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Depletion of O^6-alkylguanine-DNA alkyltransferase activity in mammalian tissues and human tumor xenografts in nude mice by treatment with O^6-methylguanine

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Summary. We have previously shown that exposure of cells in culture to O^6-methylguanine significantly reduces their level of the repair protein, O^6-alkylguanine-DNA-alkyltransferase (AGT), thus rendering cells more sensitive to the cytotoxic effects of chemotherapeutic chloroethylating agents. Experiments were carried out in mice to determine whether the AGT content of tissues and tumors could be reduced by in vivo treatment with O^6-methylguanine. There was a dose-dependent decrease in AGT activity in liver tissues of CD-1 mice to 24% of basal levels after four hourly intraperitoneal injections of O^6-methylguanine (110 mg/kg). Although the decline in AGT activity in the liver was reversible, the activity remained at 75% of basal levels for up to 25 h after the final injection. The effect of O^6-methylguanine treatment on AGT activity was measured in mouse tissues as well as human colonic carcinoma tumors (HT29 and BE) grown in Swiss athymic nude mice. The activity in the liver, kidney, and spleen of these mice decreased to 33%–35% of control levels, whereas the activity in HT29 tumors was likewise diminished to 25% of control levels after four hourly injections of O^6-methylguanine (100 mg/kg). There was no enhancement of the tumoricidal effectiveness of chloroethylating agents on the HT29 tumor after O^6-methylguanine treatment, probably due to a disproportionately higher level of AGT in human tissue than in murine tissue. However, these studies suggest that O^6-methylguanine can be given in vivo to examine the role of the AGT protein in protecting against the toxic and carcinogenic effects of alkylating agents.

Introduction

The chemotherapeutic chloroethylating agents, such as the chloroethylnitrosoureas, are effective against brain tumors and advanced lymphomas [2, 38]. Their antitumor activity appears to be a consequence of DNA alkylation and subsequent interstrand cross-linking of complimentary DNA molecules [16, 21, 37]. The mechanism by which this occurs involves an initial spontaneous decomposition of the alkylating agent to form an active chloroethylating intermediate, followed by the formation of a chloroethyl monoadduct at the O^6-position of guanine. The conversion of monoadducts to lethal DNA cross-links then proceeds slowly and involves an intramolecular rearrangement that is followed by an intermolecular reaction with cytosine on the opposite strand of DNA. This mechanism of cytotoxicity is supported by the findings of Erickson et al. [13, 15], which reveal that cells that are highly sensitive to the chloroethylnitrosoureas exhibit a greater degree of interstrand cross-links.

The aforementioned conversion of monoadducts to cross-links may be prevented by the presence of a DNA repair protein, O^6-alkylguanine-DNA alkyltransferase (AGT), which occurs in mammalian tissue in varying amounts, depending on the species and the cell type [18, 28, 29, 40]. AGT transfers an alkyl group from the O^6-position of guanine in DNA onto one of its own cysteine residues. The alkyl groups known to be accepted by this protein include methyl, ethyl, hydroxyethyl, propyl, and butyl adducts on guanine [25, 26, 31]. There is indirect evidence that the chloroethyl group is also repaired by the AGT protein, since the amount of AGT present within a cell or tumor is inversely proportional to sensitivity to the chloroethylnitrosoureas [5, 14, 32, 34]. The alkylated form of AGT is not regenerated in the reaction; hence, the number of O^6-alkylguanine lesions that can be repaired is limited by the quantity of AGT present. Nonetheless, numerous studies have demonstrated that AGT plays an important role in the protection of cells from damage produced by chemotherapeutic alkylating agents [40].

One way in which levels of AGT have been effectively diminished is through exposure of the cells to O^6-alkylguanines [7, 20]. Specifically, O^6-methylguanine acts as a weak substrate for the AGT protein, thereby depleting cells of active AGT [8, 41]. Thus, depletion of AGT in tumor cells through demethylation of the free base leaves less AGT available for the repair of cytotoxic lesions introduced by chloroethylating agents; consequently, cytotoxicity is enhanced [7, 9, 10, 17, 41]. These observations have led to the possibility that O^6-methylguanine could be used to deplete the AGT activity of tumors and enhance the effect of the chloroethylating agents in vivo. In the present study we determined the effect of O^6-methylguanine on the reduction of AGT activity in both murine tissues and human colonic carcinoma xenografts in nude mice. We also studied the contribution of O^6-

Abbreviations: AGT, O^6-alkylguanine-DNA alkyltransferase; MeCCNU, 1-(2-chloroethyl)-3-(4-methylcyclohexyl)-1-nitrosourea; Clomesone, 2-chloroethyl(methylsulfonyl) methanesulfonate; HPLC, high-pressure liquid chromatography.

