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ANTITUMOUR IMIDAZOTETRAZINES AND GENE EXPRESSION

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Abstract

The effect of 3-alkyl substituted imidazotetrazinones on methylation of DNA has been studied in drug sensitive and resistant cell lines. The 3-methyl analogue (Temozolomide) has been shown to cause a decrease in the level of 5-methylcytosine in newly synthesized DNA in both cell lines, although the effect occurred at lower drug concentrations in the drug sensitive cell line. In order to investigate the mechanism of hypomethylation of DNA, calf thymus DNA was alkylated in vitro by both Temozolomide and the 3-ethyl analogue, CCRG 82019, and the alkylated DNA was shown to inhibit the transfer of methyl groups from S-adenosyl-L-methionine to M. lysodeikticus DNA by purified eukaryotic DNA methylase. Neither free drug alone or unmodified DNA affected the methylase reaction. Calf thymus DNA modified with CCRG 82019 was more effective as a methylase inhibitor than DNA modified with Temozolomide, which was a reverse of the order of potencies of the free drugs against tumour cells in culture. CCRG 82019 modified DNA also formed a more stable complex with nuclear proteins. Alterations in the level of 5-methylcytosine in DNA may be important in the alteration of gene expression by these agents.

Key words: Chemotherapy; antitumour imidazotetrazines, gene expression.

The imidazotetrazines are a novel group of synthetic heterocycles bearing NNN linkages in a cyclic arrangement (22), which display potent and broad spectrum antitumour activity against murine tumours (14, 21). Substitution of the heterocyclic ring at position 3 with either 2-chloroethyl (Mitozolomide) or methyl (Temozolomide) yields highly active agents, while 3-ethyl (CCRG 82019) or higher alkyl substitution leads to a loss of antitumour activity (21) (Fig. 1). Under alkaline conditions ring opening occurs to form the corresponding alkyl triazenes (1), which are the active biological moieties (15). Mitozolomide ring opens to produce 5-(3-(2-chloroethyl)triazen-1-yl) imidazole-4-carboxamide (MCTIC) a potent antitumour agent against mouse L1210 leukaemia (20).

The pharmacological activity of the antitumour triazenes has been attributed to their reaction with DNA. At equimolar concentrations both Mitozolomide and MCTIC have similar in vitro cytotoxicities, and at equitoxic concentrations they produce similar levels of DNA interstrand cross-linking (12). Cells deficient in their ability to repair O^6-alkylguanine lesions (Mer^-) were shown to be more sensitive to the cytotoxic effects of Mitozolomide than repair proficient cells (Mer^+) (13). Furthermore, a concentration-dependent DNA interstrand cross-link formation was observed for both Mitozolomide and MCTIC in Mer^- cells, while in Mer^+ cells little or no interstrand cross-link formation was detected (11, 13). Thus DNA interstrand cross-linking induced by Mitozolomide is probably a consequence of an initial alkylation at the O^6-position of guanine followed by a delayed reaction with the opposite DNA strand.

Alkylation of guanine at the O^6-position is probably also involved in the mechanism of cytotoxicity produced by Temozolomide, which is, however, chemically incapable of cross-linking DNA. As with Mitozolomide Mer^+ cell lines are less sensitive to this agent than Mer^- cell lines and depletion of the DNA repair protein O^6-methylguanine-DNA methyltransferase (O^6MeGMT) in Mer^+ cells by treatment with the free base O^6-methylguanine leads to an increased sensitivity to both Mitozolomide and Temozolomide, but not to the 3-ethyl analogue (CCRG 82019) (25). In vitro Temozolomide is 10-fold more potent at inhibiting the growth of the K562 human leukaemia cell line than is CCRG 82019 (23). Furthermore, growth inhibition produced by Temozolomide is accompanied by a proportional increase in haemoglobin producing cells in the culture, while CCRG 82019 is ineffective in the induction of haemoglobin synthesis even at concentrations which have an equivalent effect on cell growth (23). A number of experi-

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ments have suggested a role for DNA methylation in the regulation of gene expression in eukaryotic cells. In K562 cells treated with Temozolomide the concentration of 5-methylcytosine in DNA decreased 3 days after treatment and was directly proportional to the number of benzidine-positive cells in the cultures, suggesting a direct correlation between hypomethylation of DNA and the induction of haemoglobin synthesis (24). This report investigates the mechanism of hypomethylation of DNA and the generality of this phenomenon.

