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# ANTITUMOUR IMIDAZOTETRAZINES—XVIII

## MODIFICATION OF THE LEVEL OF 5-METHYLCYTOSINE IN DNA BY 3-SUBSTITUTED IMIDAZOTETRAZINONES

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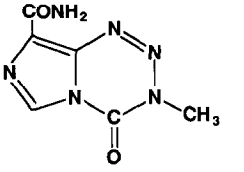
**Abstract**—The effect of 3-methyl(temozolomide) and 3-ethyl (CCRG 82019) substituted imidazotetrazinones on cytosine methylation has been studied in the human lymphoblastoid cell line GM892. There was a decrease in the 5-methylcytosine content of newly synthesized DNA in cells treated with the 3-methyl and a small increase in cells treated with the 3-ethyl analogue, which was maximal 4 days after drug treatment. There was a progressive decrease in nuclear DNA methyltransferase after treatment with temozolomide with complete inhibition at 11–12 hr after drug addition, followed by a re-establishment of enzyme levels towards control values. While the free drugs had no effect on DNA methyltransferase activity *in vitro*, DNA isolated from GM892 cells previously treated with temozolomide inhibited the transfer of methyl groups from *S*-adenosyl-L-methionine to *M. lysodeiktious* DNA. The maximum effect was observed at 6 hr after drug addition and was proportional to the concentration of temozolomide to which the cells had previously been exposed. These results suggest that temozolomide may induce a block in cellular replication as a result of an indirect inhibition of DNA methylation and cells which escape this block progress with hypomethylated DNA.

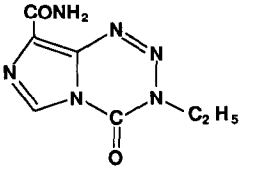
In the series of 3-alkyl substituted imidazotetrazines, antitumour activity is only observed with 3-(2-chloroethyl) (mitozolomide, CCRG 81010) and 3-methyl (CCRG 81045, temozolomide) groups, while 3-ethyl (CCRG 82019) and higher alkyl substituents lead to a decrease in activity (Fig. 1) [1]. In order to understand the structure–activity relationship among the simple alkyl homologues we have been studying the K562 human erythroleukaemia cell line, which undergoes an increase in the number of haemoglobin-producing cells three days after drug treatment in the presence of the 3-methyl but not the 3-ethyl analogues [2]. In addition the concentration of 5-methylcytosine (5-MC) in the DNA of CCRG 81045 treated cells decreases three days after drug treatment, and is directly proportional to the number of haemoglobin producing cells [3].

Hypomethylation of DNA is associated with the induction of erythroid differentiation by 5-azacytidine, 5-aza-2′-deoxycytidine [4] and L-ethionine [5]. In addition the antileukaemic action of 5-aza-2′-deoxycytidine has been correlated with its effects on DNA methylation [6]. This suggests the possibility that the tumour inhibitory activity of temozolimide is exerted via its effect on DNA methylation.

In order to assess the generality of the hypomethylation of DNA produced by temozolomide and its role, if any, in the inhibition of cellular replication, the effect of this agent on the 5-methylcytosine content of DNA isolated from GM892 cells has been determined. This cell line does not undergo morphological transformation in the presence of temozolomide, but is highly sensitive to its growth inhibitory effect. In addition CCRG 82019 is much less potent as a growth inhibitor than is temozolomide.




**Temozolomide**




**CCRG 82019**

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

### MATERIALS AND METHODS

*S*-Adenosyl-L-[methyl-$^3$H]methionine (sp. act. 80 Ci mmol$^{-1}$), [6-$^3$H]uridine (sp. act. 20 Ci mmol$^{-1}$) and eukaryotic DNA methylase were purchased from Amersham International, Bucks. Tissue culture medium and foetal calf serum were purchased from Gibco Europe Ltd. (Paisley, Scotland). CCRG 81045 (temozolomide) and CCRG 82019 were synthesized by May and Baker Ltd. (Dagenham, Essex). *M. lysodeiktious* DNA and all other chemicals were obtained from Sigma Chemical Co. (Poole, Dorset).