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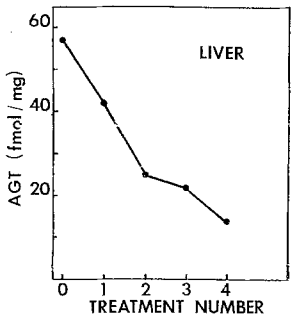

Fig. 1. AGT depletion in mouse liver after treatment with O^6-methylguanine. CD-1 mice were administered hourly i.p. injections of 110 mg/kg O^6-methylguanine. The amount of AGT in the liver was determined as described in *Materials and methods*. Results represent the means of measurements from four animals for each treatment

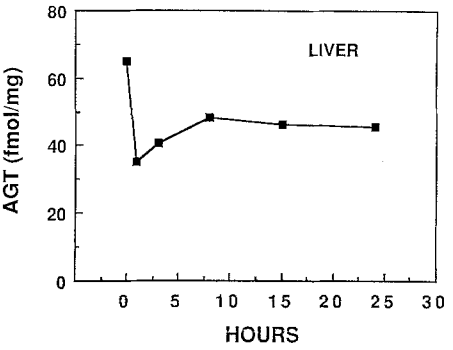

Fig. 2. Time course of mouse liver AGT activity after treatment with O^6-methylguanine. CD-1 mice were treated at hourly intervals with 80 mg/kg. After the fourth injection, animals were sacrificed at various time points and their livers were removed to analyze AGT activity. Results represent the means of measurements from two animals per time point

methylguanine pretreatment on the cytotoxic effects of CCNU and Clomesone chemotherapies in vivo. The objective was to determine whether the increased sensitivity of pretreated cells to the cytotoxic effects of chloroethylating agents observed in vitro could also be seen in vivo.

Materials and methods

Animals. Female CD-1 mice (20 g) were purchased from Charles River Breeding Laboratories, Inc. (Wilmington, Mass). Female NIH Swiss, random-bred athymic mice (4–6 weeks) were obtained from the Frederick Cancer Research Center (Frederick, Md). All animals were maintained under a controlled 12-h light 12-h dark cycle and allowed free access to water and food. Athymic mice were housed in animal isolators consisting of flexible, clear vinyl plastic in which the air supply was filtered. All food water, and supplies were sterilized before passage into the isolator.

Xenograft tumors. Human colonic carcinoma cell lines HT29 and BE were kindly provided by Dr. L. C. Erickson (Loyola Stritch School of Medicine, Maywood, Ill). Both cell lines were maintained in culture as previously described [9, 13]. HT29 or BE cells (10^7 cells) were injected subcutaneously into the flank area of each animal, and within 6–8 days palpable tumors appeared. The animals were weighed and the tumors were measured every 48 h with a caliper. The tumor-growth data are expressed as tumor volumes (mm^3), which are derived from the sum of length $\times$ width^2 $\times 0.5$. When tumors were in the range of 500–750 mm^3, tissues and tumors were removed to analyze AGT activity. Animals were treated with O^6-methylguanine and/or chloroethylating agent when tumors reached the range of 250–400 mm^3.

Drug treatment. MeCCNU (NSC 95441) and Clomesone (NSC 338947) were kindly provided by the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute (Bethesda, Md). Synthesis of O^6-methylguanine followed the procedure of Balsiger and Montgomery [3]. Hoechst dye was obtained from Calbiochem (San Diego, Calif), and all other biochemicals were purchased from Sigma Chemical Co. (St. Louis, Mo).

Each treatment consisted of an intraperitoneal injection of a sterile solution of 2 mg O^6-methylguanine dissolved in 1 ml 0.9% (w/v) saline such that the final concentration was 80–110 mg/kg. Due to the limited solubility of O^6-methylguanine, the compound was initially dissolved in saline (pH 2) and slowly brought up to pH 7.4 with NaOH. Solutions were sonicated for 10 min prior to each injection. Between one and four treatments of O^6-methylguanine were given at hourly intervals, and animals were killed by cervical dislocation 1 h after the final injection. Tissues and tumors were removed, immediately frozen in liquid nitrogen, and stored at $-70°$ C until the time of assay.