Material and Methods

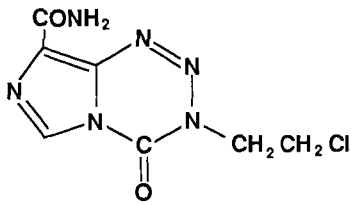
[6-^{3}H]Uridine (sp. act. 20 Ci mmol^{-1}), S-adenosyl-L-[methyl-^{3}H]methionine (sp. act. 80 Ci mmol^{-1}), and eukaryotic DNA methylase were purchased from Amersham International, Bucks. Tissue culture medium and foetal calf serum were purchased from Gibco Europe Ltd. (Paisley, Scotland). Temozolomide and CCRG 82019 were synthesized by May and Baker Ltd. (Dagenham, Essex). M. lysodeikticus 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°C in RPMI 1640 medium containing 10% foetal calf serum under an atmosphere of 5% CO_2 in air. Both Temozolomide and CCRG 82019 were dissolved in dimethyl sulphoxide (DMSO) at 20 mg ml^{-1}, such that the final concentration of DMSO in culture medium did not exceed 0.35%. For labelling of DNA [6-^{3}H]uridine (1 μCi ml^{-1}) was added to the culture medium 24 h prior to harvesting the cells.

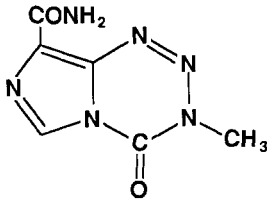
Isolation and hydrolysis of nucleic acids. This was carried out as previously described (24). Separation of bases was achieved by high-performance liquid chromatography on a Partisil 10 SCX column (0.6 × 25 cm, Whatman) eluted isocratically with 0.035 M KH_2PO_4, pH 2.5 in an Altex hplc system. Bases 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 3 separate determinations were made for each concentration of drug and day of incubation. The extent of enzymatic methylation was calculated according to the formula:

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

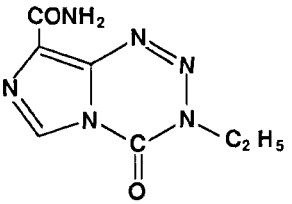
Alkylation of DNA in vitro. E. coli or calf thymus DNA (600 μg) was treated with the indicated concentrations of drugs for 2 h at 37°C in a total volume of 1 ml. The solution was saturated with sodium acetate and the DNA was precipitated with 2.5 volumes of cold absolute ethanol. The precipitation process was repeated twice and the DNA was extensively washed with ethanol. The DNA was redissolved in distilled water at a concentration of 2 mg ml^{-1} and 10 μg was routinely added to the DNA methylase assays.



Mitozolomide



Temozolomide



CCRG 82019

Fig. 1. Structures of the imidazotetrazinones.

DNA methylase assay was similar to that described by Bolden et al. (5). The standard assay contained 20 mmol Tris-HCl, pH 7.5, 5 mmol dithiothreitol, 1 mmol EDTA, 0.1 mmol EGTA, 16 μmol S-adenosyl-L-(methyl-^{3}H)methionine (sp. act. 2.5 Ci mmol^{-1}), 20 μg heat denaturated M. lysodeikticus DNA and enzyme in a total volume of 50 μl. Incubations were routinely carried out for 4 h at 37°C and were terminated by cooling the samples to 0°C and unlabelled S-adenosyl-L-methionine was added to a final concentration of 1.5 mmol. Aliquots (40 μl) of the reaction mixture were spotted onto 2.3 cm discs of Whatman DE81 paper and the discs were washed 5 times in 20 mmol Na_2HPO_4, pH 8.4 (20 ml/filter), twice in distilled water and once in absolute ethanol and ether. The discs were air dried and the radioactivity was determined in a toluene, 2,5-diphenyloxazole (PPO) mixture.

