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ORIGINAL ARTICLE

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Modulation of cyclophosphamide activity by O^6-alkylguanine-DNA alkyltransferase

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Abstract *Purpose*: The human medulloblastoma cell line D283 Med (4-HCR), a line resistant to 4-hydroperoxycyclophosphamide (4-HC), displays enhanced repair of DNA interstrand crosslinks induced by phosphoramide mustard. D283 Med (4-HCR) cells are cross-resistant to 1,3-bis(2-chloroethyl)-1-nitrosourea, but partial sensitivity is restored after elevated levels of O^6-alkylguanine-DNA alkyltransferase (AGT) are depleted by O^6-benzylguanine (O^6-BG). Studies were conducted to define the activity of 4-HC and 4-hydroperoxydidechlorocyclophosphamide against D283 Med (4-HCR) after AGT is depleted by O^6-BG. *Methods*: Limiting dilution and xenograft studies were conducted to define the activity of 4-HC and 4-hydroperoxydidechlorocyclophosphamide with or without O^6-BG. *Results*: The activity of 4-HC and 4-hydroperoxydidechlorocyclophosphamide against D283 Med (4-HCR) was increased after AGT depletion by O^6-BG preincubation. Similar studies with Chinese hamster ovary cells, with or without stable transfection with a plasmid expressing the human AGT protein, revealed that the AGT-expressing cells were significantly less sensitive to 4-HC and 4-hydroperoxydidechlorocyclophosphamide. Reaction of DNA with 4-HC, phosphoramide mustard, or acrolein revealed that only 4-HC and acrolein caused a decrease in AGT levels. *Conclusions*: We propose that a small but potentially significant part of the cellular toxicity of cyclophosphamide in these cells is due to acrolein, and that this toxicity is abrogated by removal of the acrolein adduct from DNA by AGT.

Key words Alkylating agents · Antineoplastic agents · Cyclophosphamide · Drug resistance · Nitrogen mustard compounds

Abbreviations *4-HC* 4-hydroperoxycyclophosphamide · *4-HDC* 4- hydroperoxydidechlorocyclophosphamide · *AGT* O^6-alkylguanine-DNA alkyltransferase · *CHO* Chinese hamster ovary · O^6-*BG*O^6-benzylguanine · *PM* phosphoramide mustard

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Introduction

Cyclophosphamide is an alkylating agent commonly used against a broad constellation of human malignancies

including leukemia, lymphoma, germ cell tumors, medulloblastomas, and carcinomas of the breast, lung, or cervix [5]. Cyclophosphamide is a prodrug, which, after it is hydroxylated by the mixed-function oxidase system of hepatic cytochrome P-450, is converted to 4-HC[3] in a tautomeric equilibrium with aldophosphamide. Aldophosphamide generates the active alkylating metabolite PM, which produces the DNA interstrand crosslinks critical for tumor cytotoxicity [5]. Resistance to cyclophosphamide may result from a constellation of cellular alterations including elevated glutathione, glutathione-*S*-transferase, or aldehyde dehydrogenase and enhanced repair of DNA interstrand crosslinks [8].

We now present the first evidence that the DNA repair protein, AGT, which plays a major role in resistance to alkylnitrosourea and methylator therapy by transferring the alkyl group from DNA bases to an active site cysteine residue in the enzyme with consequent suicide inactivation [3, 19], is also involved in decreasing the antitumor activity of cyclophosphamide.

Materials and methods

Cell culture

D283 Med, a human medulloblastoma cell line, and D283 Med (4-HCR), a subline with laboratory-generated resistance to 4-HC and elevated AGT levels, were maintained as previously described [8, 9].

Transfected CHO studies

CHO cells transfected with the pCMV-neo-*Bam* vector without an inserted cDNA sequence, and CHO cells transfected with the same plasmid expressing human AGT under the control of the immediate-early gene promoter of cytomegalovirus, were derived and cultured as previously described [17].

