphosphotriester. The observation in this study that the most significant decrease in DNA polymerase activity extracted from GM892A cells was after treatment with CCRG 82019 rather than temozolomide, that CCRG 82019 treated calf thymus DNA inhibited DNA methylation to a much greater extent than temozolomide treated calf thymus DNA (relative to the number of alkyl groups bound) (Tisdale, in press), and that the largest proportion of the alkyl adducts formed after CCRG 82019 treatment of calf thymus DNA is speculatively ethylphosphotriester (in contrast to temozolomide), may suggest that the phosphotriester lesion is responsible for these effects. Furthermore, Marushige and Marushige (1983b) have also reported that ENU is more effective than MNU in inhibition of the ability of treated calf thymus DNA to support RNA polymerase activity, relative to the number of alkyl groups bound to the DNA.

The observed increased affinity of nuclear proteins for CCRG 82019, rather than temozolomide, treated calf thymus DNA has been suggested to result from an increased electrostatic attraction caused by a neutralization of some of the negative charges on the DNA phosphate backbone (Tisdale, in press).

The observation that the inhibition of DNA polymerase activity was only observed in the GM892A cells and not in Raji cells may implicate a role for O^6-alkylguanine in the decrease of enzyme activity. The fact, however, that the only reported difference in repair capabilities between the two cell lines is for the repair of O^6-alkylguanine lesions still leaves open the suggestion that GM892A cells are deficient (or Raji cells proficient) in some hitherto undescribed repair process(es) for alkylphosphotriesters. The significance of these observations is obscured when taken in the context that the rank order of the efficacies of each of the three drugs in affecting nuclear proteins does not correlate with cytotoxicity .

Furthermore the lesion(s) responsible for the observed effects on nuclear proteins may require a physiologically complete chromatin structure, or to be presented in a particular conformation of DNA structure, to manifest such effects. This may be suggested by the observation that DNA extracted from treated cells, which would presumably contain the same number and types of alkyl lesions as the DNA in the nuclei from which the proteins were extracted, but which is essentially

deproteinized and structurally deformed, is not affected in its ability to support exogenously added RNA polymerase.

It was postulated that if a lesion produced by temozolomide (for example O^6-methylguanine or methylphosphotriester) was responsible for the effects on nuclear proteins, then other protein/enzymes, for example DNA glycosylases, might be similarly affected, and in turn this might potentiate the cytotoxic effect of methyl methanesulphonate, an agent which produces negligible amounts of O^6-methylguanine or methylphosphotriesters, but which produces 7-methylguanine and 3-methyladenine as its major alkylated products (Beranek et al, 1980, Lawley and Warren, 1981).

Analysis of the isobolograms, constructed in the light of the effects of both drugs on the growth of Raji and GM892A cells, showed that the combined effects of the two drugs were marginally sub-additive to that predicted if the two drugs acted independently, but the proximity of the experimental data points to the envelope of additivity implied that there was no negative interaction between the effects of the two drugs (based on the definition of Steel, 1979, Steel and Peckham, 1979), and that the cytotoxic effects of the two drugs are mediated by different events.

As mentioned previously, Tisdale has recently shown that temozolomide induces hypomethylation in both Raji and GM892A cells, the effects being more pronounced in the

GM892A cells (Tisdale, in press), and is known to cause both hypomethylation and differentiation of K562 cells (Tisdale, 1985, Tisdale, 1986). Since DNA methylation is thought to play an important role in the governing of gene function and expression (Razin and Riggs, 1980, Razin and Cedar, 1984), hypomethylation of DNA may represent reactivation of certain genes. In an attempt to monitor the effects of temozolomide on gene expression, the pattern of de novo protein synthesis in whole treated cells was investigated, utilising polyacrylamide gel electrophoresis.

It was found, however, that even using computer enhancement techniques that changes in de novo protein synthesis could not be determined with any great precision by the methods employed, and did not allow for a finer determination of the differential effects on de novo protein synthesis in the two cell lines. Two-dimensional electrophoretic techniques may have proved a more sensitive tool to resolve any differential effects in protein synthesis. However the protocol used in this study did not provide for a high enough specific activity radiolabel with which to visualise the proteins by autoradiography, and conventional gel staining techniques does not allow for a true determination of de novo protein synthesis.

The one- and two-dimensional techniques developed and utilised in this study may prove to be useful tools in the assessment of drug effects on nuclear proteins. Pulse-

labelling of cells with ^{35}S-methionine following drug treatment, followed by analysis of the nuclear proteins would elucidate the effects of drug treatment on de novo synthesis of such proteins. Labelling of cells over a longer period of time, followed by drug treatment and extraction and analysis of nuclear proteins would possibly provide information about the affinity of a range of nuclear proteins for DNA in treated cells.

Flow cytometry provides a rapid method of assessing the effects of drugs on the cell cycle. The results presented are in the form of histograms of cellular DNA content, which generally reflect the proportion of the cell population in a given stage of the cell cycle. Studies have been previously made of the effects of a range of drugs on the cell cycle (Krishnan et al, 1975, Tobey et al, 1979), and it has been found that broad distinctions can be made, using the DNA histograms, to indicate the mechanism of action of drugs. Whereas antimetabolites tend to cause an accumulation of drugs at the G1/S phase boundary, with slippage into early S phase (Tobey and Crissman, 1975), antimitotic agents produce a G_2 block (Krishnan et al, 1975), while alkylating agents cause an accumulation of cells in late S and G_2/M, primarily as a result of a gross increase in the duration of the phases of the cell cycle (Tobey and Crissman, 1975, Hill et al, 1981).

Observations made of the effects of temozolomide and CCRG 82019 on the Raji and GM892A cell cycle, especially

with respect to the Raji cells, were masked by the effects of the drug vehicle, DMSO, which at the concentrations required to dissolve the drugs applied to the Raji cells, caused a build up of cells in G1 phase (figures 52-57). However the GM892A cells were less affected by the DMSO due to the 10-fold lower concentration required to dissolve the drugs, as observed by the minimal effect of the vehicle alone on the control cells.

The effects of the drug/vehicle treatment on the GM892A cell cycle therefore provides a truer reflection of the effects of drug alone. Both temozolomide and CCRG 82019 would appear to block the cells in late $S/G_2/M$, as is the general case for alkylating agents (Tobey and Crissman, 1975, Hill et al, 1981). Furthermore it has been previously reported that mitozolomide causes an increase in the late $S/G_2/M$ population of the Lewis lung carcinoma, 24-48hr after treatment (Broggini et al, 1986). It would therefore appear that mitozolomide, temozolomide and CCRG 82019 cause a similar perturbation of the cell cycle, but it is unclear whether this is due to a similar mechanism of action. Unfortunately this present study cannot differentiate between the effects on the two cell lines due to the perturbations of the cell cycle caused by the drug vehicle alone, and may be resolved in part by using lower concentrations of drugs in future studies.

Catapano et al (1987) have recently reported the effects of temozolomide on DNA damage and repair in L1210 cells sensitive to chloroethylnitrosoureas and in an L1210 cell line resistant to these agents (they described the cell line as L1210/BCNU). They found that whilst the level of the DNA repair enzyme 7-methylguanine glycosylase was similar in the two cell lines, the L1210/BCNU cells had a three fold higher level of the O^6-alkylguanine-DNA alkyltransferase repair protein than the sensitive L1210 cell line. Furthermore they described that the number of alkali-labile sites formed in both cell lines after temozolomide treatment was similar, and that their rate of repair is similar in both cell lines and was unlikely to be the cause of cell death.

They further described the progression of temozolomide treated cells through the cell cycle and found that temozolomide caused a dose-dependent block of the sensitive L1210 cells in late $S/G_2/M$, while the L1210/BCNU cells required a higher dose of drug, and a longer interval after treatment, for the same effects to be manifested. These observations are in agreement with those made in this study.

The HPLC method utilised for the analysis of specific bases formed in calf thymus DNA following treatment with either temozolomide or CCRG 82019 was not suitable for the determination of the specific bases formed in the DNA of cells in vitro. If the amount of specific bases formed in the DNA of treated cells was of a similar proportion to

that formed in calf thymus DNA, some 5-10mg of cellular DNA would be required per analysis. Assuming that the cells contained about 10μg of DNA per 10^6 cells, then about $0.5\text{-}1x10^9$ cells would be required per sample, which was thought to be impractical.

As an alternative, immunochemical analysis of the amount of O^6-methylguanine and methylphosphotriesters in the DNA of cells following temozolomide treatment was determined by Dr. G. Margison, Christie Hospital, Manchester, and some preliminary results are available.

The amount of O^6-methylguanine formed in the DNA of GM892A cells (figures 60 and 61) would appear to increase in a time- and dose-dependent manner, with a maximum somewhere between 8 and 24hr after drug treatment, and with a subsequent decrease in the amount of lesion per mg of DNA. As this decrease is probably not due to some repair capacity, it is more likely to be explained by both the death of cells and subsequent apparent dilution of the lesion by de novo DNA synthesis in the remaining cell population. The limited data available for the formation of O^6-methylguanine in Raji cells after temozolomide administration would appear to indicate that the amount of the lesion remaining in DNA increases initially in a time- and dose-dependent manner (figures 58 and 59).