Athymic mice were randomized into four groups consisting of no treatment, O^6-methylguanine alone, saline plus chloroethylating agent, or O^6-methylguanine followed by chloroethylating agent. MeCCNU and Clomesone dissolved in 10% ethanol/10% cremophor/80% saline (by vol.) were given i.p. 30 min after the fourth injection of O^6-methylguanine in a volume of 0.01 ml/g animal wt., to give final concentrations of between 5 and 18 mg/kg MeCCNU or 17 and 50 mg/kg Clomesone. Tumor measurements and animal weights were determined every 48 h after treatment.

AGT determination. Crude extracts were prepared from mouse tissue and human tumor xenografts by previously described methods [12, 30]. Briefly, these were homogenized, sonicated, and centrifuged in buffer containing 50 m*M* TRIS-HCl, 0.1 m*M* EDTA, and 5 m*M* dithiothreitol. After incubation of crude extracts with [^{3}H]-methylated DNA for 30 min at 37° C, ice-cold perchloric acid at a final concentration of 0.4 *N* was added and the pelleted DNA was hydrolyzed in 0.1 *N* HCl at 70° C. The modified bases were separated using reverse-phase HPLC, and radioactivity was determined after the addition of 10 ml ACS II. Protein was determined by the method of Bradford [4], and DNA content was determined by the Hoechst 33258 method [22].

Results

When CD-1 mice were injected with O^6-methylguanine, there was a dose-dependent decrease in AGT activity in

Table 1. AGT levels in neoplastic and nonneoplastic tissues in athymic mice

	Alkyltransferase activity	
	(fmol/mg protein)	(fmol/mg DNA)
HT29 tumor	277	1,796
BE tumor	6	34
Liver	45	1,188
Kidney	13	257
Spleen	35	101

Table 2. Effect of O^6-methylguanine on AGT activity in tissues of athymic mice

	% AGT activity remaining:		
Treatment	Liver	Kidney	Spleen
None	100	100	100
Saline	105	82	114
O^6-methylguanine	35	35	33

Athymic mice were treated with four hourly injections of 100 mg O^6-methylguanine/kg and sacrificed 1 h after the final injection. Tissues were removed, extract was prepared, and AGT content was determined as described in Materials and methods. Each number represents the average value obtained from 4–5 animals

the liver as the number of hourly injections of 110 mg/kg increased (Fig. 1). There was no significant loss of activity after four injections of the same volume of saline or the same concentration of guanosine (data not shown). Furthermore, treatment with four injections of O^6-methylguanine resulted in no observable host toxicity, using animal body weight as the indicating parameter.

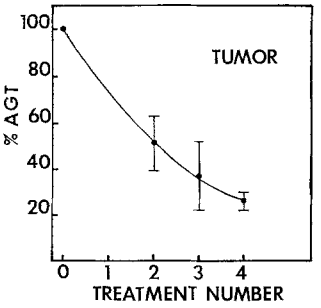

Fig. 3. AGT depletion in HT29 xenograft tumors carried in athymic mice after treatment with O^6-methylguanine. Athymic mice were treated hourly for up to 4 h with 100 mg/kg O^6-methylguanine. Animals were sacrificed 1 h after each treatment, tumors were excised, and AGT activity was analyzed as described. Results represent the means of measurements from at least four animals per treatment group

A time course of the recovery of AGT activity after depletion by O^6-methylguanine treatment was carried out (Fig. 2). CD-1 mice received four hourly injections of 80 mg/kg, and AGT activity was determined in livers removed between 1 and 25 h after the final injection. Within the 1st h, AGT levels were rapidly reduced to 50% of pretreatment values, followed by a steady rise to plateau at 75% of pretreatment levels. The plateau was first achieved at 8 h and was maintained at 14 and 25 h.

Athymic nude mice were injected in the flank region with 10^7 HT29 or BE cells. Histological sections of the tumors as well as morphological examination of the respective cell lines showed that, although the tumors were similar to their respective cell line in terms of growth characteristics, there were distinct differences between the HT29 and BE tumors. HT29 was well-differentiated and grew in a more ordered pattern as a tumor and in culture than did the undifferentiated BE adenocarcinoma of the colon, which grew in an unorganized pattern. This is in agreement with earlier reports on these cell lines [27]. AGT