The ability of drugs to kill these cells was determined using a colony-forming assay [17]. Briefly, the cells were plated at 10^6 cells per 25-cm^2 flask and were grown for 24 h. The drug (4-HC or 4-HDC) was then added and, after the cells were exposed to it for 1 h, the medium was changed and the cells were allowed to grow for 8 or 16 h as indicated. The cells were replated at densities of 100–1000 cells per 5-cm^2 flask and were grown for 7–8 days until discrete colonies could be stained and counted. The plating efficiency of untreated cells was about 44%.

Limiting dilution analysis

The cytotoxicity of 4-HC, PM and 4-HDC against D283 Med and D283 Med (4-HCR), with and without prior treatment with O^6-BG, an excellent substrate for AGT [6] (treating cells with 100 μ*M* O^6-BG for 10 min prior to alkylator exposure and subsequently plating them in 10 μ*M* O^6-BG) was determined by limiting dilution assays as previously described [12].

Animals

Male and female athymic BALB/c mice (*nu*/*nu* genotype, 6 weeks or older) were used for all xenograft studies and were maintained as described previously [2].

Drugs

Cyclophosphamide was purchased from Sigma (St. Louis, Mo). PM was provided by the Drug Synthesis and Chemistry Branch, National Cancer Institute (Bethesda, Md.). 4-HC and O^6-BG were synthesized as previously described [6, 11]. 4-HDC was synthesized by modifying the synthesis of 4-HC [21].

Measurement of AGT activity

Quantitation of AGT levels was carried out as previously described [6]. Briefly, alkyltransferase activity was measured as removal of O^6-[^{3}H]methylguanine from a [^{3}H]methylated DNA substrate (5.8 Ci/mmol) after the substrate was incubated with extract or pure protein at 37 °C for 30 min. The DNA was precipitated by adding ice-cold perchloric acid (0.25 *N*) and was hydrolyzed by the addition of 0.1 *N* HCl at 70 °C for 30 min. The modified bases were separated by reverse-phase high-pressure liquid chromatography with 0.05 *M* ammonium formate (pH 4.5) containing 10% methanol. Each assay was performed with a positive control cell line (DAOY cell extract). Protein was determined by the method of Bradford [1], and the results are expressed as femtomoles of O^6-methylguanine released from the DNA substrate per milligram of protein.

Drug effects on AGT activity

Calf thymus DNA (Sigma) was treated with pancreatic ribonuclease that was extracted with phenol, precipitated with ethanol, and dissolved in deionized water prior to reaction. The resulting DNA (1 mg/ml) was incubated at 37 °C for 1 h with 5 m*M* 4-HC, PM, or acrolein, or without alkylating agent in 0.1 *M* Na_3PO_4 (pH 7.0). The DNA was purified from each unreacted drug by spin chromatography through Sephadex G-25 followed by ethanol precipitation and was then suspended in deionized water.

Purified, recombinant, human AGT was preincubated with 50 mg DNA from each drug treatment described above for 30 min at 37 °C in a total volume of 0.5 ml 0.05 *M* Tris-Cl (pH 7.5), 5 m*M* dithiothreitol, 0.1 m*M* EDTA. The AGT activity of each was then determined by a 30-min incubation with DNA substrate that had been methylated by reaction with *N*-[^{3}H]methyl-*N*-nitrosourea (Amersham, Arlington Heights, Ill.) essentially as described previously [6, 7].

Subcutaneous tumor transplantation and measurements

Subcutaneous tumor transplantation into the right flank of animals and tumor measurements were conducted as previously described using isovolumetric inoculations of a tumor brei [10].

Xenograft

The Mer$^+$ human glioblastoma xenograft D456 MG was used for all animal studies as previously described [13].