Results

Two cell lines, GM892 (Mer$^-$) and Raji (Mer$^+$) (25) have been utilized to investigate the effects of Temozolomide

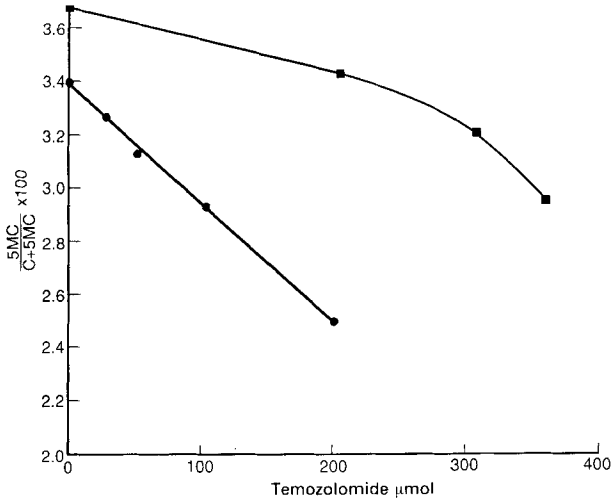


Fig. 2. Relationship between the 5-methylcytosine content of DNA of GM892 (●) and Raji (■) cells 4 days after treatment with Temozolomide. The experiment was repeated 4 times and the results are representative of a single experiment.

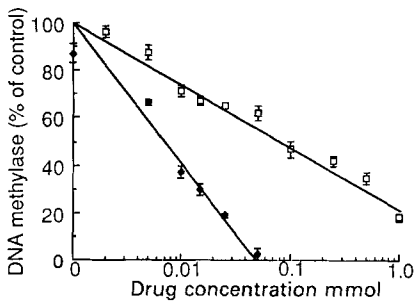


Fig. 3. Effect of calf thymus DNA modified with Temozolomide (□) or CCRG 82019 (◆) on the methylation of M. lysodeikticus DNA by eukaryotic DNA methylase. Calf thymus DNA was treated as described in methods and 10 μg of treated DNA was added to the methylation assay containing 20 μg of M. lysodeikticus DNA. There was no methylation of the calf thymus DNA alone under the conditions of the assay. The results are expressed as a percentage of the methylation of M. lysodeikticus DNA observed with untreated calf thymus DNA, and are expressed as mean ± SEM.

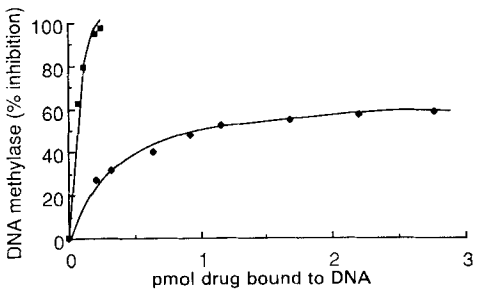


Fig. 4. Relationship between the total amount of Temozolomide (◆) and CCRG 82019 (■) bound to calf thymus DNA and the inhibition of methylation of M. lysodeikticus DNA by eukaryotic DNA methylase. The extent of drug binding to DNA was determined as described (7).

and CCRG 82019 on DNA methylation. These cell lines show a differential response to the cytotoxic effects of Temozolomide (ID_{50} 10±7 and 206±20 μmol respectively), but less so to CCRG 82019 (ID_{50} 229±20 and 360 ±15 μmol respectively). Treatment of both cell lines with Temozolomide is accompanied by a marked reduction in the level of 5-methylcytosine in newly replicated DNA 4 days after drug addition (Fig. 2). The inhibitory effect of Temozolomide on cytosine methylation is more pronounced in GM892 than in Raji cells. No change in methylation pattern is observed during the first 48 h after drug addition. Since the degree of hypomethylation is greater in GM892 cells which lack O^6MeGMT one possibility for inhibition is that the cytosine 5-methyltransferase acts as a suicide enzyme to remove O^6MeG from alkylated DNA in a similar manner to O^6MeGMT, since both enzymes share a similar structure with a sulphydryl group at the active site (8, 9). However, purified cytosine 5-methyltransferase had no ability to remove methyl groups from the O^6-position of guanine in DNA which had been alkylated with [^{3}H-methyl]-N-nitrosourea and partially depurinated as described (25), either in the presence or absence of 20 μmol S-adenosyl-L-methionine.