*Cell culture conditions*. Cells were maintained in static suspension culture at 37° in RPMI 1640 medium containing 10% foetal calf serum under an atmosphere of 5% $CO_2$ in air. Both temozolomide and

Table 1. Effect of temozolomide and CCRG 82019 on the enzymatic methylation of DNA* in GM 892 cells

| Treatment | Day 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| None | 3.40 ± 0.21 | 3.55 ± 0.11 | 3.43 ± 0.1 | 3.41 ± 0.08 | 3.53 ± 0.13 |
| Temozolomide 26 μM | 3.90 ± 0.3 | 3.22 ± 0.14 | 3.13 ± 0.08 | 3.23 ± 0.13 | 3.46 ± 0.12 |
| Temozolomide 52 μM | 3.90 ± 0.2 | 3.22 ± 0.08 | 3.29 ± 0.13 | 3.14 ± 0.10 | 3.29 ± 0.09 |
| Temozolomide 103 μM | 3.5 ± 0.1 | 3.65 ± 0.12 | 3.37 ± 0.05 | 2.94 ± 0.12 | 3.23 ± 0.11 |
| Temozolomide 206 μM | — | — | — | 2.50 ± 0.3 | 2.95 ± 0.12 |
| CCRG 82019 240 μM | 3.6 ± 0.14 | 3.8 ± 0.14 | 3.31 ± 0.15 | 3.82 ± 0.25 | 3.4 ± 0.35 |
| CCRG 82019 480 μM | — | — | 3.42 ± 0.22 | 3.38 ± 0.14 | 2.95 ± 0.25 |

* Results are means of at least three determinations for each time point and are given ± SEM.

The figures refer to percent of total cytosines methylated $\frac{5MC}{C + 5MC} \times 100$.

CCRG 82019 were dissolved in dimethyl sulphoxide (DMSO) at 20 mg ml$^{-1}$ such that the final concentration of DMSO in the culture medium did not exceed 0.3%. For labelling of DNA [6-$^3$H]uridine (1 μCi ml$^{-1}$] was added to the culture medium 24 hr prior to harvesting the cells.

*Isolation and hydrolysis of nucleic acids.* This was carried out as previously described [3]. Separation of bases was achieved by high-performance liquid chromatography on a Patisil 10 SCX column (0.6 × 25 cm, Whatman) eluted isocratically with 0.035 M $KH_2PO_4$, pH 2.5, in an Altex HPLC system. Bases were identified relative to the elution of authentic compounds and their quantity determined by measurement of the base peak area at 280 nm. The eluted material was collected in scintillation vials and the radioactivity was determined in Optiphase scintillation fluid (Fisons, Loughborough) using a Beckman Tri-carb 2000 CA scintillation analyzer. The 5-methylcytosine (5-MC) content of the samples was determined in triplicate and at least three separate determinations were made for each concentration of drug and day of incubation. The extent of enzymatic methylation was calculated according to the formula

$$\frac{100 \times \text{5-MC}}{\text{Cytosine} + \text{5-MC}}$$

*DNA methylase assay.* The standard assay contained 20 mM Tris–HCl, pH 7.5, 5 mM dithiothreitol, 1 mM EDTA, 0.1 mM EGTA, 16 μM *S*-adenosyl-L-(methyl-$^3$H)-methionine (sp. act. 2.5 Ci m mol$^{-1}$) 20 μg heat denaturated *M. lysodeiktious* DNA and enzyme in a total volume of 50 μl. Incubations were routinely carried out for 4 hr at 37° and were terminated by cooling the samples to 4° and calf thymus DNA (40 μg) was added as carrier. The samples were treated with 200 μl of 1 N NaOH and heated at 60° for 15 min, cooled, and the DNA was precipitated with 85 μl of cold 5 N $HClO_4$. The DNA was washed onto glass fibre discs with cold 5% TCA and washed with absolute ethanol and the radioactivity was determined in Luma-Gel scintillation fluid (M & B, Loughborough) using a Packard Tri-carb 2000 CA scintillation analyzer. The protein content of nuclear extracts was determined by the method of Lowry *et al.* [7] using bovine serum albumin as a standard.