Xenograft therapy

Groups of ten randomly selected mice were treated when the median tumor volume exceeded 200 mm^3. Cyclophosphamide was given to one group via i.p. injection at a single dose of 232 mg/kg in 0.9% saline, which represents 50% of the dose lethal to 10% of treated animals (LD_{10}). A second group received cyclophosphamide after they were pretreated 1 h earlier with O^6-BG at a dose of 30 mg/kg in 40% polyethylene glycol-400 in 0.9% saline which depletes xenograft AGT levels by >90% [13]. A third group received O^6-BG followed by 0.9% saline, and the control group received 0.9% saline alone.

Assessment of response

The response of s.c. xenografts was assessed by delay in tumor growth (T−C), the difference in time between the median of drug-treated tumors and the median of vehicle-treated tumors to reach a volume five times the volume at initial treatment, and tumor regression as previously described [10].

Results

D283 Med/D283 Med (4-HCR) cytotoxicity studies

D283 Med and D283 Med (4-HCR) displayed differential responses to 4-HC as previously reported [8]. Preincubation of the cells with O^6-BG increased the cytotoxicity of 4-HC against D283 Med (4-HCR) but not against D283 Med (Fig. 1). PM cytotoxicity was not altered by O^6-BG in either cell line, whereas the cytotoxicity of 4-HDC was increased with O^6-BG pretreatment, similar to that of 4-HC, only in D283 Med (4-HCR).

AGT activity

The AGT activity in D283 Med and D283 Med (4-HCR) was 76 ± 96 and 466 ± 164 (mean ± SD, $n = 18$) fmol/mg protein, respectively. The AGT activity was more than sixfold higher for the 4-HCR-resistant cell line ($P < 0.01$). Treatment with O^6-BG produced >90% depletion of AGT.

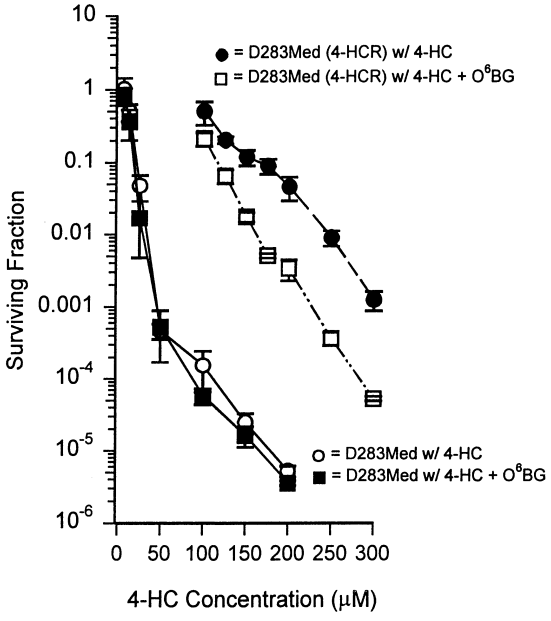


Fig. 1 Surviving fraction of D283 Med and D283 Med (4-HCR) cells treated with 4-HC for 1 h prior to exposure to O^6-BG (100 μ*M* for 10 min) and subsequently plated for limiting dilution analysis

Transfected CHO studies

To test whether the presence of AGT alone is able to provide protection against the toxicity of 4-HC, we used CHO cells that were stably transfected with a plasmid expressing the human AGT protein and compared these with the parental CHO cells that were transfected with a control plasmid and do not express AGT. As shown in Fig. 2, there was a clear difference in response between these two isogenic cell lines, with the cells expressing AGT being significantly less sensitive to killing by 4-HC. This result was highly reproducible in a number of experiments, and curves from two representative experiments are shown in Fig. 2 A and B. A similar difference between the sensitivity of control CHO cells and AGT-expressing CHO cells was seen when 4-HDC was used (Fig. 2C and D). Treatment of the AGT-expressing CHO cells with O^6-BG abolished this reduced sensitivity to 4-HC and 4-HDC (data not shown).

Drug effects on AGT activity

To examine whether alkylated DNA had an effect on AGT activity, we reacted DNA with 4-HC, PM, or acrolein and then incubated the DNA with AGT protein. As shown in Table 1, DNA alkylated with 4-HC caused a decrease in AGT activity. In aqueous solution, 4-HC decomposes to form PM and acrolein. Alkylation of DNA by PM had the opposite effect on AGT activity, actually stimulating the enzyme activity. Acrolein-treated DNA decreased AGT activity to an extent similar to that caused by 4-HC.

