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Influence of O^6-methylguanine on DNA damage and cytotoxicity of temozolomide in L1210 mouse leukemia sensitive and resistant to chloroethylnitrosoureas

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Temozolomide is a new anticancer agent which in the early clinical investigation has shown promising antitumor activity. It decomposes spontaneously to the active metabolite of DTIC (MTIC). Temozolomide is more cytotoxic against L1210 than against a subline L1210/BCNU, resistant to chloroethylnitrosoureas. Using [methyl-^{3}H] temozolomide we found that after 1 h exposure the amount of O^6-methylguanine (O^6mGua) was twice as high in L1210 than in L1210/BCNU whereas the amount of N^7 mGua was approximately the same in the two cell lines. O^6-alkylguanine DNA alkyltransferase (AT) levels were higher in L1210/BCNU than in L1210, supporting the view that the resistance to methyltriazenes is probably related to the efficient repair of O^6mGua in L1210/BCNU. Exposure of L1210/BCNU cells to 0.4 mM O^6mGua for 24 h resulted in a depletion of AT and in a higher temozolomide-induced cytotoxicity. In the sensitive cell line L1210, temozolomide activity was not potentiated by O^6mGua pretreatment. Moreover, in L1210/BCNU, O^6mGua increased DNA single-strand breaks caused by temozolomide, suggesting that O^6-guanine alkylation induces an excision repair mechanism in cells depleted in AT.

Key words: Chloroethylnitrosoureas, L1210 mouse leukemia, O^6-methylguanine, temozolomide.

Introduction

5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide (DTIC) is a very effective drug against several rodent tumors,[1] and has proven activity against human melanoma and some other human neoplasms.[2–4] It undergoes oxidative *N*-demethylation by liver cytochrome P450-dependent monooxygenase forming 5-(3-methyl-1-triazeno)imidazole-4-carboxamide (MTIC) which is a potent alkylating agent probably responsible for its antitumoral activity. It has been hypothesized that the relative low activity against human tumors of some *N*-methyl containing antitumoral agents (e.g. DTIC, hexamethylmelamine)[5,6] requiring *N*-demethylation for their activation is due to the relative inefficiency of this metabolic reaction in humans.

8-Carbamoyl-3-methyl-imidazo[5,1-*d*]-1,2,3,5-tetrazin-4(3H)-one (temozolomide) decomposes spontaneously at pH 7.4 to MTIC without requiring metabolic activation and it was selected for clinical investigation. Temozolomide crosses the blood–brain barrier as demonstrated by distribution studies in mice and by the recently reported clinical efficacy against tumors localized in the central nervous system (CNS).[7] It is known that MTIC can alkylate mainly N^7 and O^6 positions of guanine[8] but their relative importance in terms of drug cytotoxicity and antitumor activity is still debatable. In a previous study we reported that temozolomide induced a similar amount of DNA alkaline labile sites (ALS), probably due to N^7-guanine alkylation, in the L1210 parental cell line and in the subline L1210/BCNU resistant to chloroethylnitrosoureas and to methyltriazenes.[9] The observation that L1210/BCNU had a higher content of O^6-alkylguanine DNA alkyltransferase (AT) than L1210 led us to hypothesize that the higher repair capacity of O^6-guanine in L1210/BCNU was the basis of the relative resistance to methyltriazenes shown by this cell line.

The present study was designed to verify this hypothesis and to evaluate whether the inhibition of AT can counteract the resistance to methyltriazenes.

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Materials and methods

Cell culture

L1210 and L1210/BCNU mouse leukemia cells were maintained in suspension culture at 37°C in RPMI 1640 medium (Gibco Europe, Paisley, UK) containing 10% heat inactivated (56°C, 30 min) fetal bovine serum (Flow laboratories, Irvine, Scotland), 2 mM L-glutamine, 100 U/ml pencillin, 100 mg/ml streptomycin, and 10^{-5} and 5×10^{-5} M mercaptoethanol, respectively. Stock cultures were maintained in exponential growth at a density between 0.2×10^6 and 1×10^6 cells/ml.

Drug

Temozolomide and [methyl-^{14}C]temozolomide (specific activity 26.3 mCi/mmol) were kindly provided by Dr CG Newton of May & Baker Ltd, Dagenham, Essex, UK. The drug was dissolved in 1% dimethylsulfoxide (DMSO) immediately before use. O^6-methylguanine (O^6mGua) was synthesized by reacting 2-amino-6-chloropurine (Sigma, St Louis, MO, USA) and sodium methoxide; the product obtained, containing about 2–2.5% of unreacted material, was purified to over 99.5% (HPLC analysis) by repeated recrystallization from water.[10] O^6mGua was dissolved in 0.1 N HCl and stored at −20°C.