*Isolation of DNA.* DNA was isolated from GM892 cells by the method of Warren [8]. Cells were treated with 6% 4-aminosalicylic acid and lysed with 10% SDS. After extraction with phenol reagent the DNA was precipitated with 2-ethoxyethanol, washed extensively with 70% ethanol followed by absolute ethanol, and allowed to dry overnight.

## RESULTS

The human lymphoblastoid cell line GM892 was selected for study because of a high degree of sensitivity to temozolomide ($ID_{50}$ 17.5 ± 1.3 μM) while CCRG 82019 was much less cytotoxic ($IC_{50}$ 229 ± 20 μM). The effect of both agents on the extent of methylation of newly synthesized DNA was determined directly by measuring the concentration of 5-methylcytosine in DNA following incorporation with [6-$^3$H]uridine. The results in Table 1 show that when temozolomide was present in the medium of GM892 cells at different initial concentrations the overall extent of enzymatic methylation decreased in a dose-dependent manner from 2 days after drug addition with some reversion to control values by 5 days. No change in methylation pattern was observed during the first 24 hr after drug addition. In contrast CCRG 82019 appears to have a small stimulatory effect on cytosine methylation at a concentration of 240 μM.

The inhibition of DNA methylation by temozolomide would be expected to produce hemimethylated DNA which could function as an efficient substrate for maintenance DNA methyltransferase *in vitro* as is the case for 5-aza-2′-deoxycytidine treatment of cells [6]. Thus, the extent of inhibition of DNA methylation may also be determined by assaying DNA, isolated from treated cells, for its ability to accept methyl groups from *S*-adenosyl-L-methionine in the presence of exogenous DNA methyltransferase. The methyl acceptor activities of DNA from GM892 cells treated with various concentrations of temozolomide at 4 and 5 days after drug treatment is shown in Fig. 2. The methyl acceptor activities were dependent upon the concentration of temozolomide and were higher at 4 days after drug administration than after 5 days. The methyl acceptor activity of DNA from GM892 cells was increased almost 2-fold at 206 μM temozolomide

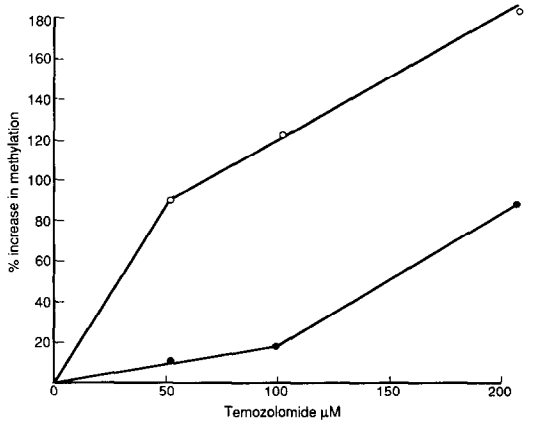



Fig. 2. *In vitro* methylation of DNA isolated from temozolomide treated GM892 cells 4 (○) and 5 (●) days after drug treatment by eukaryotic DNA methylase. DNA was extracted from drug treated cells as described in Materials and Methods and 10 μg was used as a substrate for the methylase enzyme. The results are expressed as the percentage increase in methylation of DNA from drug-treated cells over that of non drug-treated cells. DNA isolated from non drug-treated cells incorporated 0.09 pmol of methyl groups per μg of DNA.