Measurements of the amount of O^6-methylguanine remaining in the DNA of GM892A and Raji cells 12hr after temozolomide treatment (in a separate experiment) shows an increase in the amount of lesion present in a dose-

dependent manner (figure 62). In GM892A cells there is almost a linear relationship between the concentration of drug administered and the amount of O^6-methylguanine remaining in the DNA, while for Raji cells there is a non-linear relationship, with a disproportionate increase in the number of lesions remaining after treatment with higher concentrations of drug than with lower concentrations.

This may be largely explained by the relative proficiencies of either cell line in the O^6-AGAT repair protein. The GM892A cells have very low constitutive levels of the protein, thus most of the O^6-methylguanine formed will remain in the DNA, while the Raji cells contain higher levels of the protein, which will repair the O^6-methylguanine formed after treatment with lower doses of the drug. O^6-AGAT, as stated earlier (section 1.10), is stoichometrically inactivated by repairing O^6-alkylguanine adducts, and is regenerated only by subsequent protein synthesis. Thus it may be anticipated that after administration of higher concentrations of drug, the constitutive repair protein is inactivated, allowing any further lesion formed to remain in the DNA until more O^6-AGAT is synthesised. What is presumably observed in the Raji cells is that after an initial titration of the protein against the O^6-methylguanine formed in the DNA, a linear relationship exists between the amount of temozolomide administered to the Raji cells and the amount of O^6-methylguanine remaining in the DNA,

in both the time course experiment (figures 58 and 59) and the 12hr time point experiment (figure 62).

While there is a greater amount of O^6-methylguanine remaining in the DNA of GM892A cells as compared to Raji cells following temozolomide treatment, preliminary results may suggest that there may be more methylphosphotriesters formed in the DNA of Raji cells as compared to GM892A cells (figure 63). Caution must be exercised in interpreting this result as it was derived from a single experiment. However if the amount of methylphosphotriesters formed in Raji cells is greater than or equal to the amount formed in GM892A cells, then this may imply that the O^6-methylguanine lesion is of far greater biological significance.

The amount of O^6-methylguanine remaining in the DNA of Raji and GM892A cells 12hr after temozolomide treatment can be correlated with the growth curves of both cell lines following temozolomide treatment. The inhibition of growth curve for GM892A cells treated with the drug is almost linear at low doses (figure 7), whereas the inhibition of growth curve for Raji cells has a shoulder (figure 6). It can be predicted from figures 59 and 62, (ie plots of O^6-methylguanine formed in cellular DNA after temozolomide treatment), that the Raji growth inhibition curve will become linear at a concentration of greater than 100μM. This is the concentration at which the constitutive O^6-AGAT is depleted, and the amount of O^6-methylguanine remaining in the DNA increases in a

linear relationship with increasing drug concentration. This is indeed what is observed.

If the amount of O^6-methylguanine remaining in the DNA after the administration of a given concentration of temozolomide is plotted against the inhibition of cell population growth (figure 64), an apparent biphasic response is observed. A linear relationship is found in both cell lines over the first 70-80% of growth inhibition, after which there is a relative plateau in the relationship. This may imply that both cell populations are heterogeneous in their response to the effects O^6-methylguanine: A certain proportion of the cell population may be sensitive (or relatively hypersensitive) to O^6-methylguanine as a putative cytotoxic lesion, whereas the remainder are insensitive (or hyposensitive) to O^6-methylguanine as a cytotoxic lesion. It would be this insensitive/ hyposensitive population which would be more prone to mutation, possibly by the putative G:C to A:T transition. Heterogeneity of the population in the response to O^6-methylguanine would therefore explain why this lesion has been previously thought to be both a mutational/carcinogenic lesion by some workers, and a cytotoxic lesion by others. Conversely such a plateau may be artefactual, and may arise by an inability to measure greater than 90% inhibition of cell population growth, with accuracy in this study.

The amount of O^6-methylguanine remaining in the DNA of Raji cells at intervals after treatment with various concentrations of temozolomide.

Raji cells were treated with various concentrations of temozolomide and the DNA isolated at the stated time intervals. The amount of O^6-methylguanine remaining in the DNA of the cells was kindly measured by Dr. G. Margison of the Christie Hospital, Manchester, using an immunochemical method.

In fig. 52 these results are expressed as the amount of O^6-methylguanine remaining in the DNA, with respect to the time after drug addition, while in fig. 53 the same results are expressed with respect to the concentration of temozolomide used to treat the Raji cells.

Figure 58.

The amount of O^6-methylguanine remaining in the DNA of Raji cells at intervals after treatment with various concentrations of temozolomide.

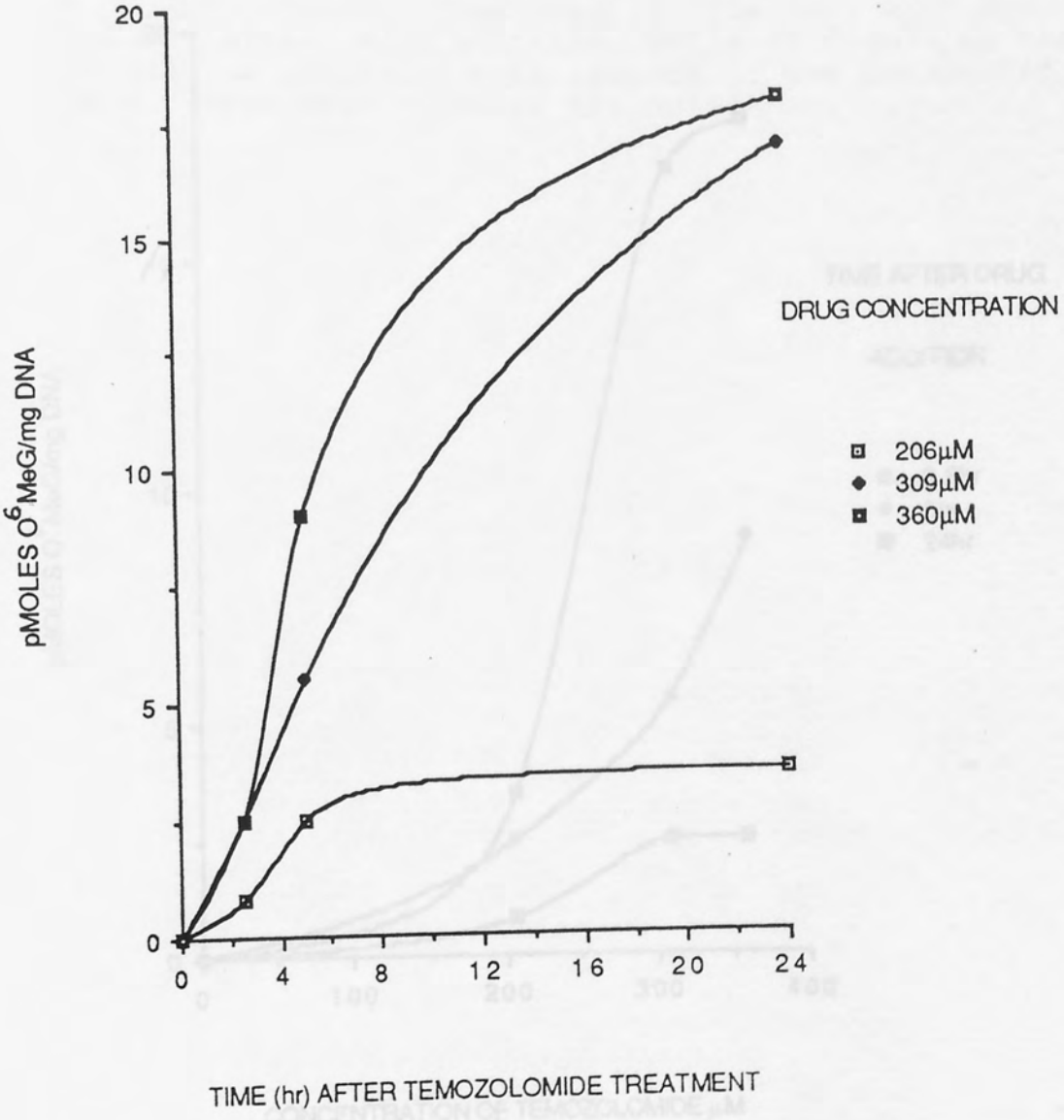
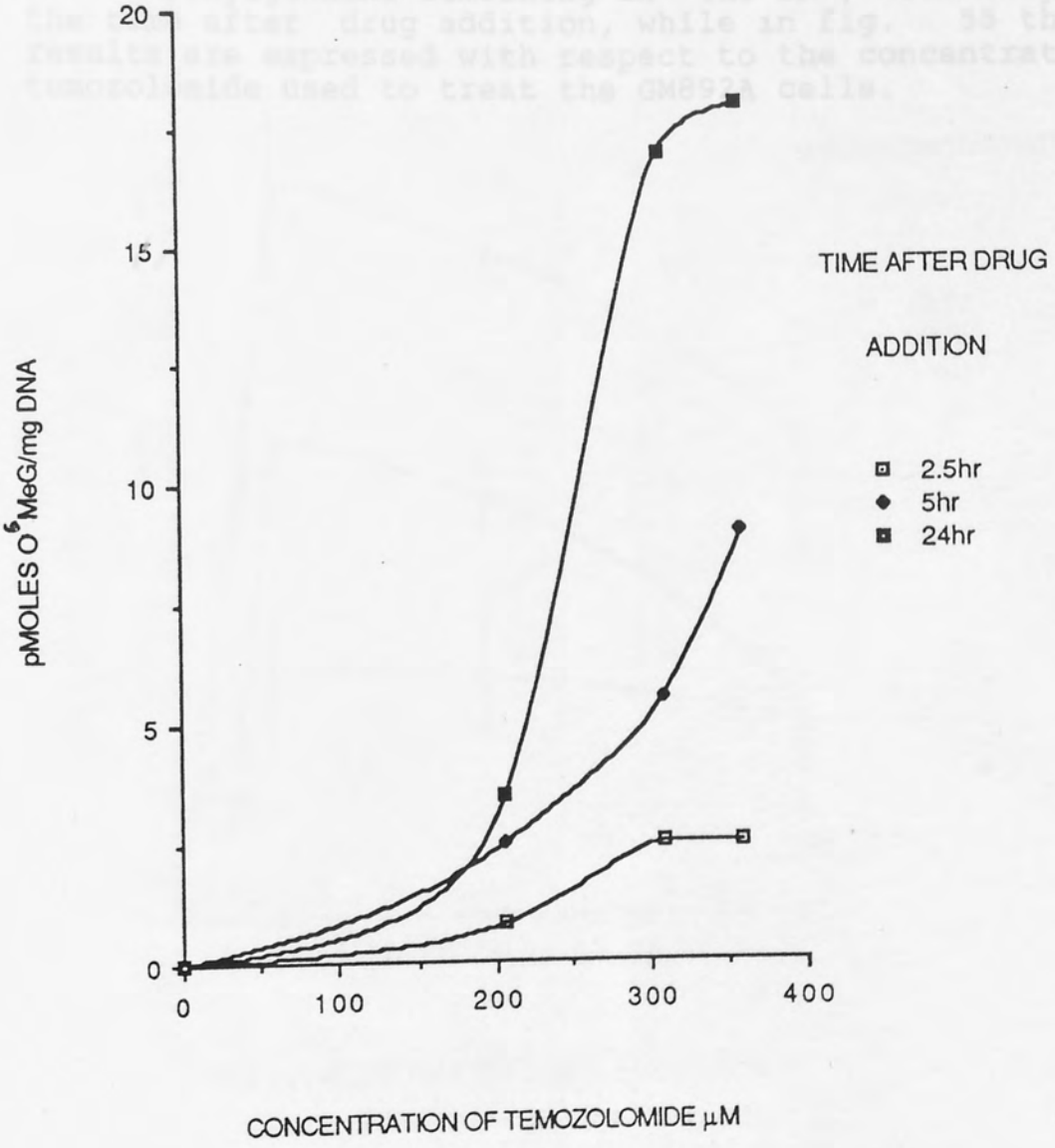


