In Vitro and in Vivo Methazolastone-induced DNA Damage and Repair in L-1210 Leukemia Sensitive and Resistant to Chloroethylnitrosoureas

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ABSTRACT

DNA damage caused by methazolastone [an analogue of 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide which does not require metabolic activation] was investigated in L-1210 leukemia which is sensitive to this drug and in a L-1210 subline (L-1210/BCNU) which is resistant to both chloroethylnitrosoureas and methazolastone. Both in vitro and in vivo methazolastone caused formation of DNA alkali-labile sites (assessed by alkaline elution techniques) which were present in similar amounts and repaired at a similar rate in L-1210 and L-1210/BCNU. This suggests that these lesions are not crucial to methyltriazenes activity. DNA alkali-labile sites may be due to the removal of 7-methylguanine by 7-methylguanine-DNA glycosylase which showed the same activity in L-1210 and L-1210/BCNU.

Flow cytometry studies revealed that in L-1210 but not in L-1210/BCNU methazolastone induced an arrest of cells in S+G2+M phases. This blockade was delayed, occurring after at least two cell divisions after drug treatment and therefore appeared temporally unrelated to the presence of DNA alkali-labile sites.

There was three times more O'-methylguanine-DNA methyltransferase in L-1210/BCNU than in L-1210 suggesting that methylation of O'-guanine is an important lesion for methyltriazenes activity and resistance to this drug may be linked to its repair.

INTRODUCTION

DMT are compounds with antineoplastic activity in rodent and human tumors (1-3). Since 1970 DTIC has been in clinical use; it is the drug of choice for human melanoma (4) and is employed in combination with other drugs in sarcomas and lymphomas (5, 6). DTIC requires metabolic activation through oxidative N-demethylation leading to the formation of the N-desmethyl derivative (MTIC), a potent alkylating agent (7, 8).

Though the mode of action of DMT is still to be elucidated, the current hypothesis is that the alkylating species (MTIC) causes DNA methylation. From a quantitative point of view most DNA methylation occurs on N1 of guanine (8-10), but how crucial this reaction is for the antineoplastic effect of DMT is still unknown. Comparative investigations in tumors with different degrees of sensitivity to DMT may cast light on the molecular events which are critical for the antitumoral effects of these drugs. We investigated DNA damage induced by methazolastone [a new compound which spontaneously decomposes to MTIC without requiring metabolic activation (see Fig. 1), now under clinical investigation in Europe] in mouse L-1210 leukemia which is sensitive to DMT and in a L-1210 subline (L-1210/BCNU) resistant to chloroethylnitrosoureas and cross-resistant to DMT. In L-1210 and L-1210/BCNU, in vitro or in vivo, methazolastone induced a similar amount of DNA ALS which were also repaired at a similar rate in both lines. Marked differences in MT activity in the two cell lines suggests that methylation of the O'-position of guanine plays a crucial role in the antitumoral activity of DMT.

MATERIALS AND METHODS

Cell Culture

L-1210 and L-1210/BCNU mouse leukemia cells were grown at 37°C in suspension culture in RPMI 1640 medium (GIBCO Europe, Glasgow, Scotland) supplemented with 10% heat-inactivated (56°C, 30 min) fetal calf serum (GIBCO Europe), 2 mM l-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10-3 M and 5 x 10-8 M mercaptoethanol, respectively, for L-1210 and L-1210/BCNU cells. Stock cultures were maintained in exponential growth at a density between 0.2 x 106 and 1 x 106 cells/ml.

Drug

Methazolastone was kindly provided by Dr. C. G. Newton, May & Baker Ltd., Dagenham, Essex (RM107XS). For in vitro studies the drug was dissolved in 1% dimethyl sulfoxide immediately before administration. For in vivo studies methazolastone was dissolved in dimethyl sulfoxide: saline (1:5). MNU was dissolved in 95% ethanol immediately before use. The final ethanol concentration in the medium never exceeded 1%.

