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ANTITUMOUR IMIDAZOTETRAZINES—XV

ROLE OF GUANINE O^6 ALKYLATION IN THE MECHANISM OF CYTOTOXICITY OF IMIDAZOTETRAZINONES

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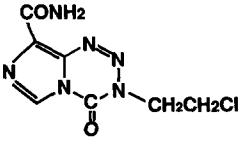
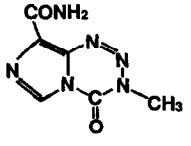
Abstract—Cell lines with constitutive levels of the DNA repair protein O^6-methylguanine-DNA methyltransferase (O^{6}MeGMT) (Mer$^+$ phenotype) were less sensitive to the cytotoxic effects of the imidazotetrazinone mitozolomide and the methyl analogue (CCRG 81045) than cells lacking the repair enzyme (Mer$^-$). In contrast neither chlorambucil or the ethylimidazotetrazinone (CCRG 82019) showed differential toxicity between Mer$^+$ and Mer$^-$ cell lines. When Mer$^+$ cell lines were incubated with the free base O^6-methylguanine (O^{6}MeG) for 16 hr there was a depletion of O^{6}MeGMT, which was dose-related. Such cells showed an increased sensitivity to both mitozolamide and CCRG 81045, but not to CCRG 82019. The only Mer$^+$ cell line not showing increased sensitization with O^{6}MeG pretreatment was Raji, where O^{6}MeGMT was shown to reappear after addition of CCRG 81045. These results suggest that the chloroethyl and methylimidazole-triazinones are similar to the nitrosoureas and triazenes in that cytotoxicity correlates with alkylation of the O^6-position of guanine, while the ethyl analogues appear to produce an alternate cytotoxic lesion.

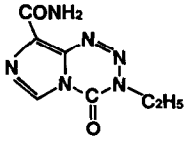
The imidazoletetrazinones are a new group of broad spectrum antitumour agents with essentially curative activity against a range of murine tumours and human tumour xenografts [1–3]. One member of the series, mitozolomide (Fig. 1; R = CH_2CH_2Cl; CCRG 81010) has recently completed a phase 1 clinical study [4] and another member (Fig. 1; R = CH_3; CCRG 81045) is also scheduled for clinical trial. Both chemical [1] and biological [5] studies suggest that the imidazotetrazinones may act as prodrug modifications of the acyclic triazenes with ring opening occurring in alkaline conditions. Mitozolomide displays cross-resistance to a triazene-resistant TLX5 lymphoma, but is not cross-resistant to an L1210 leukaemia with derived resistance to cyclophosphamide [2]. Structure–activity studies in this series of compounds show a similarity to the triazenes in that R = CH_2CH_2Cl and CH_3 are active, whilst R = C_2H_5 (Fig. 1, CCRG 82019) and higher homologues are inactive [6]. The cytotoxicity of mitozolomide has been attributed to interstrand cross-linking of DNA [7, 8] and a cell line proficient in the repair of O^6-methylguanine (O^{6}MeG) lesions (Mer$^+$, IMR-90) is much less sensitive to both cross-linking of DNA and cytotoxicity produced by mitozolomide than a repair deficient cell line (Mer$^-$, VA-13) [8]. Such a correlation betweeen the ability to repair alkyl lesions at the O^6-position of guanine and cytotoxicity has also been observed with both the nitrosoureas [9] and triazenes [10] and suggests that this base modification is a potentially cytotoxic lesion. DNA cross-links produced by chloroethylnitrosoureas can be suppressed by a partially purified extract of O^6-methylguanine-DNA methyltransferase (O^{6}MeGMT) [11], while pretreatment of O^{6}MeGMT-repair proficient cells with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), which inhibits the repair process which these cells use to prevent chloroethylnitrosourea (CNU) induced DNA interstrand cross-linking, results in a synergistic increase in cell kill [12]. In contrast, MNNG pre-treatment does not appreciably increase the cell kill produced by a typical bifunctional alkylating agent [13]. This suggests a different mechanism of cytotoxicity by these two groups of alkylating agents.



CCRG 81010
(Mitozolomide)



CCRG 81045



CCRG 82019

Fig. 1. Structure of imidazotetrazinones.