Xenograft therapy

Tumors in vehicle-treated mice reached five times the treatment size in 18.0–21.7 days (median 20.8 days). Treatment of D-456 MG with cyclophosphamide produced a growth delay of 14.4 days with ten tumor regressions out of ten mice treated. Treatment with O^6-BG alone produced a growth delay of 0 days with no tumor regressions. The combination of cyclophosphamide and O^6-BG produced a growth delay of 18.4 days with seven tumor regressions out of eight mice treated. These values were statistically significant ($P \leq 0.001$) compared with the results produced by vehicle alone. The difference between cyclophosphamide plus O^6-BG and cyclophosphamide alone demonstrated a *P*-value of 0.07.

The mortality in each of the groups was as follows: cyclophosphamide, no deaths; O^6-BG, no deaths; cyclophosphamide plus O^6-BG, two deaths.

Discussion

Resistance to cyclophosphamide is multifactorial and has been associated with elevations of glutathione,

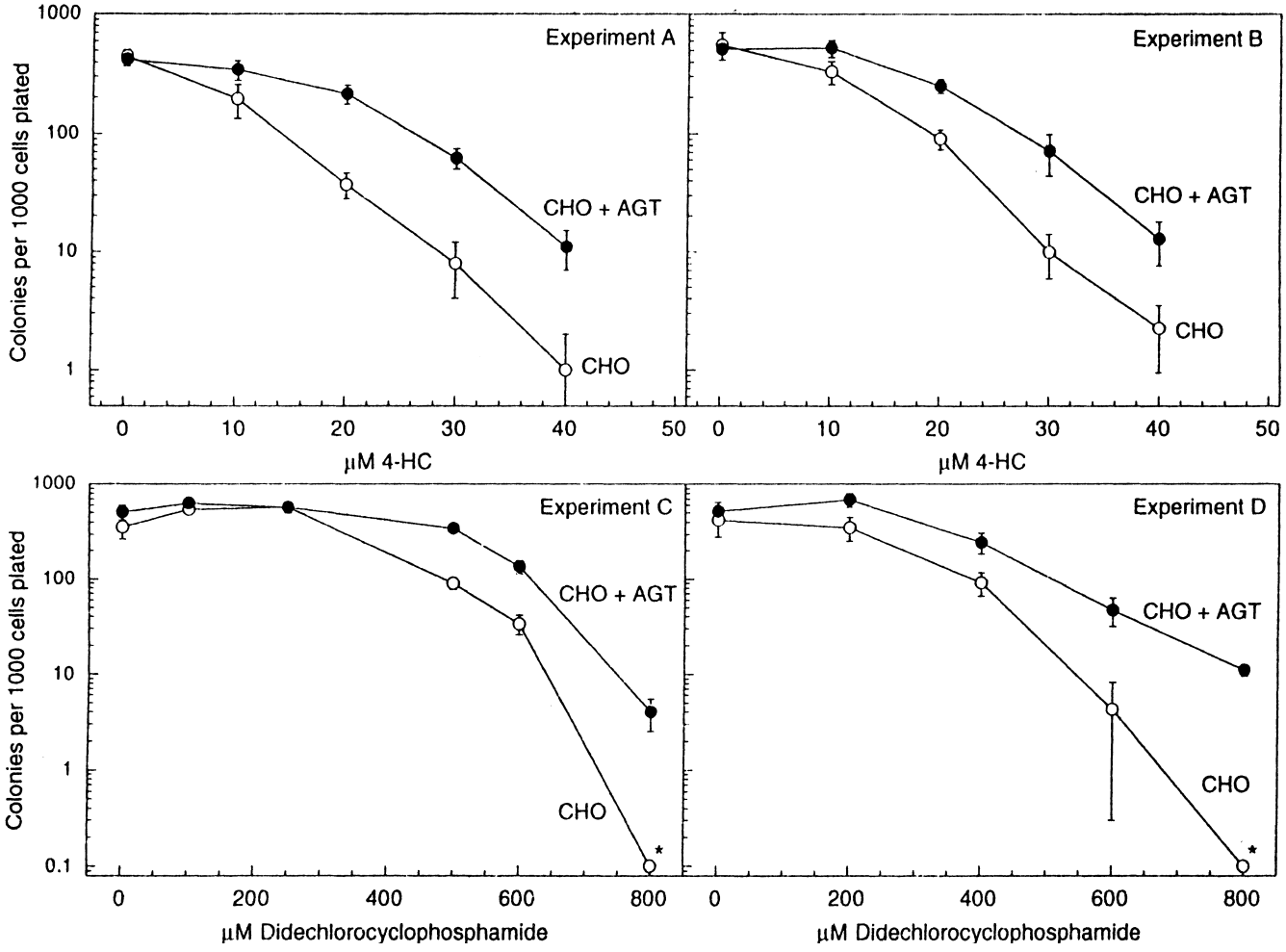


Fig. 2A–D Effect of 4-HC and 4-HDC treatment on AGT levels in CHO cells. Cells were treated for 1 h at the concentrations shown. The medium was then replaced, and the cells were allowed to grow for 16 h (**A** and **C**) or for 8 h (**B** and **D**) before replating at densities of 100–1000 per 5-cm^2 flask. Cells were grown for 7–8 days until discrete colonies could be stained and counted. The points marked * had no detectable surviving colonies

glutathione-*S*-transferase, or aldehyde dehydrogenase, as well as with repair of DNA interstrand crosslinks [8]. A direct relationship between cyclophosphamide antineoplastic activity and/or resistance to AGT has, to our knowledge, not been previously described.

The studies reported here indicate that 4-HC cytotoxicity is enhanced when AGT levels are reduced by pretreatment with O^6-BG. These results were seen only in the cell line with elevated AGT levels, D283 Med (4-HCR), and not with D283 Med, which demonstrates very low AGT activity. 4-HC is also more active in the CHO cell line missing the human AGT gene than in the same line expressing this gene. Together, these experiments support an interaction between 4-HC and AGT. Since it seemed unlikely that AGT acts on the initial N7 alkylation of guanylic acid produced by PM, we turned our attention to this as well as other potential mechanisms of cyclophosphamide-induced toxicity that might be susceptible to AGT. Additional experiments were therefore conducted to determine if PM, the metabolite critical for crosslink formation, or acrolein was the 4-HC metabolite modulated by AGT.