Colony-forming Assay

Colony-forming ability was determined by the soft-agar technique described by Chu and Fischer (11). After 1 h drug treatment at 37°C, cells were washed in PBS and seeded into polylysine culture tubes (Falcon 2054; Becton Dickinson, Oxnard, CA). The colony-forming efficiency of untreated control cells in forming colonies was approximately 30-40% in this system.

Cell Growth Inhibition Assay

L-1210 and L-1210/BCNU cells were seeded at 0.2 x 106 cells/ml and incubated for 24 h. The cultures were drug treated 1 h at 37°C, then washed twice in PBS by centrifugation and resuspended in fresh medium. Controls and treated samples were diluted in fresh medium 1:4 at 48 h and 1:2 at 96 h. Using these dilutions cell concentrations throughout the experiments were between 3 x 105 and 8 x 105/ml. Control growth is logarithmic in this range.

In Vivo Antitumor Activity

L-1210 and L-1210/BCNU mouse leukemia was obtained from NCI (Frederick Cancer Research Facility, Frederick, MD 21701) and maintained by weekly i.p. transplants of 106 cells/mouse in DBA/2 mice. For evaluation of the antitumor activity male CDF2 mice were treated with methazolastone (40 mg/kg) i.p. for 5 days. For flow cytometry and alkaline elution studies, 40 mg/kg was administered as a single i.v. dose 5 days after transplant.

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3The abbreviations used are: DMT, dimethyltriazenes; DTIC, 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide; MT, O'-methylguanine-DNA methyltransferase; O'-mg, O'-methylguanine; 7-mG, 7-methylguanine; DNA ALS, DNA alkali-labile sites; DNA ISC, DNA interstrand cross-links; PBS, phosphate buffered saline; MTIC, 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide; SE, S early; SM, S middle; SL, S late; MNU, N-methyl-N-nitrosourea; BCNU, N,N'-bis(2-chloroethyl)-N-nitrosourea; GC, AT, guanine, cytosine, adenine, thymine.
Flow Cytometry

For in vitro experiments L-1210 and L-1210/BCNU cells were treated with 100, 200, and 400 μM of methazolastone or 25 and 50 μM of MNU for 1 h and the effects on cell cycle phase distribution were evaluated at different time-intervals after treatment. L-1210 (0.2 ml) and L-1210/BCNU cells were centrifuged and directly stained with 2 ml of propidium iodine solution containing 50 μg/ml of propidium iodine (Calbiochem Behring, USA) in 0.1% sodium citrate and 25 μl RNase 0.5 μg/ml in water (Calbiochem Behring, USA) at room temperature for 15 min.

For the in vivo flow cytometry study mice bearing L-1210 and L-1210/BCNU were given a single i.v. dose of methazolastone on day 3 after tumor transplant and killed after 24, 48, 72, and 96 h. L-1210 (0.5 ml) and L-1210/BCNU cells were resuspended in PBS, centrifuged, then stained as for the in vitro experiments.

Flow cytometry analysis was performed using a 30-L cytofluorograph (Ortho Diagnostic Systems, USA). The fluorescence pulse was detected in a spectral range between 580 and 750 nm; the coefficient of variation of the G1 peak of the cancer cells was about 4%. Each cytofluorometric assay was performed with 2–3 × 10⁶ cells (12, 13). The percentage of cells in the different cycle phases was calculated by the method of Krishan and Frei (14).