In order to investigate the cytotoxic lesions produced by the imidazotetrazinones an assessment has been made of the response of a range of cell lines with varying capacities to repair O^6-alkylguanine modifications. In addition the effect of depletion of O^{6}MeGMT by free O^{6}MeG on cytotoxicity has been determined.

MATERIALS AND METHODS

[^{3}H Methyl]-*N*-nitrosourea (sp. act. 1 Ci mmol^{-1}) was purchased from New England Nuclear, Herts.

M. luteus DNA and 6-chloroguanine were obtained from Sigma Chemical Co., Dorset. Tissue culture medium and foetal calf serum were purchased from Gibco Europe, Paisley, Scotland. 8-Carbamoyl-3-(2-chloroethyl)-imidazo [5,1-d]-1,2,3,5-tetrazin-4-(3H)-one (mitozolomide; M & B 39565; CCRG 81010), 8-carbamoyl-3-methylimidazo [5,1,d]-1,2,3,5-tetrazin-4-(3H)-one (M and B 39831; CCRG 81045) and 8-carbamoyl-3-ethylimidazo [5,1-d]-1,2,3,5-tetrazin-4-(3H)-one (M and B 40447; CCRG 82019) were kindly supplied by Dr. C. Newton, May & Baker Ltd., Dagenham, Essex. O^6-Methylguanine (O^6MeG) and O^6-ethylguanine (O^6EtG) were synthesized from 6-chloroguanine by reaction with sodium methoxide and sodium ethoxide, respectively, as described [14]. The material was determined to be free of unaltered 6-chloroguanine and guanine by hplc analysis using a Whatman Partisil 10SCX column eluted isocratically with 50 mM ammonium formate, pH 4, containing 8% methanol at room temperature in an Altex hplc system. Stock solutions were prepared in 0.1 N HCl for use in tissue culture experiments and were stored at −20°. The concentration was determined from the extinction coefficient at 280 nm (7.9×10^3).

Cell culture. The Burkitt's lymphoma cell line, Raji, GM892A (human lymphoblastoma), K562 (human myeloid leukaemia) and MAC16 (mouse colon adenocarcinoma) were maintained in RPMI 1640 media containing 10% foetal calf serum. A549 (human lung carcinoma) and JAR (human choriocarcinoma) were cultured in Ham's F12 media containing 10% foetal calf serum. All cells were maintained under an atmosphere of 5% CO_2 in air and were passaged twice a week. Cytotoxicity was determined from the loss of colony-forming ability by an *in situ* assay in which cells were treated at cloning densities. Cells were plated into 100 mm diameter dishes at a cell density of 500–1000 cells per dish. In some cases (Raji, K562, GM892) a growth inhibition assay was used to determine the effect of the chemicals. Cells were seeded at an initial density of 5×10^4 per ml and cell counts were determined daily by means of a Coulter Electronic Particle Counter, Model D. By this means a growth curve was constructed and the degree of inhibition was determined from the linear part of the growth curve. 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%. O^6MeG in 0.1 N HCl was added to exponentially growing cultures 16 hr before drug addition.

Enzyme assays. [^{3}H]Methylnitrosourea treated *M. luteus* DNA was prepared and partially depurinated as described [14]. Cells ($1–3 \times 10^7$) were harvested by low speed centrifugation, washed with 0.9% NaCl and disrupted by sonication in 100–150 μl of 50 mM Tris HCl, pH 7.8, 10 mM EDTA, 10 mM dithiothreitol and 0.3 M KCl. A supernatant fraction for enzyme assay was produced by centrifugation for 5 min at 4° in an Eppendorf microcentrifuge. The protein content of the cell extract was determined by the method of Lowry *et al.* [15] using bovine serum albumin as a standard.

Methyltransferase activity in tumour extracts was assayed by determining the disappearance of O^6MeG from [^{3}H]methyl DNA. The reaction mixture contained [^{3}H]methyl-DNA (2–4 μg; $20–30 \times 10^3$ dpm [^{3}H]O^6MeG), 50 mM HEPES KOH, pH 7.8, 10 mM dithiothreitol, 1 mM EDTA, 5% glycerol, 50 mM spermidine–hydrochloride and enzyme extract in a total volume of 50 μl. After incubation for 30 min at 37°, the reaction was terminated by cooling to 0° and adding 30 μl ice-cold 0.8 M trichloroacetic acid. After 10 min at 0° the precipitated material was pelleted by centrifugation in an Eppendorf microcentrifuge at 4°, the supernatant was removed and the DNA was hydrolysed by heating at 70° for 30 min in 50 μl 0.1 N HCl. The concentration of O^6MeG in the acid supernatant was determined by hplc analysis on a Whatman Partisil 10SCX column eluted isocratically with 25 mM ammonium formate, pH 4, with 10% methanol at a flow rate of 1.5 ml/min. Fractions corresponding to O^6MeG were collected and the radioactivity was determined in Optiphase scintillation fluid (Fisons, Loughborough) using a Beckman Tri-carb 2000CA scintillation analyzer. The concentration of [^{3}H]O^6MeG in DNA was determined from the specific activity of the [^{3}H methyl] nitrosourea.