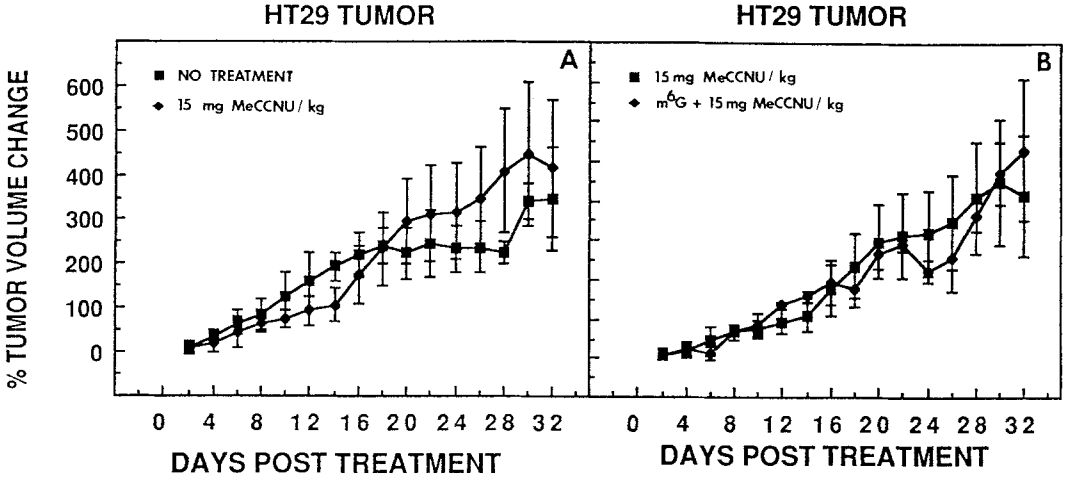

Fig. 4. Response of the HT29 tumor carried in athymic mice to MeCCNU and/or O^6-methylguanine. **A** Results for relative tumor volumes in athymic mice carrying HT29 tumors treated with vehicle (■) or 15 mg/kg MeCCNU (◆). **B** Results for relative tumor volume after treatment with 15 mg/kg MeCCNU (■) or O^6-methylguanine plus 15 mg/kg MeCCNU (◆). Data points represent the mean (±SEM) percentage of change in tumor volumes of five animals

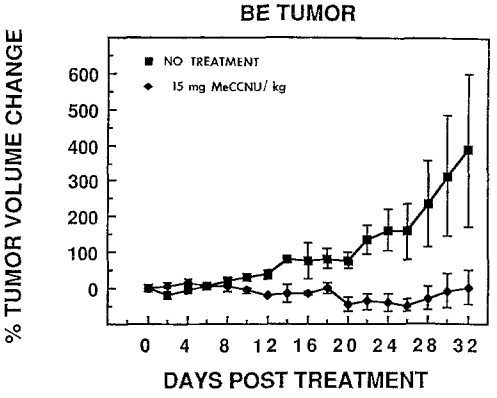

Fig. 5. Response of the BE tumor carried in athymic mice to MeCCNU. Relative tumor volumes of athymic mice carrying BE tumors treated with vehicle (■) or 15 mg/kg MeCCNU (◆). Data points represent the mean (±SEM) percentage of change in tumor volume

levels in HT29 and BE lines, their resultant tumors, and nonneoplastic tissues of the mice were analyzed and expressed in terms of protein and DNA content (Table 1). The tumors had the same activity as their respective cell lines. The AGT activity of the HT29 tumor was 46 fold greater than that found in the BE tumor and 6 fold greater than that in the mouse liver (Table 1). The AGT activity in HT29 tumor xenografts carried in athymic mice was depleted by treatment with O^6-methylguanine (Fig. 3). After four hourly injections of 100 mg/kg O^6-methylguanine, AGT levels were decreased to 25% of pretreatment levels (Fig. 3). Similar injections of the saline vehicle alone did not cause a decrease in the activity in the tumor (data not shown). The AGT activity in the maximally depleted HT29 tumor was 16-fold greater than that in the BE tumor.

Table 2 illustrates the effect of four injections of saline or 100 mg/kg O^6-methylguanine on the AGT activity in the liver, kidney, and spleen of athymic mice. The level of AGT did not change significantly after treatment with saline, but in each case it was reduced to approximately 35% of control values after O^6-methylguanine treatment.