MT Assay

Extracts of L-1210 and L-1210/BCNU leukemia cells were prepared by sonication (5 × 10 s) of the cellular pellet resuspended in 50 mM Tris-HCl (pH 7.5 at 4°C), 1 mM DTT, 0.01 mM EDTA (at 70 × 10⁶ cells/ml). After centrifugation (700 × g/5 min) to remove cellular debris, the supernatant, containing 2.5–5 mg/ml protein, was employed for determination. 3H-Methylated substrate was prepared by reacting 2 mg of calf thymus DNA (Miles Laboratories) in 0.5 ml of 10 mM Tris-HCl buffer, pH 8, with 0.5 μCi of [3H]MNU (Miles, 5.0 Ci/mg). The incubation mixture was adjusted to 1.7 mM diethiothreitol, 72 μM Tris-HCl, pH 8. The samples were incubated, with or without cell extracts (control), at 37°C for 1 h, then the DNA was precipitated by adding cold 1 N HCl and analyzed by high-performance liquid chromatography as described before. Controls for spontaneous hydrolysis of 7-mG were performed by incubating DNA at 37°C without extracts for identical intervals.

Alkaline Elution Assays

In Vivo Assay. The method of alkaline elution was recently reviewed in detail (16). Cells were labeled for 24 h using a medium supplemented with 0.05 μCi/ml [3H]thymidine (specific activity, 20 Ci/mmol; Amer sham) and 10⁻⁴ M unlabeled thymidine. Postlabeling 18–24 h chasing in medium without [3H]thymidine was performed before drug treatment. Some standard controls were irradiated with 450 rads. After 1 h treatment or after the indicated intervals of postdrug incubation, cells (5–10 × 10⁶) were washed with cold PBS and layered on polycarbonate filters, 10-μm pore size and 25-mm diameter (Nucleopore Corp., Pleasanton, CA). Cells were then lysed with a solution containing 2% sodium dodecyl sulfate, 0.02 M Na₂EDTA, 0.1 M glycine, pH 10.0 (lysis solution), which was flowed through the filter by gravity. After connecting the outlet of the filter holder to the pumping system, proteinase K, 2 ml of 0.5 mg/ml (EM Laboratories, Darmstadt, West Germany) dissolved in the lysis solution, were added to a reservoir over the polycarbonate filters and pumped for approximately 1 h at a rate of 0.35 ml/min.

DNA was eluted from the filters by pumping 0.02 M EDTA solution adjusted to pH 12.1 or 12.6 with tetrpropylammonium hydroxide (Fluka, West Germany) containing 0.1% sodium dodecyl sulfate through the filters at approximately 2 ml/h. Three-h fractions were collected, and fractions and filters were processed as described previously (16). In Vivo Assay. Experiments were performed on day 5 after i.p. tumor transplant when normal cells in peritoneum were less than 5% of the leukemic cells. Cells of untreated or treated mice were obtained from the peritoneal cavity, washed and suspended in cold PBS. Cell viability was determined by the Trypan blue exclusion test. Approximately 1.2 × 10⁶ cells were layered on polycarbonate filters, 2-μm pore size and 25-mm diameter (Nucleopore Corp., Pleasanton, CA). Cells were then lysed with a solution containing (see in vitro assay); then the funnel outlets were plugged and 2 ml proteinase K (0.5 mg/ml) were gently added. The funnel containing the filters were kept in the dark at room temperature for 1 h. The plugs were then removed and the solution allowed to drip from the outlet of each funnel.

To remove sodium dodecyl sulfate the filters were washed with 10 ml of 20 mM EDTA, pH 10.0. Funnels were then connected to the elution apparatus (eight-channel peristaltic pumps and fractions collector model 202; GILSON, France), filled almost to the edge with the elution buffer (20 mM EDTA, pH 12.2) and covered with Parafilm. The elution rate was 2 ml/h for 15 h. Three-h fractions were collected. In these conditions the pH varied less than 0.1 in 15 h. The DNA content in each fraction and in sonicated filters was determined by fluorometric detection using Hoechst 33258, as previously described (17).