RESULTS

The toxicities of the nitrogen mustard type alkylating agent, chlorambucil, and the three imidazotetrazinone derivatives against a range of human and murine tumour cell lines are shown in Table 1. All of the cell lines show about equal sensitivity towards chlorambucil with the ID_{50} values only varying between 1 and 5 μM. In contrast there is a wide variation in response to the imidazotetrazinones with the ID_{50} values for mitozolomide varying 165-fold and CCRG 81045 30-fold. In contrast the ethyl analogue, CCRG 82019, shows only a 3-fold difference in ID_{50} values between the various cell lines. The toxicity of both mitozolomide and CCRG 81045 correlates with the level of the repair enzyme O^6MeGMT in the cell (Table 1 and Fig. 2). Thus cell lines with low levels of O^6MeGMT such as GM892A are sensitive, while cell lines with high enzyme levels such as Raji, JAR, MAC16 and A549 are much more resistant to these agents. In contrast the toxicity of CCRG 82019 does not show any correlation with the level of the O^6MeGMT protein in the cell. These results suggest that the primary cytotoxic lesion produced by both mitozolomide and CCRG 81045 involves alkylation at the O^6-position of guanine in DNA.

When Raji cells are incubated with CCRG 81045 there is a rapid depletion of O^6MeGMT (Fig. 3). This again suggests an interaction of CCRG 81045 with the O^6-position of guanine residues in DNA, followed by suicide attack of the repair protein on the O^6 alkylated guanines, since no inhibition of enzyme activity was observed in *in vitro* incubations.

The activity of O^6MeGMT in Raji cells has been shown to be reduced by the inclusion of free O^6MeG in the culture medium [16]. Similar results were obtained with A549, JAR, MAC16 and Raji cells in the present study (Table 2). Neither O^6MeG or O^6EtG had any effect on cell growth at concentrations below 1 mM. Depletion of O^6MeGMT is dependent on the concentration of O^6MeG in the

Table 1. O^6-Alkylguanine-DNA alkyltransferase activity and sensitivity of cell lines to various alkylating agents

Cell line	O^6MeGMT fmol/mg protein (± SEM)	ID_{50} μM* (± SEM) Chlorambucil	Mitozolomide	CCRG 81045	CCRG 82019
MAC 16	320 ± 75	3.0 ± 0.2	62 ± 4	245 ± 8	360 ± 15
Raji	634 ± 80	1.0 ± 0.5	20 ± 7	206 ± 20	229 ± 30
A549	391 ± 60	5.0 ± 0.4	24 ± 5	299 ± 30	200 ± 15
K562	87 ± 40	2.0 ± 0.5	0.8 ± 0.3	15 ± 5	128 ± 30
GM 892	10 ± 5	2.6 ± 0.3	2 ± 0.5	10 ± 7	229 ± 20
JAR	504 ± 70	5.0 ± 0.5	132 ± 15	293 ± 30	504 ± 40

* Concentration required to give 50% inhibition of cell growth or colony forming ability.

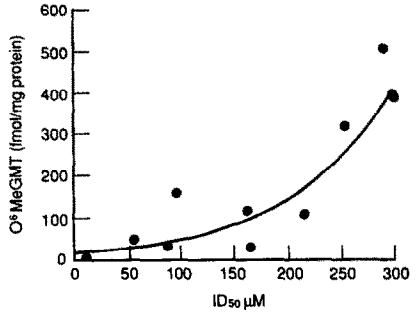


Fig. 2. Correlation of O^6MeGMT with the ID_{50} of CCRG 81045 towards MAC16, JAR and A549 cells in the presence or absence of O^6MeG. (Correlation coefficient 0.86.)