Figure 59.

The amount of O^6-methylguanine remaining in the DNA of Raji cells at intervals after treatment with various concentrations of temozolomide.



The amount of O^6-methylguanine remaining in the DNA of GM892A cells at intervals after treatment with various concentrations of temozolomide.

GM892A cells were treated with various concentrations of temozolomide and the DNA isolated at the stated time intervals. The amount of O^6-methylguanine remaining in the DNA of the cells was kindly measured by Dr. G. Margison of the Christie Hospital, Manchester, using an immunochemical method.

In fig. 54 these results are expressed as the amount of O^6-methylguanine remaining in the DNA, with respect to the time after drug addition, while in fig. 55 the same results are expressed with respect to the concentration of temozolomide used to treat the GM892A cells.

Figure 60.

The amount of O^6-methylguanine remaining in the DNA of GM892A cells at intervals after treatment with various concentrations of temozolomide.

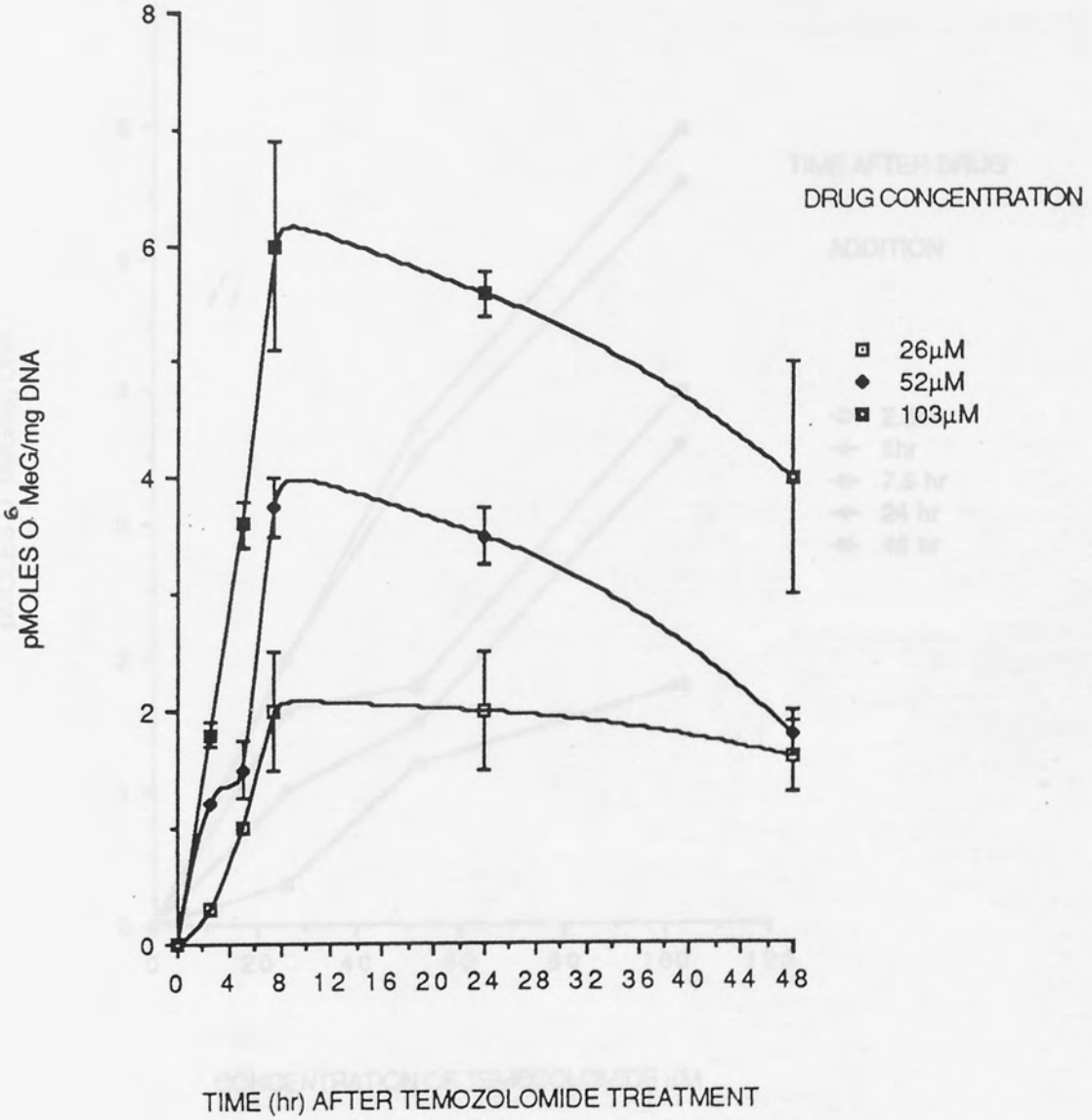


Figure 61.

The amount of O^6-methylguanine remaining in the DNA of GM892A cells at intervals after treatment with various concentrations of temozolomide.