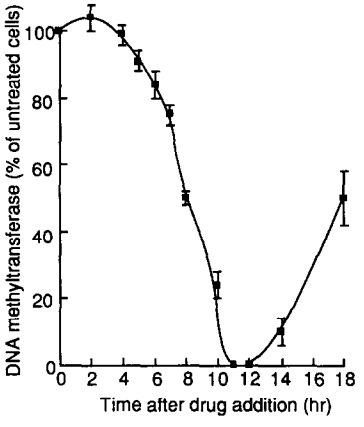



Fig. 3. Effect of temozolomide (103 μM) on the activity of cytosine methylase in GM892 cells. At the indicated time points aliquots of the cells were removed, sedimented by centrifugation and washed with 0.9% NaCl. Cells were lysed by treating with 10 mM Tris–HCl, pH 7.5, 10 mM EDTA and 0.5% Nonidet P40. The nuclear pellet was sedimented by centrifugation and the nuclear proteins were extracted with 20 mM Tris–HCl, pH 7.5, 5 mM dithiothreitol, 1 mM EDTA, 0.1 mM EGTA, 0.2 mM phenylmethyl sulphonyl fluoride containing 0.3 M NaCl. The nuclear protein extract was used directly for the measurement of DNA methylase activity as described in Materials and Methods. The results are expressed as a percentage change in enzyme activity in drug-treated cells.

4 days after drug treatment. This also shows that DNA isolated from treated cells is capable of enzymatic methylation in an *in vitro* situation.

In order to investigate the mechanism of DNA hypomethylation the effect of treatment of GM892 cells with temozolomide on the activity of nuclear DNA methyltransferase was investigated as a function of time. The results presented in Fig. 3 show

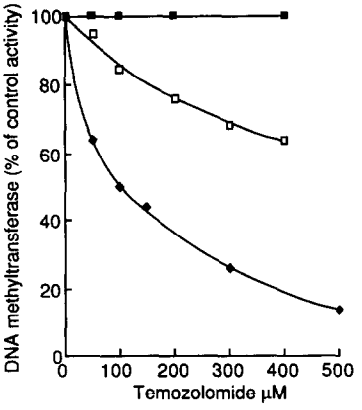



Fig. 4. Effect of DNA extracted from GM892 cells on the methylation of *M. lysodeiktious* DNA by eukaryotic DNA methylase. Cells were treated with the indicated concentrations of temozolomide and DNA was extracted at 6 hr (◆), 12 hr (□) and 24 hr (■) after drug treatment. The standard incubation assay for DNA methylase was as described in Materials and Methods but contained in addition 10 μg of cellular DNA. The results are expressed as a percentage change in methylation produced by DNA from drug treated cells relative to non-treated cells.

Table 2. Effect of temozolomide on the activity of DNA methyltransferase *in vitro**

| Temozolomide (mM) | pmole $CH_3$ group transferred per μg of DNA |
|---|---|
| 0 | 1.15 |
| 0.25 | 1.05 |
| 0.5 | 1.01 |
| 1 | 1.17 |
| 2.5 | 1.21 |
| 5 | 1.05 |
| 10 | 1.07 |
| CCRG 82019 (mM) | |
| 5 | 1.21 |
| 10 | 1.18 |

* Eukaryotic DNA methylase (IU) plus *S*-adenosyl-L-(methyl$^3$H)-methionine (16 μM) were incubated with the indicated concentrations of temozolimide for 2 hr at 37°, after which enzyme activity was determined as described in Materials and Methods.

that temozolomide produced a progressive decrease in enzyme activity which reached a minimum 11 to 12 hr after treatment, followed by a re-establishment of enzyme activity towards control values.

Although enzyme activity is reduced in treated cells, *in vitro* assays using *M. lysodeiktious* DNA as methyl acceptor and purified DNA methyltransferase show neither temozolomide or CCRG 82019 to have any direct effect on cytosine methylation at concentrations up to 10 mM (Table 2). This suggests that enzyme inhibition *in situ* arises from an indirect effect of these agents, possibly as a secondary consequence of DNA alkylation.