Table 2. Effect of exposure to either 0.1 mM (A) or 0.5 mM (B) exogenous O^6MeG for 16 hr on the level of O^6MeGMT in cell extracts

Cell line	O^6MeGMT (fmole/mg protein)* A	B
JAR	109 (22)	32 (6)
A549	158 (41)	35 (9)
MAC16	116 (38)	50 (16)
Raji	393 (62)	70 (11)

* Percentage of original O^6MeGMT left after incubation with free O^6MeG in brackets.

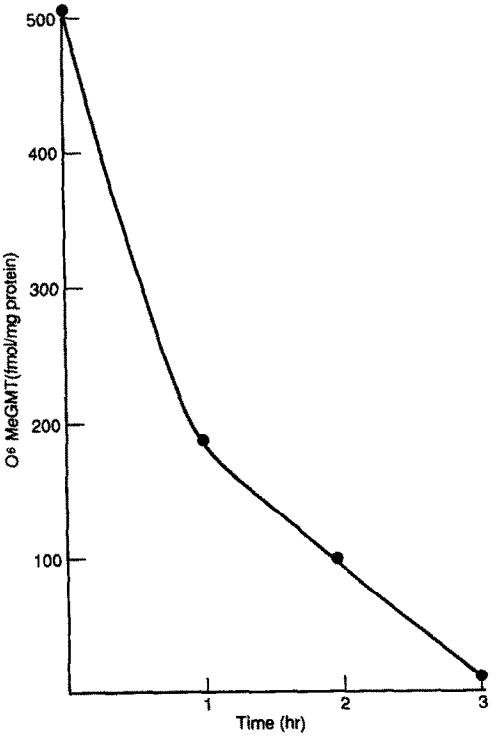


Fig. 3. Effect of CCRG 81045 on O^6MeGMT in Raji cells. Raji cells (8×10^5/ml) were incubated with 288 μM CCRG 81045 and samples were assayed for O^6MeGMT activity at the indicated times.

culture medium and is less effective in Raji cells than in the other cell lines. Both guanine and 7-methylguanine (7-MeG) are ineffective in reducing methyltransferase activity.

The effect of overnight growth in O^6MeG (0.1 or 0.5 mM) on the subsequent sensitivity of the cell lines to mitozolomide, CCRG 81045 and CCRG 82019 is shown in Table 3 and Fig. 4. For JAR, A549 and MAC16 cells the toxicity of both mitozolomide and CCRG 81045 is increased in O^6MeGMT-depleted cells, while for Raji cells growth in the presence of these agents is unaffected by prior treatment with O^6MeG. Depletion of O^6MeGMT by O^6MeG in Raji cells has also been shown not to lead to an increased sensitivity towards MNNG or the cross-linking nitrosoureas [16]. In this study Raji cells were the least responsive to O^6MeGMT-depletion by 0.1 mM O^6MeG, although at 0.5 mM O^6MeG, methyltransferase levels were reduced to a level comparable with the other cell lines. For JAR, A549 and MAC16 cells an exponential relationship exists between the ID_{50} for CCRG 81045 and the level of the repair enzyme (Fig. 2). This suggests that very high levels of the repair enzyme would be required to completely eliminate the cytotoxic effect of the imidazotetrazinones. Unlike mitozolomide and CCRG 81045 the ethyl analogue CCRG 82019 appears to be almost equally toxic in cell lines with wide variations in repair protein (Table 1) and the

Table 3. Effect of exposure to either 0.1 mM (A) or 0.5 mM (B) exogenous O^{6}MeG for 16 hr on the toxicity of mitozolomide, CCRG 81045 and CCRG 82019

Cell line	ID_{50} μM (± SEM) Mitozolomide		CCRG 81045		CCRG 82019	
	A	B	A	B	A	B
MAC16	49 ± 5	19 ± 3	162 ± 10	68 ± 8	333 ± 10	363 ± 15
Raji	14 ± 4	18 ± 2	172 ± 20	145 ± 40	230 ± 10	218 ± 20
A549	18 ± 2	11.5 ± 1	96 ± 3	86 ± 5	200 ± 20	212 ± 15
JAR	103 ± 10	54 ± 8	216 ± 20	165 ± 8	504 ± 10	520 ± 20