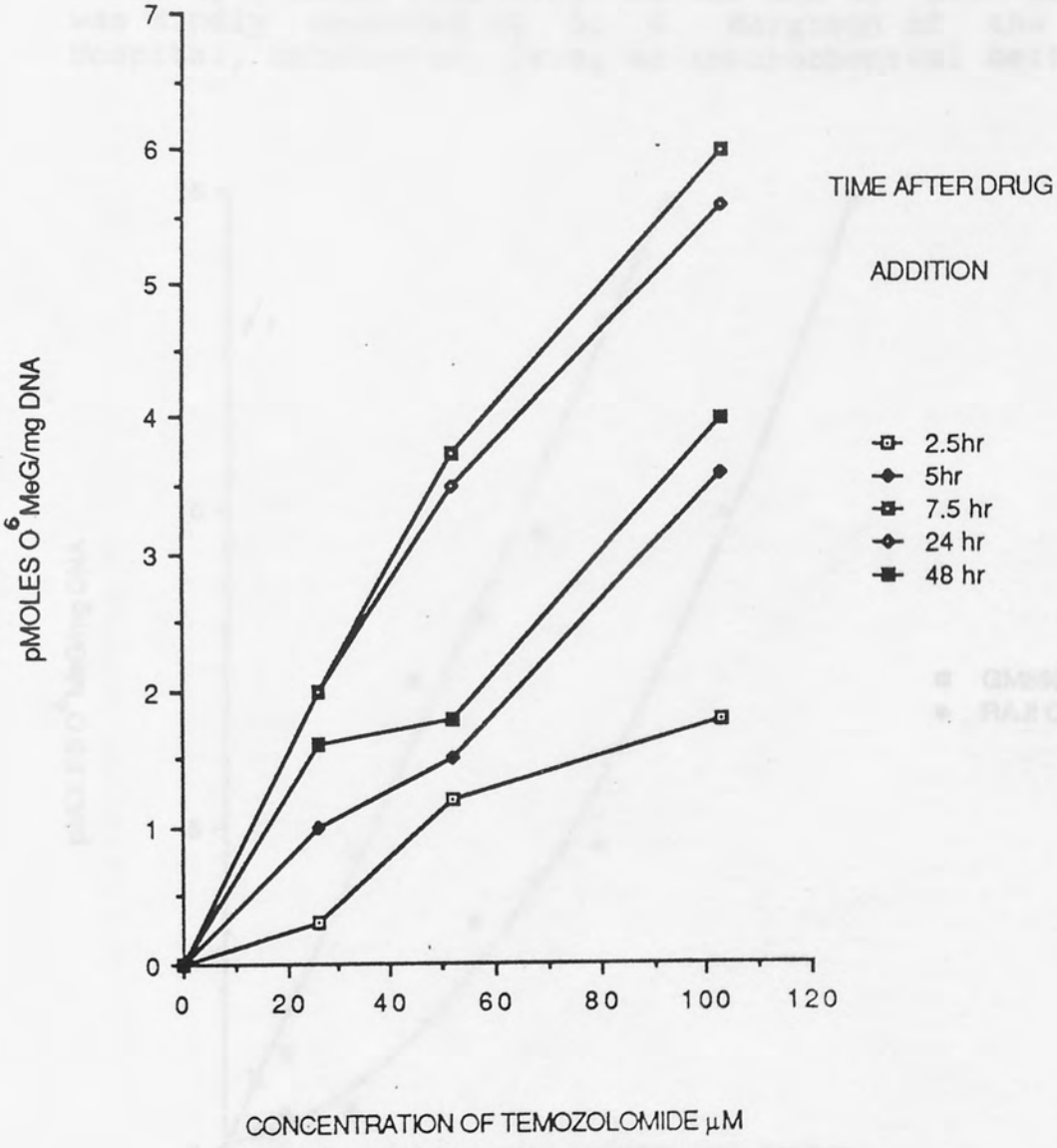


Figure 62.

<u>The amount of O^6-methylguanine remaining in the DNA of Raji and GM892A cells 12h after treatment with temozolomide.</u>

Raji and GM892A cells were treated with temozolomide for 12hr and the DNA isolated. The amount of O^6-methylguanine remaining in the DNA of both cell lines was kindly measured by Dr. G. Margison of the Christie Hospital, Manchester, using an immunochemical method.

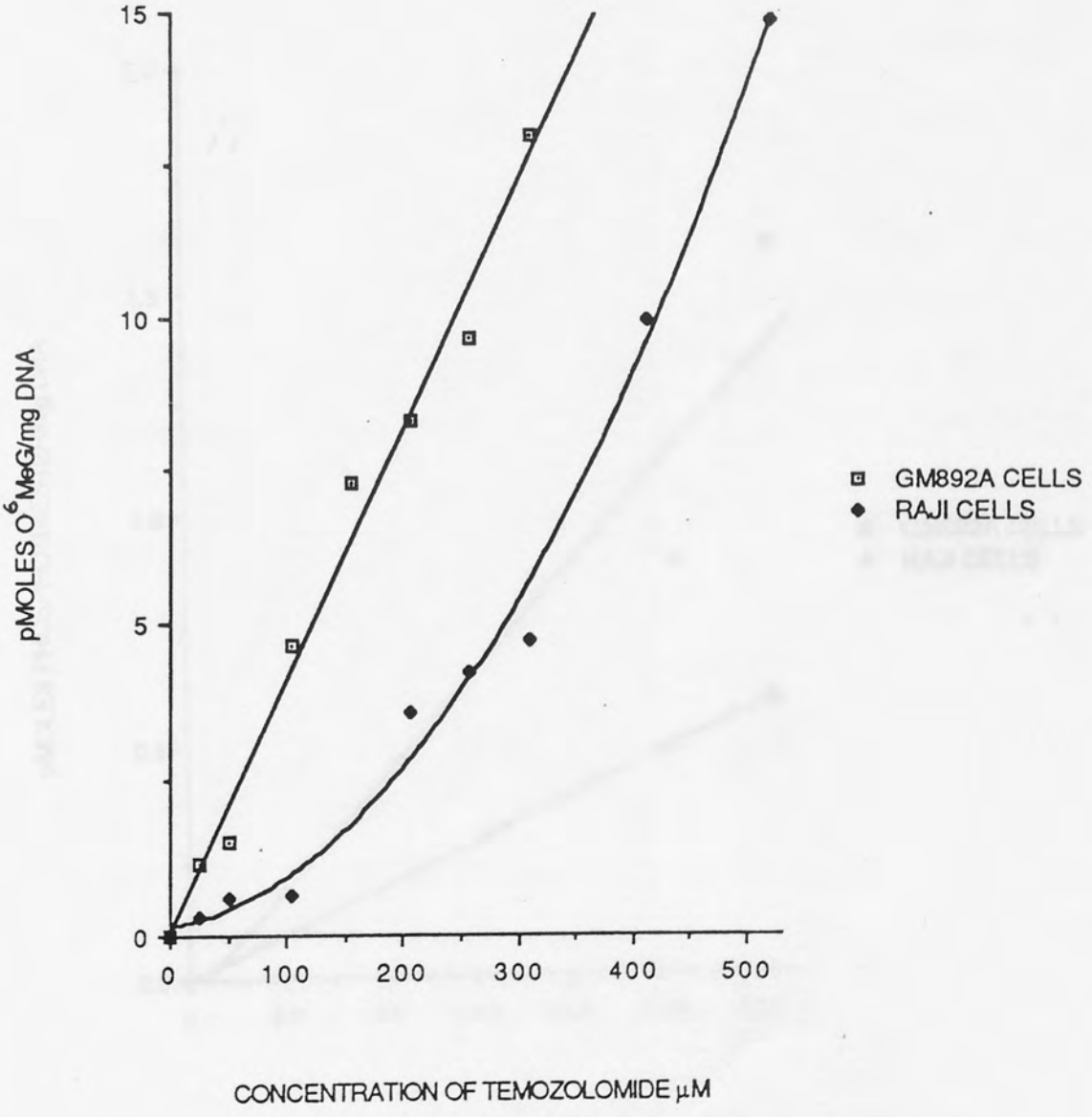


Figure 63.

The amount of methylphosphotriesters remaining in the DNA of Raji and GM892A cells 12h after treatment with temozolomide.

Raji and GM892A cells were treated with temozolomide for 12hr and the DNA isolated. The amount of methylphosphotriesters remaining in the DNA of both cell lines was kindly measured by Dr. G. Margison of the Christie Hospital, Manchester, using an immunochemical method.