The results presented in Fig. 4 show a direct inhibitory effect of DNA isolated from GM892 cells treated

Table 3. Effect of DNA extracted from GM892 cells 6 hr after treatment with CCRG 82019 on the methylation of *M. lysodeiktious* DNA by eukaryotic DNA methylase*

| CCRG 82019 μM | DNA methyltransferase (% of control) |
|---|---|
| 0 | 100 |
| 192 | 101 |
| 288 | 106 |
| 384 | 110 |
| 480 | 103 |
| 600 | 94 |
| 721 | 97 |
| 960 | 104 |

* DNA was extracted from CCRG 82019 treated cells as described in Materials and Methods. Each incubation contained 20 μg of *M. lysodeiktious* DNA and 10 μg of cellular DNA.

with temozolomide on the methylation of *M. lysodeiktious* DNA using purified DNA methyltransferase. The results are expressed relative to the methylation of *M. lysodeiktious* DNA in the presence of the same concentration of DNA from GM892 cells not exposed to temozolomide. The effect is dose-related and the maximum effect was observed at 6 hr after drug addition, after which there was a progressive decrease in inhibitory activity. Since the inhibitory effect of DNA isolated from temozolomide treated cells precedes the maximum inhibition of cellular DNA methyltransferase it suggests that the alkylated DNA may be responsible for the decrease in enzyme activity. In contrast to temozolomide, DNA isolated from GM892 cells treated with CCRG 82019 for 6 hr had no effect on the *in vitro* methylation of *M. lysodeiktious* DNA even after treatment with concentrations up to 1 mM (Table 3). These results suggest that the alkylated lesion in DNA responsible for enzyme inhibition is either repairable or is not formed in intact cells with the same kinetics as for temozolomide since DNA treated directly with CCRG 82019 is a much more effective inhibitor of DNA methyltransferase than is that treated with temozolomide [9].

## DISCUSSION

Methylation of cytosine residues at critical CpG sites may act as a regulatory sequence for the expression of some eukaryotic genes [10]. In particular double methylation has been shown to reduce the transforming activity of the human Ha-*ras* oncogene by about 80% [11], while DNA from both benign colon polyps and malignant carcinomas has been shown to be substantially hypomethylated when compared with DNA from adjacent normal tissues [12].

Alkylation of DNA with *N*-methyl-*N*-nitrosourea (MNU) impairs the ability of restriction endonucleases to cleave this substrate [13]. This suggests that modification of DNA with other alkylating agents may also affect the action of other enzymes recognizing specific DNA sequences. Since DNA methylation is also catalysed by sequence specific enzymes [14] modifications of DNA may lead to alterations in methylation patterns. Indeed treatment of DNA with a number of chemical carcinogens leads to an inhibition of DNA methyltransferase activity and this may be related to their oncogenic activity. Thus benzo[*a*]pyrene diol epoxide [15], *N*-acetoxy-*N*-acetylaminofluorene [16], nitrogen mustard, 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) and *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (MMNG) alkylation [17] of DNA leads to an inhibition of DNA methylation *in vitro*. Two agents which produce a similar alkylating moiety to temozolomide, MNU [18] and MMNG [19] produce hypomethylation of newly replicated DNA in Raji cells, which persists in the cell cycles following the treatment.

In view of the similarity of the alkylating species produced by the antitumour imidazotetrazinones and the antitumour nitrosoureas [20] the possible role, if any, of DNA methylation in the cytotoxicity of the imidazotetrazinones has been studied, with particular emphasis on structure–activity relationships for the methyl and ethyl derivatives. In GM892 cells treated with temozolomide, but not CCRG 82019, a delayed hypomethylation of DNA is seen, which follows a time course similar to that observed during the induction of haemoglobin synthesis in K562 cells [3]. In contrast CCRG 82019 at a concentration of 240 μM caused a slight increase in the 5-methylcytosine content of cellular DNA. Since GM892 cells do not appear to undergo morphological or functional differentiation in response to temozolomide this suggests a drug-specific effect, which probably results from an inhibition of the methylase enzyme at earlier time points. Indeed, there is a reduction in the total extractable nuclear DNA methyltransferase activity within 5 hr after treatment with temozolomide with complete inhibition occurring within 11–12 hr.

DNA extracted from GM892 cells 4 days after temozolomide addition is a more efficient substrate for DNA methyltransferase *in vitro* than is DNA extracted from cells 5 days after drug addition. The methyl acceptor activity of DNA *in vitro* is greater than observed in intact cells probably because the enzyme to DNA ratio *in vitro* is higher than attained in intact cells. Thus, even DNA isolated from non drug-treated cells incorporates 0.9 pmole of methyl groups per μg of DNA, although this does not happen in intact cells. However, there does appear to be a qualitative correlation between hypomethylation of DNA in intact cells and an increased methyl accepting ability of the DNA *in vitro*.