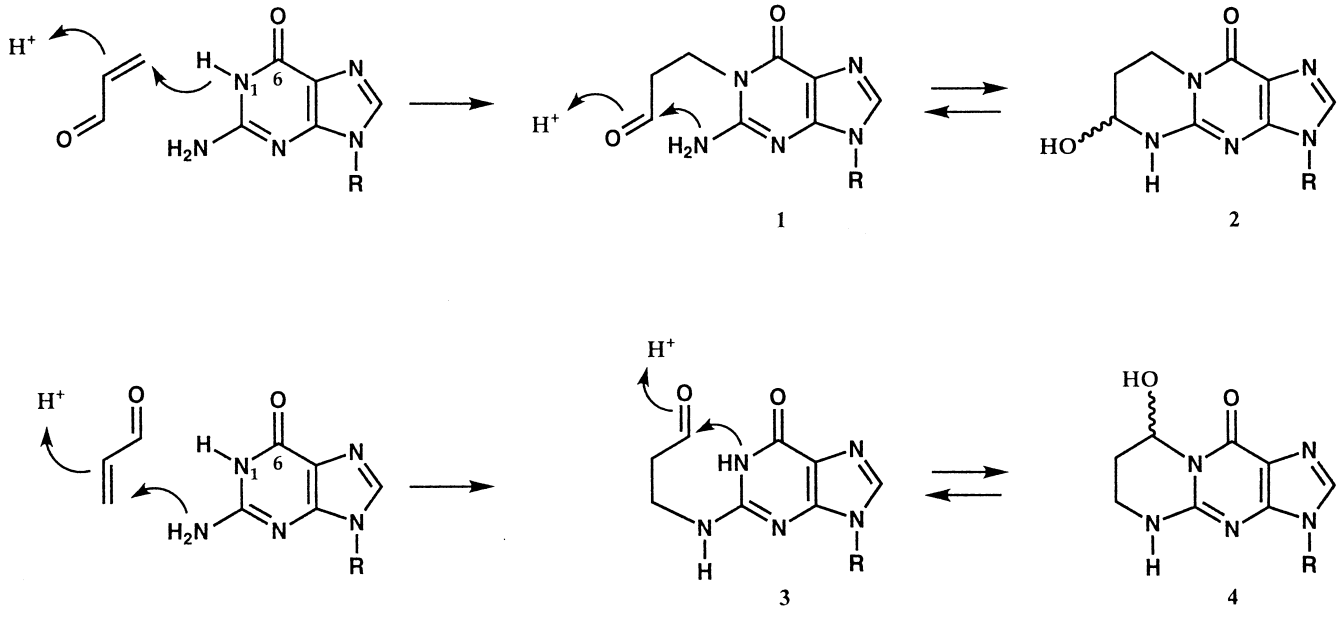
Table 1 Effect of drug-exposed DNA on AGT activity. Values are means ± SEM (n = 4)

Drug	AGT (fmol/mg)
None	2730 +/– 210
4-HC	1720 +/– 145
PM	6840 +/– 140
Acrolein	1710 +/– 180

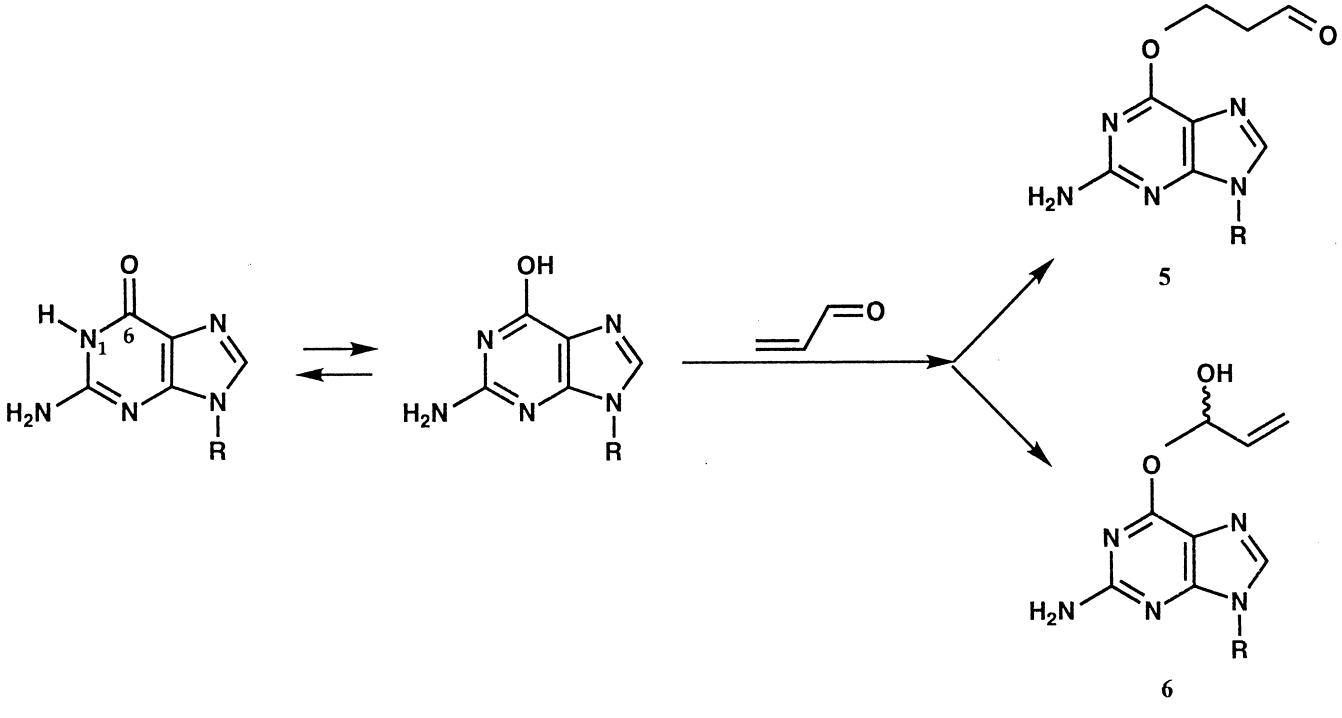
Incubation of DNA with 4-HC, PM, or acrolein demonstrated a depletion of AGT only after 4-HC or acrolein treatment. These results are consistent with those of previous studies demonstrating inhibition of AGT activity by cyclophosphamide [16] or aldehydes [14, 15]. Furthermore, Meer et al. [18] have previously suggested that cyclophosphamide alkylates the O^6 position of guanine. Although Preuss et al. [20] did not show modulation of cyclophosphamide by AGT, the in vitro studies used mafosfamide under different conditions. Cytotoxicity experiments demonstrated O^6-BG-mediated enhancement of 4-HC or 4-HDC (which