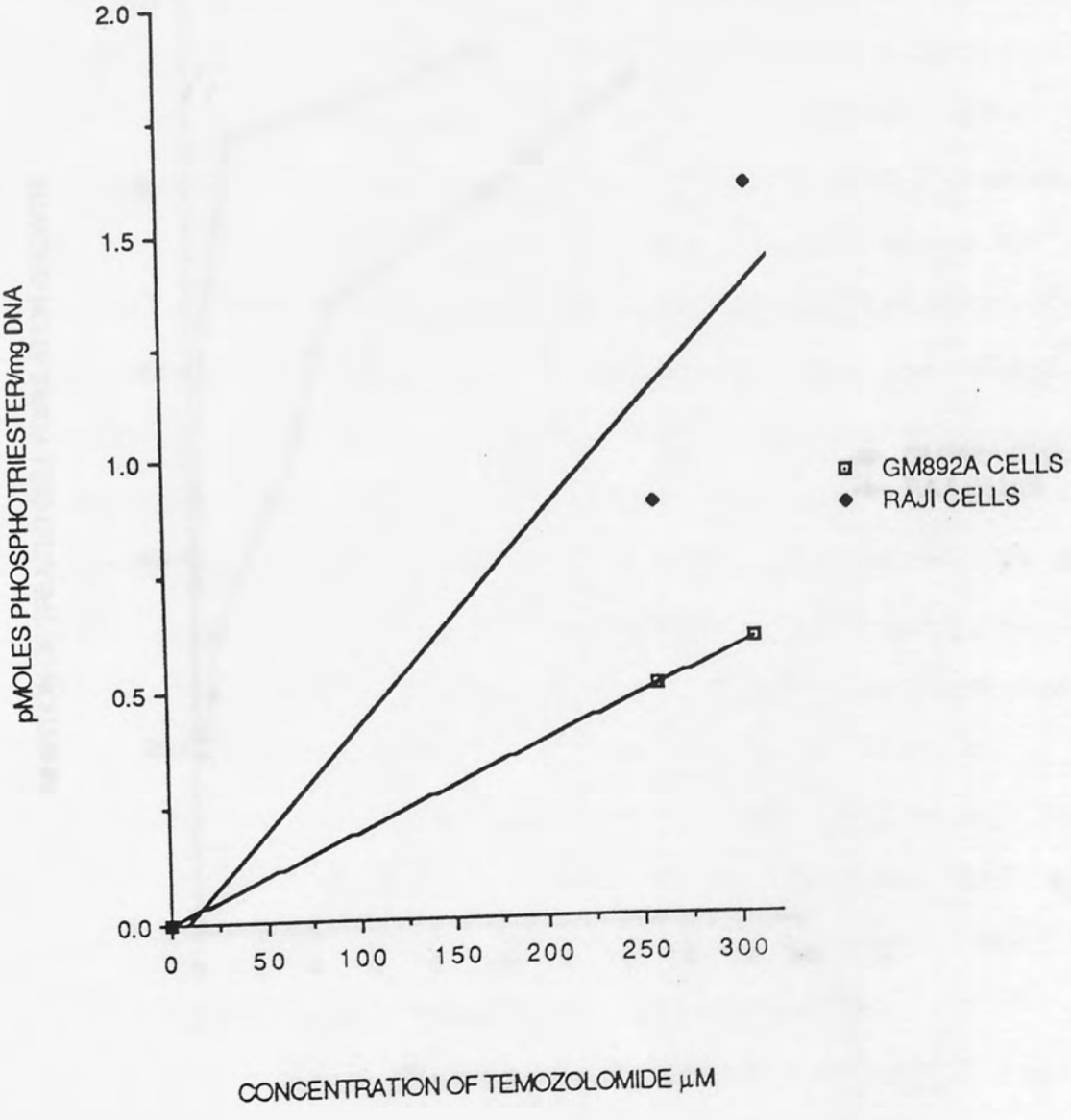
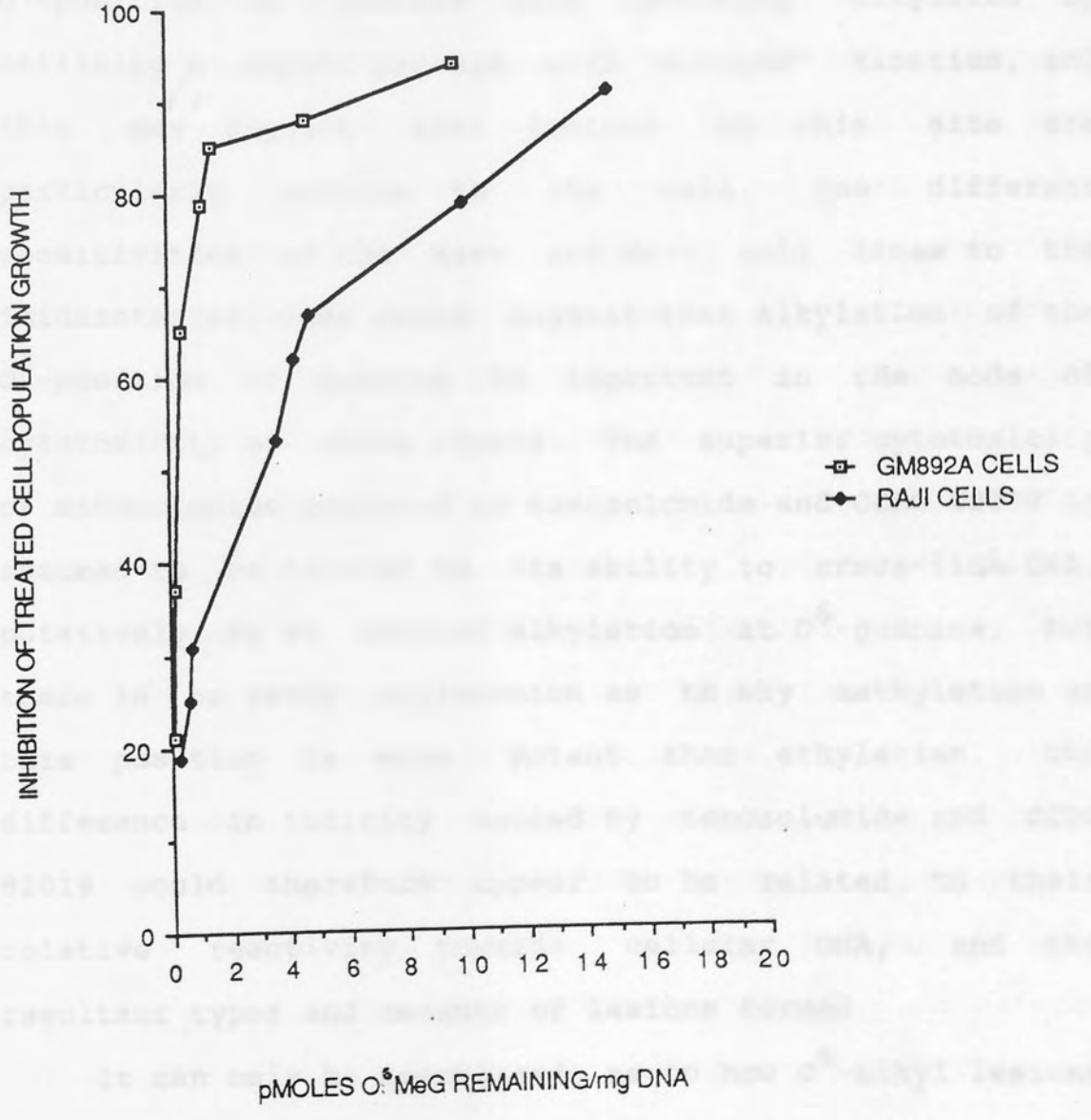


Figure 64.

A plot of the amount of O^6-methylguanine remaining in the DNA of Raji and GM892A cells 12h after treatment with temozolomide versus inhibition of cell population growth.

The amount of O^6-methylguanine remaining in the DNA of treated cells 12h after treatment with a range of concentrations of temozolomide (derived from fig. 56) was plotted against the degree of inhibition of cell population growth which was observed at those concentrations (derived from figs. 6 and 7).



The results presented in this thesis are a catalogue of the effects of imidazotetrazinones on two cell lines, and do not provide definitive answers to questions such as how these drugs (and related compounds) cause cell death, why the chloroethyl analogue is more potent than than the methyl analogue, and why the ethyl (and higher) homologues are relatively ineffective as antitumour agents.

Normal cells would seem willing to expend a considerable amount of energy in protecting the O^6-position of guanine from remaining alkylated by utilising a repair protein with "suicide" kinetics, and this may suggest that lesions at this site are particularly noxious to the cell. The different sensitivities of the Mer+ and Mer- cell lines to the imidazotetrazinones would suggest that alkylation of the O^6-position of guanine is important in the mode of cytotoxicity of these agents. The superior cytotoxicity of mitozolomide compared to temozolomide and CCRG 82019 is assumed to be related to its ability to cross-link DNA, putatively by an initial alkylation at O^6-guanine, but there is no ready explanation as to why methylation at this position is more potent than ethylation. The difference in toxicity caused by temozolomide and CCRG 82019 would therefore appear to be related to their relative reactivity towards cellular DNA, and the resultant types and amounts of lesions formed.

It can only be speculated as to how O^6-alkyl lesions may cause mutations and/or cell death. Mutations may

arise by the postulated G:C to A:T transition (section 1.12), and such mutations may be a carcinogenic event, or may cause cell death by a disruption of cellular functioning. It is less clear as to why this lesion should cause cell death.

Results presented in this thesis (and other studies) suggest that a DNA lesion (possibly O^6-alkylguanine) interferes with the functioning of nuclear proteins within the cell. However DNA extracted from imidazotetrazinone treated cells does not inhibit the ability of the DNA to act as a support for nuclear enzymes. This may suggest that the effects of the lesion are manifested only when presented in a chromosomal structure, and are not seen when the DNA is heavily deproteinised and denatured. The inhibition of nuclear proteins within the cell would be deleterious to the cell, but it is uncertain as to whether this would constitute a cytotoxic event or not.

The implications of this and other studies would appear to be that only a fraction of presented tumours will respond to the imidazotetrazinones and related compounds, by virtue of the fact that the majority of tumours would appear able to repair the DNA damage caused by these agents. A priority of future studies should be the development of assays to identify those patients to whom this type of drug treatment would be beneficial, with the consequent saving in both drug budgets and patient suffering. A greater understanding of the mode of cytotoxicity of these agents would aid both the design of

future compounds and possibly give some insight on how to selectively sensitise tumour cells to the effects of these drugs.

APPENDIX.

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ANTITUMOUR IMIDAZOTETRAZINES—XVI
MACROMOLECULAR ALKYLATION BY 3-SUBSTITUTED IMIDAZOTETRAZINONES

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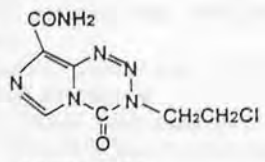
Abstract—The extent of macromolecular alkylation by three imidazotetrazinones, 8-carbamoyl-3-(2-chloroethyl)imidazo[5,1-*d*]-1,2,3,5-tetrazin-4-(3H)-one (mitozolomide) and the 3-methyl CCRG 81045) and 3-ethyl (CCRG 82019) analogues has been studied both in intact cells and with isolated DNA, RNA and protein. Towards isolated DNA and RNA CCRG 81045 was about twice as reactive as mitozolomide and 5–10-fold more reactive than CCRG 82019. Two cell lines were chosen to study macromolecular alkylation, GM892A and Raji, the latter being 10–20-fold less sensitive to mitozolomide and CCRG 81045 than the former, but only one-and-a-half-fold less sensitive to CCRG 82019. Drug uptake into both cell lines was shown to be by a rapid diffusion process with a cell medium distribution ratio not far from unity. For all three agents intracellular radioactivity became associated with macromolecules, and the level found at any time is a balance between the rate of alkylation and the rate of alkyl group removal by repair processes. Both CCRG 81045 and CCRG 82019 produced approximately the same level of alkyl groups bound to DNA, RNA and protein over a 24-hr period, whereas mitozolomide produced a greater extent of alkylation. All three agents left more alkyl groups bound to DNA and RNA in GM892A than in Raji cells, but there was no difference in the level of alkyl groups remaining bound to proteins. However, in GM892A cells the overall level of alkylation of DNA by CCRG 81045 exceeded that of CCRG 82019 only after 24 hr of drug incubation despite the twenty-fold difference in potency of these agents. These results suggest that specific base alkylations rather than total macromolecular alkylation may be more important in determining relative cytotoxicity.