Alkylation products analysis

Cells suspended in RPMI 1640 medium without serum at 7×10^6 cells/ml were treated for 1 h at 37°C with 200 μCi of [methyl-^{14}C]temozolomide (800 μM DMSO solution). Then the cells were rapidly pelleted at 4°C and the medium discarded. The cellular pellets were washed twice with phosphate buffered saline (PBS) and then stored at −80°C until analysis. DNA was recovered from the cellular pellets by chloroform/iso-amyl alcohol extraction according to the method described by Umbenhauer and Pegg.[11] The amounts of labeled O^6mGua and N^7mGua in the extracted DNA were determined, after mild acidic hydrolysis, by a radiochromatographic method (HPLC-LSC) as described previously.[12]

Cell growth inhibition assay

L1210 and L1210/BCNU cells were seeded at 0.2×10^6 cells/ml and incubated for 24 h. The cells were pretreated or not with 0.4 mM O^6mGua for 24 h and then treated with various concentrations of temozolomide for 1 h at 37°C. The cultures were washed in PBS by centrifugation and resuspended in fresh medium. Controls and treated samples were diluted in fresh medium 1:4 at 48 h; using these dilutions, cell concentrations throughout the experiments were between 3×10^5 and 8×10^5/ml. Control growth is logarithmic in this range.

O^6-alkylguanine DNA alkyltransferase assay

The AT activity was determined following the procedure of Margison *et al.*[13] Briefly, pellets of cells, washed with PBS, were resuspended in buffer containing 50 mM Tris-HCl, pH 8.3, 1 mM EDTA and 3 mM dithiothreitol. Samples were sonicated for 2×10 s periods at 14 μm peak to peak and 87 μg phenylmethylsulfonyl fluoride was added in a volume of 10 μl of ethanol. Sonicates were centrifugated for 10 min at 13 000 r.p.m. and the supernatants were analyzed for AT activity. Increasing amounts of cell extracts were combined with 10 μg [methyl-^{3}H]DNA (a generous gift from Dr GP Margison, Paterson Institute for Cancer Research, Manchester, UK) that had been reacted with *N*-[methyl-^{3}H]-*N*-nitrosourea, 20 Ci/mmol, and incubated for 60 min at 37°C. The reaction was stopped adding 1 M perchloric acid (final concentration). Samples were then incubated for 40 min at 80°C to hydrolyze DNA. The protein was collected by centrifugation for 10 min at 3000 r.p.m. and the pellet was washed with a further 4 ml of 1 M perchloric acid. Finally the pellet was dissolved in 10 mM NaOH and liquid scintillation mixture for the subsequent assay of radioactivity. The efficiency of counting for ^{3}H was about 30%. The results were expressed as fmole methyl transferred per mg of protein content in the sample assayed. The protein content of each sample was determined by analyzing 10–20 μl of the corresponding supernatant according to the method of Bradford.[14]

Alkaline elution assay

Temozolomide-induced DNA damage was analyzed by the alkaline elution procedure of Kohn *et al.*[15] The cells, labeled for 24 h with 0.05 mCi/ml [^{3}H]thymidine (specific activity 83 Ci/mmol) and 10^{-6} M unlabeled thymidine, were pretreated or not with 0.4 mM O^6mGua during the postlabelling

24 h chasing in medium without [^{3}H]thymidine. After a 1 h drug treatment, cells (5–10 × 10^5) were washed with cold PBS and layered on polycarbonate filters, 0.8 μm pore size and 25 mm diameter (Nucleopore Corp., Pleasanton, CA, USA). Some standard controls were irradiated with 450 rad at 0°C. Cells were then lysed with a solution containing 2% sodium dodecylsulfate (SDS), 0.02 M Na_2 EDTA, 0.1 M glycine, pH 10.0, which flowed through the filter by gravity. After connecting the outlet of the filter holders to the pumping system, 2 ml of 0.5 mg/ml proteinase K (EM Laboratories, Darmstadt, Germany) dissolved in the lysis solution was added to a reservoir over the polycarbonate filters. DNA was eluted from the filters by pumping 0.02 M EDTA solution adjusted to pH 12.1 with tetrapropylammonium hydroxide (Fluka, Switzerland) containing 0.1% SDS through the filters at approximately 2 ml/h. Fractions were collected at 3-h intervals, and fractions and filters were processed as described previously.[15]

Results

Table 1 illustrates the levels of [^{14}C]O^6mGua and of [^{14}C]N^7mGua in L1210 and L1210/BCNU exposed for 1 h to [methyl-^{14}C]temozolomide. In both L1210 and L1210/BCNU cells the levels of N^7mGua were much greater than those of O^6mGua. The alkylation of N^7-guanine was similar in the two cell lines whereas the relative alkylation of O^6-guanine was approximately 2-fold increased in L1210 as compared with L1210/BCNU.