Another possibility is the direct inhibition of cytosine 5-methyltransferase by the chemically modified DNA. To investigate the effect of DNA modification by Temozolomide and CCRG 82019 on the methylation of M. lysodeikticus DNA by eukaryotic DNA methylase, calf thymus DNA was alkylated in vitro and, after extensive washing, the alkylated DNA was allowed to compete with M. lysodeikticus DNA in the methylase assay. The results presented in Fig. 3 show an increasing inhibition of the methylation reaction by a constant concentration of calf thymus DNA, which had previously been treated with an increasing concentration of both agents. There was no effect of unmodified DNA on the methylation of M. lysodeikticus DNA or of free drug alone at concentrations up to 10 mmol. While agents, such as N-methyl-N-nitrosoureas (MNU) have been shown to inhibit DNA methylase directly, 50% inhibition was only achievable with 47 mmol MNU (8). DNA modified with CCRG 82019 was more effective in inhibiting the transfer of methyl groups from S-adenosyl-L-methionine to M. lysodeikticus DNA than that modified with Temozolomide. This is surprising since CCRG 82019 is much less effective in alkylating DNA than is Temozolomide, the ovarall extent of reaction being 3-fold greater with Temozolomide (7). When the inhibition of methylation of M. lysodeikticus DNA by alkylated calf thymus DNA is plotted against the number of pmoles of bound drug an exponential relationship is observed (Fig. 4), with CCRG 82019 treated DNA being approximately 3 times more effective in inhibiting methylation than an equivalent weight of Temozolomide treated DNA. Although the overall level of alkylation of DNA by CCRG 82019 is less than that with Temozolomide the pattern of alkylation might be expected to be different in

comparison with the nitrosoureas where the predominant alkylation product by ethyl nitrosourea is phosphotriesters, whereas N^7-methylguanine is the predominant product after methyl nitrosourea treatment (2). A similar situation occurs with the imidazotetrazinones where 70% of the alkylated DNA is N^7-methylguanine with Temozolomide, whereas CCRG 82019 gives only 24% of N^7-ethylguanine, the predominant product (66%) being phosphotriesters (V. Bull, unpublished results).

Nuclear proteins form more stable non-covalent complexes with DNA substituted with residues from CCRG 82019 than with Temozolomide (Table). DNA methyltransferase together with a variety of other non-histone nuclear proteins were isolated with GM892 cell nuclei by treatment with 0.3 mol NaCl. The complex between the DNA and nuclear proteins causes its quantitative retention on nitrocellulose filters, since neither substituted DNA bound to the filter in the absence of nuclear protein. The binding of Temozolomide treated DNA to the filter was reduced by 65% on addition of 1 mol NaCl, while the binding of CCRG 82019 treated DNA was reduced by only 21%. The CCRG 82019 DNA protein complexes were also more stable when exposed to 0.6% sarkosyl and 0.5 mol NaCl. However, complexes between nuclear proteins and substituted DNA could be dissociated in the presence of sodium dodecyl sulphate (SDS) showing that the increased resistance of CCRG 82019 DNA protein complexes to dissociation by salt and sarkosyl did not result from formation of stable covalent complexes.

Table

Stability of preformed alkylated calf thymus DNA: Nuclear protein complexes to treatment with salt and detergents[a]

Dissociating agent	% DNA bound to filter[b] (Temozolomide) DNA	[CCRG 82019] DNA
None	100	100
1.0 M NaCl	35±3	79±2
0.6% Sarkosyl + 0.5 M NaCl	22±3	50±2
0.5% SDS	4±1	14±2

[a] Calf thymus DNA was reacted with either 1 mmol [3-^{14}C] Temozolomide on 5 mmol 3-[^{14}C] CCRG 82019 in 50 mmol phosphate buffer, pH 8.4, for 2 h at 37°C and the DNA was isolated as described in Material and Methods. Nuclear extracts were prepared from GM892 cell nuclei by lysing the cells in 10 mmol Tris pH 7.5, 10 mmol EDTA containing 0.5% Nonidet P40 and the pelleted nuclei were washed and extracted with 100 µmol imidazole, pH 7.5, 20 mmol EDTA, 0.5 mmol dithiothreitol, 0.3 mol NaCl, and the extract was dialysed. Complexes were formed during a 20 min incubation of the substituted DNA with 50 µg of the nuclear extract in a final volume of 200 µl of 100 µmol imidazole, pH 7.5, 20 mmol EDTA, 0.5 mmol dithiothreitol and 10 µmol S-adenosyl-L-methionine. The complexes were incubated for 10 min at 4°C prior to being washed onto a Millipore HA 0.45 µmol filter either with the incubation buffer or the dissociating agents indicated.

[b] Results are expressed as a percentage of the dissociation in the absence of salt or detergent, and are expressed as a mean ± SEM (n=4).