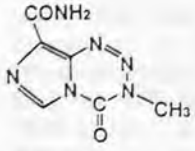
The imidazotetrazines are a new group of broad spectrum antitumour agents which are highly active against both transplantable animal tumours [1, 2] and human tumour xenografts [3]. A strict structure–activity relationship is observed for substituents in the 3-position and against the murine TLX5 lymphoma only R = CH_2CH_2Cl (Mitozolomide, CCRG 81010) and R = CH_3 (Temozolomide CCRG 81045) show activity [2]. A similar relationship is observed with the antitumour triazenes [4], and both chemical [5] and biological [6, 7] studies suggest that the imidazotetrazines may act as pro-drugs of the active triazenes with ring opening occurring *in vivo*. In addition both groups of drugs show selective toxicity to cell lines lacking O^6-methyl-guanine methyltransferase (Mer^- phenotype) [8, 9], suggesting that alkylation of DNA guanine in the O^6 position may be the primary cytotoxic event.

The cytotoxicity of mitozolomide has been correlated with interstrand cross-linking of DNA [10, 11], which probably arises after an initial alkylation at the O^6 position of guanine residues in DNA. However, CCRG 81045 is incapable of cross-link formation, but still shows antitumour activity, suggesting that binding of the alkyl group at the 3-position of the imidazotetrazine nucleus to macromolecules may be sufficient for activity. In this case it is not clear why the higher homologue (R = C_2H_5, CCRG 82019; Fig. 1) should be inactive.

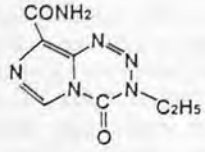
The greater carcinogenic potency of *N*-methyl-*N*-nitrosourea (MNU) in adult animals when compared with ENU has been attributed to differences in relative reactivities towards DNA and proteins [12]. In the imidazotetrazine series CCRG 81045 (R = CH_3; Fig. 1) has a greater inhibitory effect on the template activity of isolated DNA than CCRG 82019 (R = C_2H_5; Fig. 1) [13]. This suggests that the difference in reactivity of the two analogues towards cellular macromolecules may be responsible for the difference in biological activity. This study determines the extent of reaction of different 3-substituted imidazotetrazines with DNA, RNA and protein and attempts to correlate the differences in reactivity with the biological effects.



CCRG 81010
(Mitozolomide)



CCRG 81045



CCRG 82019

Fig. 1. Structures of drugs used in this study.

MATERIALS AND METHODS

8-Carbamoyl-3-[^{14}C]methylimidazo[5,1-*d*]-1,2,3,5-tetrazin-4(3H)-one (sp. act. 20.2 mCi mmol^{-1}) and 8-carbamoyl-3-[^{14}C]ethylimidazo[5,1-*d*]-1,2,3,5-tetrazin-4(3H)-one (sp. act. 12.9 mCi mmol^{-1}) were synthesized by ICI and kindly donated for this study by May & Baker Ltd. (Dagenham, U.K.) 8-Carbamoyl-3-[^{14}C]-2-chloroethylimidazo[5,1-*d*]-1,2,3,5-tetrazin-4-(3H)-one (sp. act. 15.8 mCi mmol^{-1}) and the unlabelled imidazotetrazines were synthesized and donated by May & Baker Ltd. Tissue culture medium and foetal calf serum were purchased from Gibco Europe Ltd. (Paisley, Scotland). Calf thymus DNA, calf liver RNA and bovine serum albumin were purchased from Sigma Chemical Co. (Dorset, U.K.). Labelled drugs were made up in dimethyl sulphoxide (DMSO) at a concentration of 5 mg ml^{-1} and stored frozen at −20°.

In vitro *reactions of imidazotetrazinones with DNA, RNA and protein.* Radiolabelled drugs were incubated with 600 μg of DNA, RNA or bovine serum albumin in 300 μl of 50 mM KCl plus 700 μl of 50 mM Tris–HCl, pH 7.8, 10 mM EDTA and 10 mM dithiothreitol for 2 hr at 37°. The reactions with DNA and RNA were terminated by chilling on ice, adding sodium acetate to give a 2% solution, followed by precipitation of the macromolecules with 3 vol. of ice-cold absolute ethanol. The DNA and RNA were collected by centrifugation and non-reacted drug removed by two washes in ice-cold absolute ethanol. The reaction with protein was terminated by chilling the solution on ice followed by the addition of 3 ml of ice-cold 10% trichloroacetic acid. The protein was collected by centrifugation and washed and recentrifuged twice in 3 ml ice-cold trichloroacetic acid.

The DNA and RNA precipitates were dissolved in 150 μl of 50 mM KCl and the radioactivity was determined in Luma-Gel scintillation fluid (M & B, Loughborough U.K.) using a Packard Tri-carb 2000 CA scintillation analyzer. A portion was assayed for DNA by the method of LePecq and Paoletti [14] and RNA was determined by the absorption at 260 nm. The protein was dissolved in 150 l of 0.01 M NaOH and the concentration determined by the method of Lowry *et al.* [15]. The solution was neutralised with 1 M HCl prior to radioactivity detection. All solutions of macromolecules were solubilized with hyamine hydroxide prior to counting. To establish that the agents were covalently attached to the macromolecules portions of the drug-treated DNA, RNA and protein after incubation with 0.1 mM drug for 2 hr at 37° were precipitated and washed as above and redissolved in 50 mM KCl (DNA and RNA) or 0.01 M NaOH protein. A portion of each sample was then dialysed against water for 24 hr at 4°. The undialysed and dialysed samples were then reprecipitated, washed and the macromolecular bound radioactivity was determined. After dialysis the percentage of radioactivity remaining bound to DNA, RNA and protein was for mitozolomide, 130, 99 and 88, for CCRG 81045 93, 108 and 120 and for CCRG 82019 97, 94 and 98 respectively. This suggests that all of the macromolecular bound radioactivity is covalently bound drug.

Extent of reaction of imidazotetrazinones with macromolecules in intact cells. Both Raji and GM892A cells were routinely grown in RPMI 1640 medium containing 10% foetal calf serum under an atmosphere of 5% CO_2 in air. For incorporation experiments cells (8×10^6 ml^{-1}) were treated with sodium formate to give a final concentration of 20 mM 30 min prior to the addition of 0.1 mM (final concentration) of 3-^{14}C side-chain labelled imidazotetrazinones at the specific activities indicated in the materials section. At time intervals a portion of the cell suspension was removed, sedimented by centrifugation and washed three times in 1 ml of phosphate-buffered saline. The cell pellet was lysed in 0.5 ml of ice-cold 0.2 M perchloric acid and sedimented by centrifugation at 4°. The precipitate was washed twice with 0.5 ml of ice-cold 0.2 M perchloric acid at 4° and the washings plus the original supernatant were used to measure the acid-soluble pool. The radioactivity in the RNA in the cell pellet was determined by hydrolysis in 0.7 ml of 0.3 M KOH for 1 hr at 37° followed by precipitation with 0.5 ml of 1 M perchloric acid at 4°. The precipitate was collected by centrifugation and washed twice with 0.2 M perchloric acid. The supernatant and washings were neutralised and the radioactivity determined in Luma-Gel scintillation fluid.

The radioactivity in DNA in the pellet from the RNA determination was assayed by hydrolysis (twice) in 0.75 ml of 0.5 M perchloric acid for 30 min at 80°. On cooling the protein precipitated and the supernatant when neutralised was used for the determination of radioactivity in DNA. The remaining acid-insoluble fraction was dissolved in 1 ml of 0.01 M NaOH and the radioactivity determined after addition of 0.2 ml of hyamine hydroxide. The concentration of DNA was determined by the reaction with diphenylalanine and RNA by reaction with orcinol according to the method of Munro and Fleck [16]. To determine the efficiency of extraction of macromolecules and the extent of contamination of the various macromolecules with one another both GM892A and Raji cells were incubated with 1 μCi/ml of either (methyl-^{3}H) thymidine, (sp. act. 5.0 Ci/mmol) [5-^{3}H]uridine (sp. act. 29 Ci/mmol) or L-[4,5-^{3}H] leucine (sp. act. 67 Ci/mmol) and the DNA, RNA and protein were extracted as above. In both cell lines 96% of the (methyl-^{3}H) thymidine was associated with the DNA fraction, 97% of the [5-^{3}H] uridine was associated with the RNA fraction and 94% of the ^{14}C leucine was associated with the protein fraction.