It has previously been shown [9] that calf thymus DNA alkylated *in vitro* by both temozolomide and CCRG 82019 is an effective inhibitor of the transfer of methyl groups from *S*-adenosyl-L-methionine to *M. lysodeiktious* DNA by eukaryotic DNA methylase. When the extent of alkylation of DNA by these two agents is taken into account DNA modified with CCRG 82019 was found to be approximately seven times more effective as a methylase inhibitor than DNA modified with temozolomide. Using DNA extracted from GM892 cells treated with the two imidazotetrazinones a reversal of the order of potency is observed with temozolomide modified DNA being much more potent as an enzyme inhibi-

tor than CCRG 82019 modified DNA. We have previously observed that the level of alkyl groups bound to DNA by these two agents is different in intact cells than when the drugs are incubated directly with DNA [21]. Thus, when DNA is incubated *in vitro* with the two agents at a concentration of 100 $\mu$M temozolomide results in 0.15 ± 0.02 pmoles of drug bound per $\mu$g of DNA while CCRG 82019 produces only 0.045 ± 0.0005 pmoles of drug bound per $\mu$g of DNA. However, in intact cells about 0.3 pmoles of both drugs are found per $\mu$g of cellular DNA over the first 24 hr after treatment. It is therefore difficult to reconcile differences in potency of cellular DNA as an enzyme inhibitor after drug addition with the total number of alkyl groups bound, although the relative alkylation of the individual bases may be important. These results, together with the decrease in potency of DNA from temozolomide treated cells as an enzyme inhibitor with increasing time after 6 hr of drug incubation, suggest that the alkylated lesion in DNA responsible for enzyme inhibition is readily repairable. Pfohl-Leszkowicz *et al.* [22] have shown that alkylation of DNA by dimethylsulphate, which yields mostly 7-methylguanine and 3-methyl-adenine did not affect enzymatic methylation, while alkylation with MNU, which in addition to producing these two base modifications also produced methylphosphotriesters and $O^6$-methylguanine caused an inhibition of enzymatic methylation. The percentage of various methylated bases produced by the imidazotetrazinones is similar to that of the nitrosoureas with about 5.4% being $O^6$-alkylated products [23]. However, temozolomide produces predominantly 7-methylguanine whereas CCRG 82019 produces predominantly phosphotriesters. The GM892 cell line utilized in this study is deficient in the repair of $O^6$-alkylated guanines, although it does contain low levels of repair activity [24]. The greater effectiveness of the ethyl analogue utilizing directly alkylated DNA suggested that phosphotriesters may be the important lesion in inhibiting DNA methyltransferase, but phosphotriesters appear to be remarkably stable in both rat and human cells [25]. Thus the alkylated bases responsible for enzyme inhibition is not known at present, but is currently under investigation.

The potential role, if any, of the inhibition of DNA methyltransferase to the mechanism of growth inhibition produced by the imidazotetrazinones remains speculative. DNA methyltransferase is an S-phase specific enzyme [26] and in analogy with other S-phase specific enzymes inhibition might be expected to block the cells in late S phase or $G_2$ phase. Flow cytometric studies of L1210 leukaemia cells treated with temozolomide showed an arrest of cells in the late $S/G_2$ phase of the cell cycle [27]. Inhibition of DNA methylation by 5-aza-2′-deoxycytidine has been suggested as a possible mechanism of chemotherapeutic action [6], although this agent can also produce its lethal effects by incorporation into DNA [28]. Since the block to enzyme activity appears to be reversible it would also explain why temozolomide displays a better antitumour activity when given as a split-dose schedule rather than as a single dose as with mitozolomide [29]. Cells escaping the block would have hypomethylated DNA and the potential of displaying aberrant gene expression and malignant progression. Further studies are aimed at evaluating the potential role of inhibition of DNA methylation as a mechanism of growth inhibition.

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