RESULTS

Cytotoxicity and Antitumor Activity in L-1210 and L-1210/BCNU. Methazolastone was much less effective in inhibiting colony formation (Fig. 2) or in inhibiting cell growth (Fig. 3) in the L-1210 subline resistant to chloroethyl nitrosoureas (L-1210/BCNU) than in the parental L-1210. Sensitivity was similarly different for MNU. For example 1 h exposure of L-1210 to 25 μM or 50 μM MNU gave respectively 44 to 77% inhibition of cell growth assessed 72 h after drug treatment. The same treatments caused no detectable inhibition of L-1210/BCNU growth. Sensitivity to methazolastone was also different in vivo, a daily i.p. dose of 40 mg/kg for 5 consecutive days (days 1–5 after tumor transplant) increasing life-span by 86% in L-1210 and 22% in L-1210/BCNU.

Flow Cytometry Studies. As can be seen in Fig. 4, in L-1210 methazolastone caused a dose-dependent accumulation of cells
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Fig. 2. Colony assay of L-1210 (C) and L-1210/BCNU (O) after 1 h exposure to methazolastone. For details see “Materials and Methods.”

Fig. 3. Growth inhibition assay of L-1210 (open symbols) and L-1210/BCNU (closed symbols) after 1 h exposure to methazolastone at concentrations of 100 μM (C, O), 200 μM (C, D) and 400 μM (G, H). For details see “Materials and Methods.”

Fig. 4. Effects of 1 h treatment with methazolastone at concentrations of 0 μM (A, B), 100 μM (C, D), 200 μM (E, F) and 400 μM (G, H) on cell cycle phase distribution in L-1210 (left) or in L-1210/BCNU cells (right). Flow cytometry analysis was done before and after 24, 48, 72, 96, and 120 h posttreatment incubation in drug-free medium. Phases: G, G1; O, SE; M, SM; G2-M.

Fig. 5. Effects of 1 h treatment with MNU at concentrations of 0 μM (A, B), 25 μM (C, D) and 50 μM (E, F) on cell cycle phase distribution in L-1210 (left) or in L-1210/BCNU cells (right). Flow cytometry analysis was done before and after 24, 48, and 72 h posttreatment incubation in drug-free medium. Symbols are the same as in Fig. 4.

Fig. 6. Effects of 1 h treatment with MNU at concentrations of 0 μM (A, B), 25 μM (C, D) and 50 μM (E, F) on cell cycle phase distribution in L-1210 (left) or in L-1210/BCNU cells (right). Flow cytometry analysis was done before and after 24, 48, and 72 h posttreatment incubation in drug-free medium. Symbols are the same as in Fig. 4.

1210 leukemia-bearing mice were treated i.v. with methazolastone (40 mg/kg) (Fig. 6). No such effect was seen on L-1210/BCNU cells from mice given the same drug dose.

Alkaline Elution Studies. Preliminary alkaline elution studies in vitro in growing L-1210 and L-1210/BCNU treated with methazolastone showed that elution profiles were not linear but

in SL-G2-M phases of the cell cycle, but the proportion of cells in SE and SM was apparently unaffected. Interestingly, the accumulation of L-1210 cells in SL-G2-M was hardly detectable after 24 h incubation in drug-free medium but became evident at 48 h and increased further after 72 h. At this latter time cells in SL-G2-M were 32, 46, and 56% after 100, 200, and 400 μM, respectively, compared to 22% in the untreated control cells.

In L-1210/BCNU no effect was seen after 100 or 200 μM treatment; only 400 μM methazolastone produced an accumulation of cells in premitotic phase but much less than in L-1210. In L-1210/BCNU the maximum accumulation of cells in SL-G2-M was, after 48–72 h, approximately 30% as compared to 23% in untreated cells. SL-G2-M arrest was similarly delayed after 1 h exposure to 25 and 50 μM MNU (Fig. 5).

Cells accumulated in SL-G2-M occurred in vivo too when L-
INCREASE OF MT IN METHYLTRIAZENE-RESISTANT CELLS

![Graph](image)

Fig. 6. Effects of methazolastone (40 mg/kg) i.v. after 24, 48, 72, and 96 h treatment on cell cycle phase distribution in mice with L-1210 (left) or L-1210/BCNU (right). A and B, untreated controls. Symbols are the same as in Fig. 4.