Transport studies. These were performed essentially as described previously [17]. Cells were resuspended in fresh RPMI 1640 medium at a concentration of 5×10^6 cells ml^{-1}, and equilibrated for 10 min at 4° in an ice-water bath. Uptake was initiated by the addition of drug (0.1 mM, without dilution from the initial specific activity) in DMSO. At specified time points after drug addition, samples (200 μl) were removed to an Eppendorf tube which contained 100 ml of a silicon oil: corn oil (10:3) mixture and 50 μl of 90% formic acid. After centrifugation (9000 *g*) for 1 min the tube was frozen in liquid nitrogen, cut at the oil/acid boundary and the radioactivity was determined in Luma-Gel scintillation fluid. To establish the volume of the cells a 1 ml

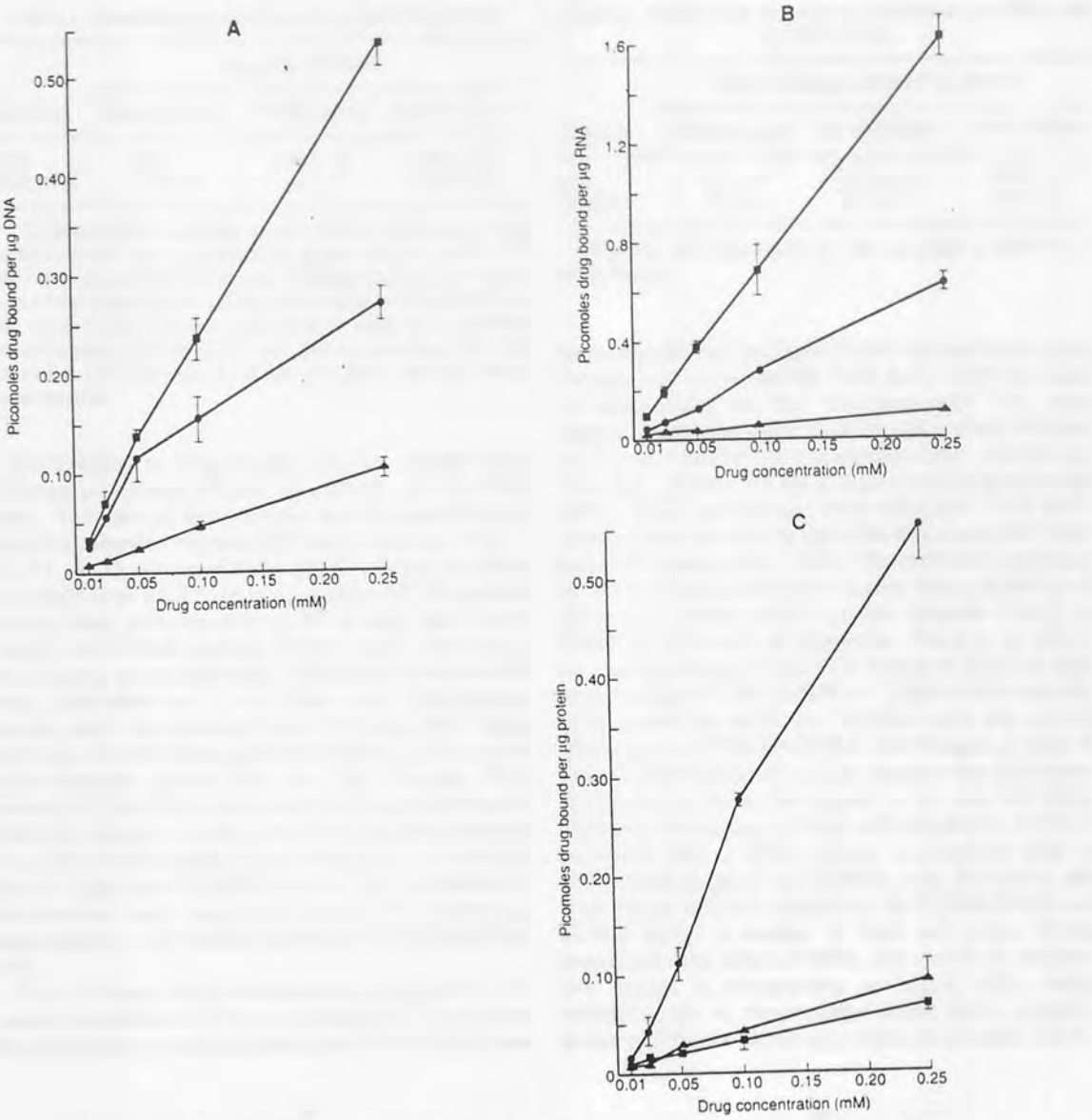


Fig. 2. Reaction of calf thumus DNA (A), calf liver RNA (B) and bovine serum albumin (C) with mitozolomide (●), CCRG 81045 (■) and CCRG 82019 (▲). Macromolecules were incubated with drugs for 2 hr at 37° and the amount of radioactivity bound was determined as described in Methods.

sample of cell suspension was treated with a mixture of ^{14}C insulin and $[^3H]$-H_2O. Once equilibrium had been established the samples were treated as above.

RESULTS

The extent of total alkylation of calf thymus DNA, calf liver RNA and bovine serum albumin by mitozolomide, CCRG 81045 and CCRG 82019 is shown in Fig. 2. The extent of reaction of all agents with all three macromolecules increases as the concentration of drug increases. For both DNA and RNA (Fig. 2 A,B) the total alkylation increases in the series CCRG 82019, mitozolomide, CCRG 81045, with CCRG 81045 being about twice as reactive as mitozolomide. However, mitozolomide is about 5–10-fold more reactive towards protein than the other two imidazotetrazinones. The alkylation of RNA exceeds that of DNA with both mitozolomide and CCRG 81045, while CCRG 82019 displays a similar low reactivity towards DNA, RNA and protein. Towards DNA, CCRG 81045 shows a five-fold greater alkylation than CCRG 82019.

To further investigate the extent of reaction of the imidazotetrazinones with macromolecules two cell lines (Raji and GM892A) were chosen with different sensitivities towards these agents (Table 1). Thus GM892A cells which have very low levels of the O^6-methylguanine methyltransferase repair protein [8] are 10- and 20-fold more sensitive to mitozolomide and CCRG 81045 than Raji cells. In contrast the ethyl analogue, CCRG 82019, shows little difference in toxicities towards the two cell lines, and in GM892A cells is 20-fold less potent than CCRG 81045 and 100-fold less potent than mitozolomide.

Table 1. Sensitivity of cell lines to imidazotetrazinones

Cell line	ID_{50} μM* (± SEM)		
	Mitozolomide	CCRG 81045	CCRG 82019
Raji	20 ± 7	206 ± 20	360 ± 30
GM892A	2 ± 0.5	10 ± 7	229 ± 20

*Concentration required to give 50% inhibition of cell growth. Cells were plated at an initial density of 8×10^4 ml^{-1} and growth inhibition was calculated from the linear part of the growth curves. Drugs were dissolved in DMSO at 10^3 times their required concentration such that the final concentration of DMSO in the culture medium did not exceed 0.1%. Clonogenic assays on either cell line were unsuccessful.

Differences in drug sensitivity may result from differential uptake of these agents into the two cell lines. The rate of drug uptake and the equilibrium values attained in the two cell lines is shown in Fig. 3 (A,B). As shown previously for the uptake of mitozolomide into TLX5 lymphoma cells [17] the initial rate of drug accumulation at 37° is very rapid with steady-state levels reached within 1 min. Therefore, to compare more accurately differential uptake cells were incubated at 4°, in which case equilibrium values were not attained until 4–8 min after drug addition. For all three agents the initial uptake rate is approximately linear over the first 2–4 min. The values for the initial rates of uptake into the two cell lines are shown in Table 2, and indicate that for each drug the initial uptake into Raji cells is 2–4-fold greater than into GM892A cells. The cell/medium distribution ratio remains constant for 30 min at approximately 1.3 in Raji cells and 1.8 in GM892A cells.

For all three drugs intracellular radioactivity becomes associated with macromolecules. The amount of radioactivity remaining bound to DNA, RNA and protein in the two cell lines at various times after drug treatment is shown in Fig. 4 (A,B,C). Incorporation of radioactivity via the "1-carbon pool" has been suppressed by the addition of 20 mM sodium formate prior to the addition of the labelled drug. In both cell lines the number of alkyl groups remaining bound to DNA, RNA and protein after treatment with mitozolomide exceeds that of the other two imidazotetrazinones by a factor of 4–7-fold. The extent of alkylation of DNA by mitozolomide is higher than alkylation of RNA and protein and is greater (almost 2-fold) in GM892A cells than in Raji cells. There is no difference in the overall level of alkylation of DNA in Raji cells by either CCRG 81045 or CCRG 82019 over the 24 hr period of study. In GM892A cells the overall alkylation of DNA by CCRG 81045 exceeds that of CCRG 82019 only after 24 hr despite the difference in potency of these two agents to the two cell lines. However, in contrast to Raji cells alkylation of DNA increases over a 24 hr period, presumably due to deficiencies in repair in GM892A cells. However, the total extent of DNA alkylation by CCRG 81045 and CCRG 82019 is similar in both cell lines. When compared with isolated DNA, the reactivity of these two agents is comparable in intact cells, while mitozolomide is about three times more reactive towards DNA in intact cells than to isolated DNA.

Table 2. Initial rates of drug accumulation into Raji and GM892A cells

Cell line	Initial velocity pmol/10^6 cells/min*		
	Mitozolomide	CCRG 81045	CCRG 82019
Raji	111 ± 4	62 ± 9.2	108 ± 7
GM892	32 ± 8	22 ± 2	66 ± 2

*Results are expressed as the average ± SEM of 3 experiments.