In order to evaluate whether the depletion of AT could increase the sensitivity to methyltriazenes, we performed experiments pretreating L1210 and L1210/BCNU with O^6mGua for 24 h before a 1 h exposure to temozolomide. This pretreatment caused a significant depletion of AT (Table 2). Figure 1 shows that in L1210/BCNU, but not in L1210, O^6mGua pretreatment enhanced the growth inhibition induced by temozolomide. This effect appeared clearer at higher temozolomide concentrations.

We then asked the question whether O^6mGua could increase the DNA breakage caused by temozolomide. Figure 2 shows the alkaline elution profiles of L1210 and L1210/BCNU; it is evident that O^6mGua caused no detectable DNA single-strand breaks (SSBs) but did cause a very significant increase in temozolomide-induced DNA SSBs in L1210/BCNU. The amount of DNA SSBs in L1210/BCNU was 7 ± 2 and 32 ± 11 rad equivalents after 200 and 400 μM temozolomide treatments, respectively; O^6mGua increased the amount of temozolomide-induced DNA SSBs in L1210/BCNU to 80 ± 12 and 303 ± 11 rad equivalents after 200 and 400 μM temozolomide treatments, respectively. In L1210 cells treatment with O^6mGua produced only a moderate increase in DNA SSBs caused by 200 μM temozolomide (11 ± 0 rad equivalents without O^6mGua pretreatment versus 52 ± 5 rad equivalents with O^6mGua pretreatment).

Table 1. [^{14}C]N^7mGua and [^{14}C]O^6mGua levels in L1210 and L1210/BCNU after 1 h exposure to [^{14}C-methyl]temozolomide

Cell line	[^{14}C]N^7mGua (fmol/μg DNA)	[^{14}C]O^6mGua (fmol/μg DNA)	Ratio O^6mGua/N^7mGua (%)
L1210	72.75	4.15	5.7
L1210/BCNU	66.71	1.9	2.85

Cells, suspended in RPMI 1640 medium without serum at 7 × 10^6 cells/ml, were treated for 1 h at 37 C with 200 μCi of [methyl-^{14}C]temozolomide (800 μM DMSO solution). The amounts of labeled O^6mGua and N^7mGua were determined by HPLC-LSC.

Table 2. Effect of O^6mGua on AT activity in L1210 and L1210/BCNU cells

Cell line	O^6mGua (0.4 mM × 24 h)	AT activity (fmol/mg protein)
L1210	–	3.28 ± 0.25
	+	3.43 ± 0.71
L1210/BCNU	–	32.28 ± 6.34
	+	2.79 ± 0.46

Cells (1 × 10^8) were sonicated and the supernatants were analyzed for AT activity by measuring the transfer of radioactively labeled methyl groups from substrate [methyl-^{3}H]-DNA (*N*-[^{3}H]-methyl-*N*-nitrosourea, 20 Ci/mmol) to the AT protein.

Discussion

We have previously reported that temozolomide causes more cytotoxicity in L1210 mouse leukemia cells than in a subline resistant to chloroethylnitrosoureas (L1210/BCNU).[9] The higher level of AT in L1210 BCNU than in L1210 suggested the hypothesis that the efficient repair of O^6mGua was the reason for the resistance to methyltriazenes

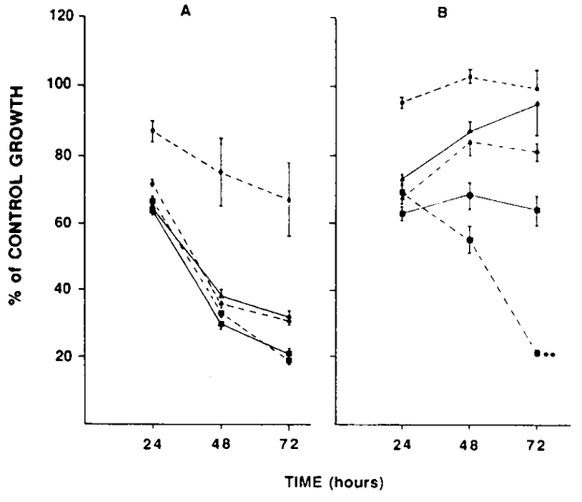

Figure 1. Effect of O^6mGua on temozolomide cytotoxicity in L1210 (panel A) and L1210/BCNU (panel B) cell lines. The cells were exposed (----) or not (———) to 0.4 mM O^6mGua for 24 h. Temozolomide concentrations used were 0 μM (●), 200 μM (▲) and 400 μM (■) for 1 h exposure. $^{**}p < 0.01$ with O^6mGua pretreatment versus without pretreatment.