Convex upward and the elution rate increased on raising the pH of the elution buffer from 12.1 to 12.6. The shape of the curves and the faster elution of pH 12.6 are indicative of the presence of DNA ALS (16) in cells treated with methazolastone (data not shown). Subsequent experiments were therefore performed at pH 12.6.

Fig. 7 shows the elution profile of DNA for L-1210 (A) or L-1210/BCNU (B) at the end of 1 h treatment or after 24 h of incubation in drug-free medium. The rate of elution was only slightly faster in L-1210 than L-1210/BCNU; after 24 h recovery there was still a significant fraction of DNA ALS not yet repaired in both cell lines.

Using the alkaline elution method coupled with fluorimetric detection we investigated DNA damage in peritoneal L-1210 or L-1210/BCNU cells at different times after an i.v. dose of methazolastone (40 mg/kg) (see Fig. 8). Considering that the figures for each time point are the average of three animals, the results do not vary much. After 1 h of methazolastone treatment DNA damage was significant in both cell lines as demonstrated by the higher elution rate than in controls. The presence of DNA ALS is again indicated by the shape of the curves.

No difference appeared in the extent of DNA damage in L-1210 and L-1210/BCNU at different times after drug treatment. Fig. 9 shows that in vitro and in vivo methazolastone-induced DNA damage was repaired slowly, with similar kinetics in both cell lines.

O\(^6\)-Methylguanine-DNA Methyltransferase and 7-Methylguanine-DNA Glycosylase. The L-1210/BCNU cell extracts were more efficient than L-1210 extracts in CH\(_3\) removal from the O\(^6\) position of guanine in the standard DNA. MT content was approximately three times higher in L-1210/BCNU than in L-1210 extracts (Table 1).

As concerns 7-mG repair, the two cell extracts produced identical kinetics of base removal after spontaneous hydrolysis subtraction, and the net diagram for the calculation of activities is presented in Table 1.

DISCUSSION

In L-1210 and in an L-1210 subline resistant to chloroethyl-nitrosoureas and to DMT (L-1210/BCNU), methazolastone induced a similar amount of DNA damage (i.e., presence of DNA ALS) that was repaired at a similar rate. The presence of DNA ALS has already been reported in L-1210 exposed to...
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Table 1 O'-Methylguanine DNA methyltransferase and 7-methylguanine DNA glycosylase activity in L-1210 and L-1210/BCNU

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<tr>
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<th>L-1210</th>
<th>L-1210/BCNU</th>
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<tr>
<td>MT activity (fmol/mg DNA)</td>
<td>1.692 ± 0.285</td>
<td>5.687 ± 0.310</td>
</tr>
<tr>
<td>Glycosylase activity (fmol/h x mg DNA)</td>
<td>23,000 ± 1.872</td>
<td>24,571 ± 1.580</td>
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another methylating agent, MNU (16, 18). The extent of methazolastone-induced DNA damage and repair in these cells was also similar in vitro in L-1210 and L-1210/BCNU bearing mice. Since L-1210/BCNU is much less sensitive to methazolastone than L-1210 either in vitro or in vivo, these data indicate that methazolastone-induced DNA ALS are not crucial for the drug's cytotoxicity and antitumoral activity.

We have not determined which adducts were at the basis of DNA ALS caused by methazolastone but they appear to be mostly due to methylation of the 7 position of guanine, for the following reasons: (a) 7-mG is the major adduct of MTIC (to which methazolastone decomposes) and other methyltriazenes (8-10); (b) repair of methazolastone-induced DNA ALS seems very slow (it was still incomplete 24 h after drug treatment) and this supports the idea that 7-mG-glycosylase is involved. Ample evidence exists that 7-mG-glycosylase is a particularly slow repair enzyme, much slower than 3-methyladenine-glycosylase which removes the alkylated adenines within a few minutes (19-22); (c) 7-mG-glycosylase activity in L-1210 and L-1210/BCNU is identical, which might explain the similar repair kinetics of DNA ALS observed in the two cell lines.