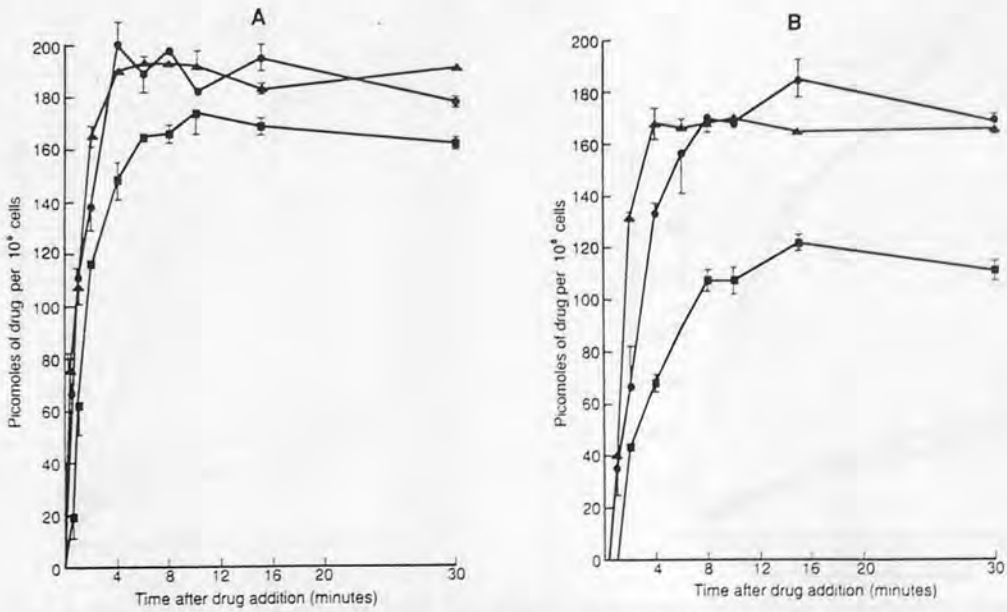


Fig. 3. Uptake of mitozolomide (●), CCRG 81045 (■) and CCRG 82019 (▲) at 4° into Raji (A) and GM892A (B) cells.

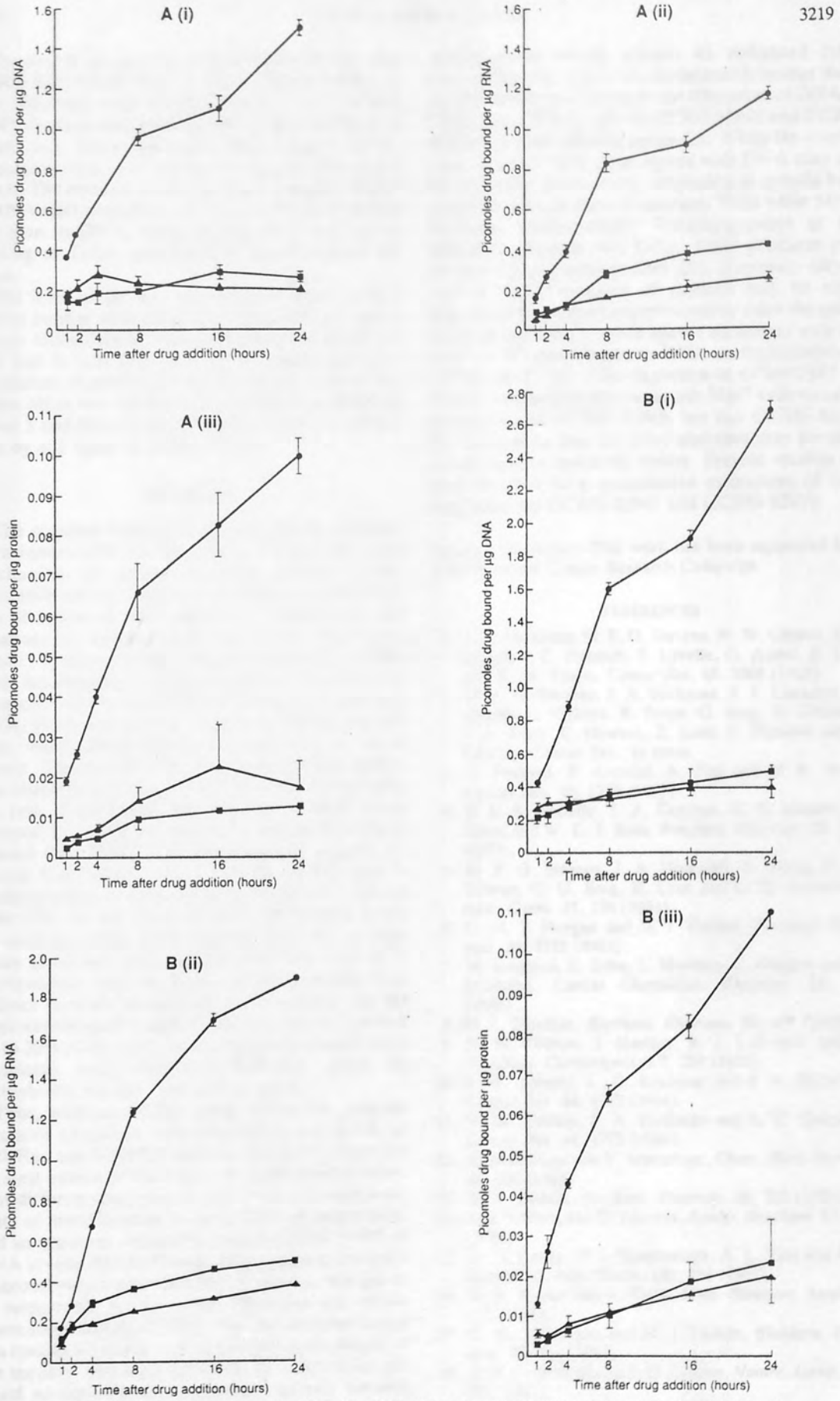


Fig. 4. Radioactivity associated with DNA (i), RNA (ii) and protein (iii) isolated from Raji (A) and GM892A (B) cells after treatment with mitozolomide (●) CCRG 81045 (■) and CCRG 82019 (▲), at stated intervals after drug addition.

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The extent of reaction of both mitozolomide and CCRG 82019 with RNA in intact cells is similar to that observed with isolated RNA, while CCRG 81045 displays less alkyl groups bound to RNA in intact cells when compared with isolated RNA, presumably due to an enhanced rate of repair of this lesion. The number of alkyl groups remaining bound to RNA after treatment with mitozolomide is slightly less than to DNA, while for the other two agents binding to DNA and RNA is approximately the same.

The level of protein alkylation in intact cells is similar to that with isolated protein with all agents except mitozolomide where alkylation is about 10-fold less in cells than with bovine serum albumin. Alkylation of proteins by mitozolomide exceeds that of the other two imidazotetrazinones by a factor of about 5 and there is no difference in protein alkylation by any agent in either cell line.

DISCUSSION

The covalent binding of the antitumour imidazotetrazinones with DNA may be a critical event in the mechanism of action of these agents. Cross-resistance studies suggest a similarity in the mechanism of action of these agents to nitrosoureas and triazenes [1], agents considered to exert their cytotoxicity by reaction with specific bases in DNA. This study has attempted to determine the relative levels of reaction of 3-substituted imidazotetrazinones with DNA, RNA and protein both alone and in two cell lines which show different sensitivities to these agents. The overall level of macromolecular alkylation observed in intact cells will be a balance between the rate of alkylation and the rate of alkyl group removal by repair processes. In human fibroblasts treated with MNU 3-methyladenine is rapidly removed from DNA with a half-life of 2 hr and 7-methylguanine is removed slower with a half-life of 30 hr [18]. At low doses of MNU about 90% of the O^6-methylguanine is removed in 10 hr, but at high doses about half still remains. Also the level of 7-alkylguanine may be lower in cells treated with sodium formate to suppress incorporation via the "one-carbon pool", since it has been shown [19] that up to 30% of the total 7-methylguanine formed after treatment with dimethylnitrosamine arose by methylation via the "one-carbon pool".

Thus, whereas CCRG 81045 shows the greatest extent of alkylation of isolated DNA and RNA, in both Raji and GM892A cells mitozolomide shows the greatest extent of alkylation of all macromolecules. This difference appears to arise from a greater reactivity of mitozolomide towards DNA in intact cells, and an apparent reduced binding of CCRG 81045 to RNA in cells. Mitozolomide decomposes to form a 2-chloroethyldiazonium species [5] which is thought to be responsible for the initial alkylation and subsequent cross-linking of DNA. The rate of formation of this species is known to be dependent upon the pH of the medium and local variations in intracellular pH could account for an increased reactivity towards DNA. Alternatively chromosomal proteins may be important in directing the alkylation of DNA in cells.

While the overall level of DNA alkylation by mitozolomide would explain its enhanced cytotoxicity over the other two imidazotetrazinones there is little correlation between the alkylation of DNA in GM892A and Raji cells by CCRG 81045 and CCRG 82019 and their relative potencies. While the overall level of reaction of these agents with DNA may not be critical for cytotoxicity, alkylation at specific base positions may be more important. Thus while MNU produces predominantly 7-methylguanine as the alkylation product with DNA, ENU produces predominantly phosphotriesters [20]. However, alkylation at the O^6-position of guanine may be more important in determining cytotoxicity since the sensitivity of cell lines to these agents correlates with the level of O^6-methylguanine-DNA methyltransferase (O^6MeGMT) [8]. Also depletion of O^6MeGMT by free O^6-methylguanine sensitizes Mer^+ cells to mitozolomide and CCRG 81045, but not CCRG 82019 [8], suggesting that the ethyl analogue may produce an alternative cytotoxic lesion. Present studies are thus directed to a quantitative estimation of base alkylation by CCRG 81045 and CCRG 82019.

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