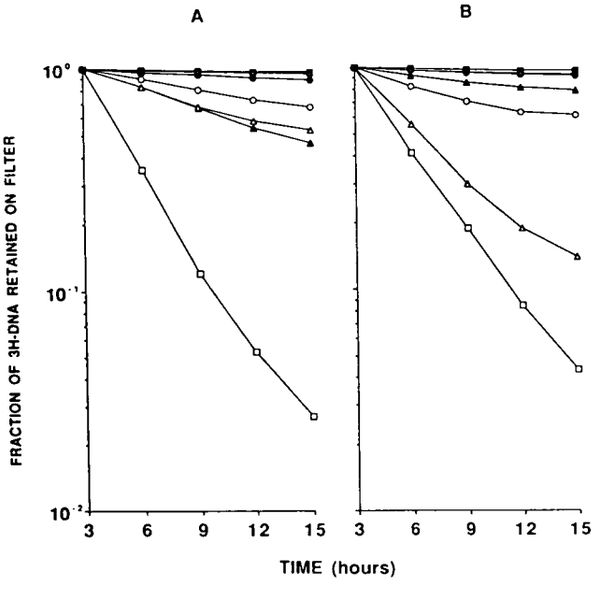

Figure 2. Effect of 24 h exposure to 0.4 mM O^6mGua on DNA SSBs after 1 h exposure to temozolomide in L1210 (panel A) and (panel B). Symbols: ■, controls; □, 450 rad; ○, 0.4 mM O^6mGua; ●, 200 μM temozolomide; ○, 0.04 mM O^6mGua + 200 μM temozolomide; ▲, 400 μM temozolomide; △, 0.4 mM O^6mGua + 400 μM temozolomide.

of L1210/BCNU. In the present study we verified this hypothesis. After exposure to [methyl-^{14}C]temozolomide, the amount of N^7mGua was, in fact, the same in the two cell lines whereas the level of O^6mGua was approximately 2-fold increased in L1210 as compared with L1210/BCNU.

The importance of the repair O^6-alkylguanine for the cytotoxicity of methyltriazenes and other methylating agents has already been hypothesized in previous studies showing that AT proficient cells (MER^+) are more sensitive to these drugs than AT deficient cells (MER^-).[16,17]

Based on the finding that O^6mGua is able to inhibit AT,[18] it is possible that it could enhance the cytotoxicity of methylating agents in the cells which are resistant to these drugs because of their relative high level of AT. The potentiation of the cytotoxicity of the methylating agents by pretreatment with O^6mGua in MER^+ cells had been previously reported,[19,20] but no studies have so far been available on the comparative effects of O^6mGua in a methylating agent-sensitive and resistant tumor cell line.

O^6-guanine alkylation and its repair by AT does not cause apparent alterations in DNA integrity.[21] In contrast to the excision repair mechanism, AT removes the alkyl group from the O^6 position of guanine without any depurination.[22]

Therefore the inhibition of AT by O^6mGua should not modify the number of DNA breaks or alkaline labile sites caused by methyltriazene treatment. Instead, in L1210/BCNU, exposure to O^6mGua significantly increased DNA SSBs produced by temozolomide.

A tentative explanation of this phenomenon is that the permanence of O^6-guanine alkylation in cells depleted of AT induced excision repair. This hypothesis could also explain the greater number of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidin induced DNS SSBs in AT defective mutants of the HeLa CCL2 cell line[23] and is supported by the observation that in *Escherichia coli* O^6mGua lesions can be repaired by the Uvr ABC excision repair system in the absence of *ada* protein (the bacterial DNA repair protein for O^6mGua lesions).[24]

In summary we provide further and more direct evidence that resistance to methyltriazenes is related to an efficient repair of O^6-guanine alkylation. O^6mGua can counteract resistance to methyltriazenes by causing a depletion of AT levels and inducing an increase in DNA SSBs by a mechanism still to be elucidated. In view of the data presented in the present study and of the reported efficacy of temozolomide against CNS tumors[7] it seems impor-

tant to design inhibitors of AT which can cross the blood–brain barrier, thus possibly increasing temozolomide activity against resistant cancer cell clones.

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