The lack of correlation between the assumed methylation of N7 guanine and the cytotoxicity of methylating agents is not surprising and is in line with recent observations by Hayward and Parsons who found a similar amount of 7-mG in a human melanoma cell line sensitive to methylating agents and in a resistant subline (10), thus supporting the idea that methylation of N7 of guanine is not a cytotoxic event.

MT was three times less active in L-1210 than in L-1210/BCNU cells. This probably explains the lower DNA ISC and cytotoxicity induced by chloroethylnitrosoureas in L-1210/BCNU (data not shown). MT can in fact remove the adducts from O6 of guanine, which otherwise undergoes intramolecular rearrangement and secondary cross-linking with a cytosine located on the opposite DNA strand, forming the DNA ISC [i.e., 1-(N2-deoxyctydyl)-2-(N4-deoxyguanosyl)ethane].

DNA ISC are reportedly crucial biochemical events for chloroethylnitrosourea cytotoxicity in several other cell lines (23-25). The present findings suggest that for methyltriazenes too, alkylation of O6 of guanine, though not convertible to a DNA ISC, may be a cytotoxic lesion since L-1210/BCNU, which showed greater MT activity, were less susceptible to these drugs. This hypothesis is supported by the recent reports of Gibson et al. (26) and of Lunn et al. (27) who found monomethyltriazenes more cytotoxic in MT deficient cells (Mer-) than in MT proficient cells (Mer+).

The molecular events underlying the antiproliferative activity of O6-mG are unknown. Much more information is available on the alkylation of O6 of guanine in relation to chemical mutagenesis and carcinogenesis. O6-alkylation appears to be a mutagenic and carcinogenic lesion and the ability of a tissue to remove an alkyl group from O6 of guanine seems inversely related to the frequency of malignant transformation induced by chemicals (e.g., methylnitrosamines) in that tissue (19, 28).

The alkylation of O6 of guanine makes this base prone to mispairing with a thymine; therefore after a subsequent cell division and DNA replication a GC:AT transition can occur...
It has been proposed that in a tissue rich in MT activity methyl groups bound on O₆ of guanine may be removed before and cytotoxicity of methylating agents are related, but from our results they appear to have in common the formation and removal of the methyl group from O' of guanine.

Like many other DNA-interacting anticancer agents, methazolastone causes an arrest of cells sensitive to its antiproliferative and antitumoral activity (i.e., L-1210) in premitotic phase. However, the other drugs, including the DNA-cross-linking agent mitozolomide (which differs from methazolastone only for a chloroethyl replacing a methyl group), induce this block a few hours after drug treatment (12) whereas methazolastone-treated cells or MNU treated cells appear to go through the cell cycle for at least two cell divisions before being arrested in SL-G₂-M. This delay suggests that cell cycle perturbation is not directly related to methazolastone-induced methylation of DNA which is presumably maximal at the end of treatment. It may be mere coincidence that the arrest in SL-G₂-M becomes evident when GC:AT transition is conceivably in cells which do not repair O'-methylguanine (O'MG) efficiently (i.e., after at least two cell divisions). Possibly when GC:AT transition occurs, a surveillance mechanism is activated in the cells, causing a premitotic blockade to prevent perpetuation of the genetic defect in daughter cells. Recently Tisdale (30) reported that methazolastone (but not mitozolomide) induced gene hypomethylation (e.g., of globin genes) in a human erythroleukemia cell line and supposedly by this mechanism caused terminal differentiation. We are currently exploring the possibility of GC:AT transition and consequent changes in cytosine-DNA-methylation being the causes of the premitotic blockade and arrest of cell proliferation caused by methazolastone.

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REFERENCES


21. Singer, B., and Brent, T. The human lymphoblasts contain DNA glycosylase activity excising N-3 and N-7 methyl and ethyl purines but not O'-alkylated guana...
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