The effect of O^6-methylguanine on the response of HT29 tumor xenografts to MeCCNU was studied by analyzing tumor growth after no treatment vs treatment with O^6-methylguanine alone, MeCCNU alone, or a combination of O^6-methylguanine and MeCCNU. The growth of the HT29 tumor was unaffected by MeCCNU (Fig. 4 A), O^6-methylguanine alone (data not shown), or the combination of MeCCNU and O^6-methylguanine (Fig. 4 B). In contrast, growth of the BE tumor was arrested after treatment with the same amount of MeCCNU (Fig. 5). As an indicator of host toxicity animal weights were measured in all groups. Animals that received alkylating agent alone exhibited slight weight losses; however, those receiving O^6-methylguanine alone exhibited no such change (data not shown).

Discussion

These experiments clearly show that a reduction in AGT activity in mammalian tissues can be achieved by treatment with O^6-methylguanine. Although extensive tests of the toxicology of this base given alone have not yet been completed, quite large doses were used in the present experiments without adverse effects being detected in either normal or athymic mice. This protocol may therefore be a less hazardous method of reducing cellular AGT levels than the pretreatments with monofunctional methylating agents such as streptozotocin or *N*-methyl-*N*-nitrosourea that have been proposed [1, 15, 42]. It is established that the presence of AGT renders mammalian tumor cell lines more resistant to the lethal effects of chloroethylating agents [1, 6–8, 14, 17, 34, 36, 41, 42]. Consequently, the possibility that the therapeutic indices of certain chloroethylnitrosoureas might be increased by combining them with substances that decrease AGT levels deserves further study.

Although the reduction of AGT activity by pretreatment with O^6-methylguanine did not demonstrate an enhancement of the therapeutic effectiveness of MeCCNU against the HT29 tumor, this is not unexpected, as this tumor contains a much higher AGT activity than normal mouse tissues. It is well established that the content of AGT is species-specific, with human cells having much higher activities than their rodent equivalents [18, 28, 29, 40]. Thus, the athymic mouse human-xenograft model provides a particularly unfavorable system in which the therapeutic protective effects of alkyltransferase depletion can be established.

At present, the administration of O^6-alkylguanine derivatives is the only feasible method available to reduce the cellular content of AGT besides the depletion brought about by treatment with methylating agents that reduce AGT by the formation of adducts in the DNA that are recognized by AGT; their repair then leads to the loss of AGT activity. However, the latter protocol obviously cannot be used in studies of the biological consequences of alkylation damage. Several groups have described experiments in which the importance of AGT to the in vitro response to alkylation damage in cells has been studied using O^6-methylguanine [6, 8, 10, 17, 34, 36, 41]. Our results indicate that these protocols can be extended to experiments in whole animals and that the role of the AGT protein in carcinogenesis or the toxic effects of alkylating agents can be examined by reduction of the AGT content by injection of O^6-methylguanine.

The major problems with the use of O^6-methylguanine for both therapeutic and experimental purposes are threefold: (1) the relatively high doses of the compound that are required, (2) its limited solubility, and (3) the lack of selectivity of AGT reduction. The large doses are required due to both the relatively poor uptake and the low affinity of the AGT protein for the free base [8, 41]. Some of these problems may be overcome by alterations in the route of administration or by the design of newer inhibitors. It is possible that some other alkyl substituents at the O^6-position might be more useful in vivo, since in cell culture, a number of other O^6-alkylguanines were active in bringing about AGT depletion [8]. These included the O^6-*n*-butylguanine, which was almost as potent as O^6-methylguanine and may exhibit enhanced cellular uptake. Unfortunately, the solubility of these derivatives is lower than that of O^6-methylguanine itself; therefore, alternative methods of administration may have to be devised. These procedures may also be able to achieve some selective uptake of the inactivator into tumor tissue. However, there is clearly a

need for the production of more potent and possibly tumor-specific inactivators of AGT. In this respect, it is interesting that Likhachev et al. [23] have recently reported that intraperitoneal administration of methylated DNA leads to an inactivation of rat liver AGT. This suggests that DNA fragments can be made available and act as substrates for AGT, thereby significantly reducing its activity.

We [11, 33] and others [19, 39] have shown that short-chain oligodeoxynucleotides containing O^6-methylguanine are substrates for AGT and that the rate of inactivation of AGT by these substrates is much faster than that achieved using O^6-methylguanine. However, the limitations of using conventional oligodeoxynucleotides as substrates are the possible degradation of these substrates due to nucleases and their poor uptake into cells. Therefore, derivatives of oligodeoxynucleotide compounds, such as methylphosphonates or phosphothioates [24, 35], containing O^6-methylguanine residues would probably have satisfactory biological stability and uptake and may be able to bring about the inactivation of the AGT protein at much lower concentrations.

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