spontaneously releases acrolein but does not produce PM since it contains no chlorine atoms) but not PM. Similar results were noted using the CHO cells (with and without the human AGT gene), with 4-HC and 4-HDC showing differential cytotoxicity. The xenograft studies demonstrated that enhancement of antitumor activity and toxicity occurs if cyclophosphamide is administered following O^6-BG-mediated depletion of tumor AGT levels.

These findings suggest that the toxicity abrogated by AGT may be due to acrolein. It has previously been demonstrated that acrolein produces cyclic adducts between the N^1 and extracyclic amino nitrogen of guanylic acid in DNA [4], as shown in Scheme 1. An initial reaction between C3 of acrolein and either N1 or the exocyclic amino nitrogen produces *1* or *3*, respectively. Ring closure in *1* and *3* occurs through the reaction of the aldehydic carbon to give *2* and *4*, respectively. The



Scheme 1



Scheme 2

hemiaminals *2* and *4* are expected to be in equilibrium with their ring-opened tautomers *1* and *3*, respectively. Adducts *1* and *3* with alkylated nitrogens may be chemically analogous to O^6-alkylated species and may be substrates for attack by AGT to remove the alkyl appendage in the cell lines used in these studies. Alternatively, formal alkylation of the O^6-position of guanine in DNA could occur by addition of the 6-hydroxy group in the enol tautomer of guanine moieties in DNA to acrolein to produce a β-carbonyethyl ether derivative (Scheme 2) which, by close analogy with O^6-methyl- and O^6-benzyl-guanines, would be predicted to be isosteric with such structures and to be a typically structured substrate of AGT. Further, addition of the aldehyde function of acrolein to the 6-hydroxy group in the enol tautomer is also possible, yielding a hemiacetal, which similarly may be a substrate for AGT. These latter two structures would not require C—N bond cleavage for repair of the adduct by AGT. Elucidation of the mechanism of adduct removal is currently under investigation by determining if structures *1–2* and *3–4* [14] are substrates for AGT in a cell-free system, which would indicate whether AGT is able to cleave a C—N bond in the putative adduct structures. Therefore, we propose that a small but potentially significant part of the cellular toxicity of cyclophosphamide is due to acrolein and that this particular cytotoxicity is abrogated by removal of the acrolein adduct from DNA by AGT. This putative mechanism of resistance is currently being investigated.

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