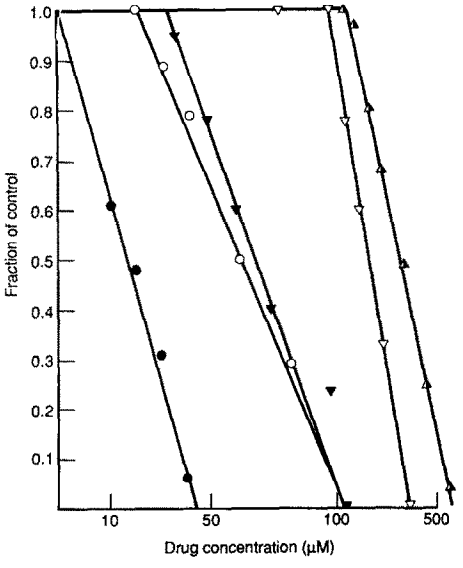


Fig. 4. Effect of mitozolomide (○), CCRG 81045 (▽) and CCRG 82019 (▲) on the growth of MAC16 cells in the abssence (open symbols) and the presence (closed symbols) of 0.5 mM O^{6}MeG.

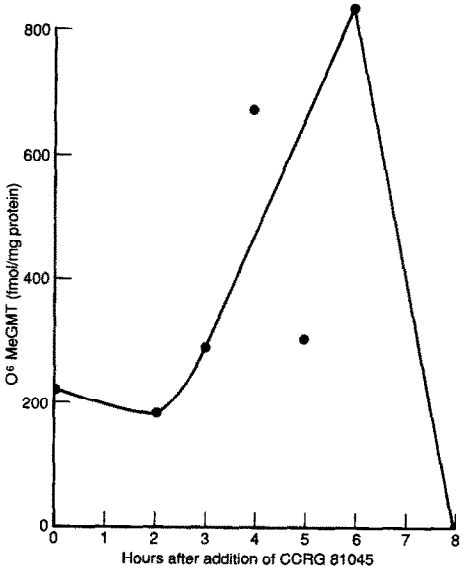


Fig. 5. Effect of 98 μM CCRG 81045 on the activity of O^{6}MeGMT in methyltransferase-depleted Raji cells. Raji cells were treated with 0.5 mM O^{6}MeG for 16 hr prior to addition of CCRG 81045. The O^{6}MeGMT in cell-free extracts was monitored by removing portions of cell suspension at various times after addition of CCRG 81045 and enzyme activity was determined as described in Materials and Methods.

cytotoxicity is not increased after a large decrease in the O^{6}MeGMT (Table 3). This suggests that this agent may exert its cytotoxic effect other than through alkylation of the O^6-position of guanine.

To investigate the apparent lack of sensitization of Raji cells to the imidazotetrazinones after treatment with O^{6}MeG the effect of CCRG 81045 on the O^{6}MeGMT in cells previously treated with 0.5 mM O^{6}MeG was determined (Fig. 5). The level of the methyltransferase increased after a 2 hr lag period reaching a peak level 6 hr after drug addition. This was followed by a rapid loss of enzyme activity during the next 2 hr, presumably due to removal of alkyl lesions from DNA. The kinetics of appearance of the O^{6}MeGMT were similar to that observed in Raji cells depleted of O^{6}MeG by washing [16].

DISCUSSION

Cellular resistance to the cytotoxic effects of the chloroethylnitrosoureas appears to be associated with an increased activity of O^{6}MeGMT [17]. The effectiveness of the chloroethyl and methylimidazotetrazinones in inhibiting the growth of cells differs from that of a conventional bifunctional alkylating agent, chloambucil, but is similar to that of the nitrosoureas in depending on the level of the repair protein O^{6}MeGMT. Thus both mitozolomide and CCRG 81045 show preferential toxicity towards O^{6}MeGMT deficient (Mer$^-$) cell lines, while the ethyl analogue CCRG 82019 shows no differential toxicity between Mer$^+$ and Mer$^-$ cell lines. A similar relationship has been shown among a series of alkyltriazenylimidazoles where the momomethyltriazene and some hydroxymethyl derivatives capable of generating the monomethyltriazene *in situ* showed preferential toxicity towards a Mer$^-$ cell line, while the monoethyltriazenes showed no differential toxicity between Mer$^-$ and Mer$^+$ cell lines [10]. Both the chloroethyltriazenes and imidazotetrazinones would be selectively toxic towards Mer$^-$ cells due to an initial chloroethylation of the O^6-position of guanine followed by cross-linking of DNA. However, the methyl analogues would be incapable of cross-linking DNA, but still show preferential toxicity towards Mer$^-$ cells. In the study of alkyltriazenylimidazoles [10] neither the formation of DNA single-strand breaks or DNA-protein cross-links could account for the differential cytotoxicity of the monomethyltriazenes to Mer$^-$ cells.