Discussion

Substantial evidence has been accumulated to indicate that methylation of cytosine residues in vertebrate DNA is implicated in the control of gene expression. A diverse range of chemical carcinogens have been shown to inhibit the methylation of DNA suggesting that chemical carcinogens may induce changes in gene expression by alteration of cellular methylation patterns (16). In particular 2 agents which decompose to produce a similar alkylating moiety to Temozolomide, N-methyl-N-nitrosourea (MNU) (3) and N-methyl-N-nitro-N-nitrosoguanidine (MNNG) (4) produce hypomethylation of newly synthesized DNA in human Raji cells in a dose-dependent manner. Both agents have also been shown to induce morphological differentiation in cultured mouse neuroblastoma cells (27). The methyl analogues were more effective in the induction of differentiation than their corresponding ethyl derivatives. Direct alkylating carcinogens, such as MNU and MNNG have been shown to directly inhibit DNA methylase by reaction with critical sulphydryl groups in the methylase molecule, although the concentration of carcinogen required for inhibition of methylase activity is much higher than was normally attainable in vivo (8). In many cases the methylation of DNA modified in vitro with chemical carcinogens is much less than the unmodified substrate suggesting that DNA damage may inhibit maintenance methylation (19).

Formation of alkali-labile sites in DNA has also been shown to reduce its ability to accept methyl groups (26), but the degree of methyltransferase inhibition produced by chemical carcinogens is greater than that expected from this damage alone. Alkylation of specific base residues may be responsible for the inhibition of DNA methyltransferase activity. Thus Pfohl-Leszkowicz el al. (17) showed that alkylation of both DNA and poly (dG-dC); poly (dG-dC) by dimethylsulphate which yields mostly 7-methylguanine and 3-methyladenine did not affect the enzymatic methylation. However, alkylation with MNU, which in addition to producing the 2 base modifications produced by dimethylsulphate also produces methylphosphotriesters and O^6-methylguanine caused an inhibition of enzymatic methylation, which was directly proportional to the extent of chemical alkylation of the substrate. The results from the present investigation would suggest that methylphosphotriesters may be more important than O^6-alkylguanine formation in the inhibition of DNA methyltransferase, since the ethyl imidazotetrazinone CCRG 82019, which forms predominantly phosphotriesters, is a much more effective inhibitor of DNA methylation than is Temozolomide. In addition nuclear proteins have a stronger affinity for such modified DNA, which may result from an increased electrostatic attraction by neutral-

ization of some of the negative charges on the DNA phosphate backbone. Ethylation of poly (dG-dC) poly (dG-dC) with ethyl methanesulphonate, which produced predominantly N^7 ethylguanine (2) stimulates methylation of the copolymer at molar ratios of base to copolymer of 6 to 7 × 10^3 and inhibits at base ratio of 9 to 10 × 10^3 (10). This suggests that alkylation of DNA may result in complex changes in overall DNA methylation.

It is possible that GC rich regions in DNA are preferentially alkylated since neighbouring bases have been shown to affect the alkylation pattern of guanine by MNU (6). Thus the O^6-methylguanine/7-methylguanine ratio of the alternating co-polymer C–G–C treated with MNU was 0.21 whereas the polynucleotide sequence G–G–C produced a ratio of only 0.1. Since 5-methylcytosine occurs predominantly in 5'CpG3' sequences in G–C rich regions of DNA (5) the higher level of O^6 methylguanine may sterically exclude the DNA methyltransferase from cytosine residues.

The inhibition of DNA methylation described in this report differs from that observed previously in that an alkylated calf thymus DNA, which is a poor substrate for the DNA methyltransferase inhibits the methylation of M. lysodeikticus DNA, which is a good substrate for the methylase enzyme. This suggests an increased interaction between the methylase enzyme and the alkylated DNA. Some support for this is provided from the increased stability of CCRG 82019 substituted DNA with nuclear proteins suggesting the formation of highly stable, but non-covalently linked complexes. It is possible that the adduct could block the progressive movement of the enzyme along the DNA helix by causing either dissociation or the formation of a tight ternary complex as suggested for 2-(acetylamino)fluorene (18) thus decreasing the effective concentration of the enzyme on the substrate and lowering the catalytic efficiency.

Further investigations are aimed at determining the specific DNA modification responsible for the inhibition of DNA methylase and the biological significance of this modification.

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