The importance of O^6-alkylation of guanine in the cytotoxicity of these agents has been investigated by the depletion of O^6MeGMT by free O^6MeG. Previous studies [18, 19] have shown a marked increase in sensitivity of human fibroblasts and tumour cells with the Mer^+ phenotype to cell killing by 1-(2-chloroethyl)-1-nitrosourea (CNU) and MNNG after pre-treatment with O^6MeG, which is probably a weak substrate for the methyltransferase [19]. However, treatment of another Mer^+ cell line, Raji a Burkitts lymphoma, with free O^6MeG did not sensitise the cells to killing by MNNG or CNU [16], which led to the suggestion that adducts at the O^6 atom of guanine in DNA are not potentially cytotoxic lesions. In the present study there was an increased sensitization of the Mer^+ cell lines, MAC16, JAR and A549 towards both mitozolomide and CCRG 81045 after depletion of the methyltransferase with free O^6MeG. In contrast there was no sensitization towards the ethyl analogue CCRG 82019 by O^6MeG suggesting a mechanism of cytotoxicity of this agent other than through *O*-alkylation of guanine. The Raji cell line appears to differ from the other Mer^+ cells used in this study in showing no increased sensitization towards either mitozolomide or CCRG 81045 after pre-treatment with O^6MeG. However, in Raji cells O^6MeGMT activity reappeared transiently after treatment with CCRG 81045, and was then lost, presumably due to removal of O^6MeG from alkylated DNA. This result is unusual in that it has been shown [20] that no induction of O^6MeGMT occurs in HeLa cells treated with multiple doses of MNNG. Thus overall these results suggest that alkylation of the O^6 position of guanine is important in the mechanism of cytotoxicity of the imidazotetrazinones.

If O^6MeG is a potentially cytotoxic lesion the question arises as to the mechanism of cytotoxicity and the reason for the ineffectiveness of the ethyl analogues. On chemical grounds the ethyl analogues should be more reactive to the O^6-position of DNA-guanine than the methyl analogues, although in comparison with the nitrosoureas this difference might be expected to be small [21]. The major difference may be the overall reaction with DNA, which with ethylnitrosourea has been shown to be 40-times less than with methylnitrosourea [22]. Although this would explain why higher concentrations of the ethyl analogue were required to inhibit growth, if O^6EtG was a cytotoxic lesion the agent should still display preferential toxicity towards cells with low levels of O^6MeGMT.

One potential biological effect produced by O^6-alkylation of guanine may be an inhibition of cytosine methylation. It has recently been shown [23] that CCRG 81045 causes hypomethylation of newly synthesized DNA in K562 cells induced to differentiate with this agent. Alkylation of DNA by methylnitrosourea also leads to a reduction in methylation of this substrate by DNA (cytosine-5)methyltransferase, which was attributed to the formation of either methylphosphotriesters or O^6MeG [24]. The site for methylation of DNA is always in the sequence 5′G-C 3′ and recent evidence [25] suggests that the relative reactivity of O^6-G with respect to N^7-G is much greater in the sequence CGC than in the sequence GGG. Inhibition of cytosine methylation may lead to an inhibition of cell growth as has been shown between the anti-leukaemic activity of 5-azacytidine and its capacity to inhibit DNA methylation [26]. Interestingly the ethyl analogue, CCRG 82019, inhibited growth of K562 cells without affecting cytosine methylation in contrast with the methyl analogue [23].

The present results would suggest that the primary cytotoxic lesion produced by both mitozolomide and CCRG 81045 is alkylation of DNA at the O^6-position of guanine. With mitozolomide elimination of the chlorine atom upon reaction of the O^6-bound chloroethyl group with the N^1 of the same guanine followed by a rearrangement of the ethylene carbon from the guanine O^6 to the N^3 of the cytosine across the double helix will lead to a second lesion which is susceptible to repair. This may account for the fact that the difference in toxicity between Mer^- and Mer^+ cells is less with mitozolomide than CCRG 81045.

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