Leukocyte O^6-Alkylguanine-DNA Alkyltransferase from Human Donors Is Uniformly Sensitive to O^6-Benzylguanine[1]

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ABSTRACT

O^6-Alkylguanine-DNA alkyltransferase (AGT) is the key DNA repair protein responsible for resistance to chloroethylating and methylating agents that attack at the O^6 position of guanine. O^6-Benzylguanine (BG), a potent inhibitor of AGT, has recently entered clinical trials. A number of point mutations and at least one human polymorphism within AGT are associated with AGT resistance to inactivation by BG. In this study, we evaluated AGT inhibition by BG in an *in vitro* assay of peripheral blood mononuclear cell AGT from 56 normal donors, 42 Caucasians, and 14 Japanese. AGT activity ranged from 2.7 to 21.9 fmol/μg DNA and was similar in Japanese and Caucasian donors. Depletion of AGT by BG was uniform in all donors with mean ED_{50}s of 0.37 μM BG in Caucasians and 0.36 μM BG in Japanese. To determine whether the gly160arg AGT polymorphism described in the Japanese population, and recently shown to be BG resistant, could be detected by this assay, we mixed purified gly160arg AGT protein with blood mononuclear cell extract and measured *in vitro* BG inactivation. The ED_{50} for the mixture of the gly160arg AGT and mononuclear cell extract was 9 μM BG. On the basis of results in 56 donors, we conclude that BG-resistant AGT, defined as an ED_{50} in mononuclear cells of >1 μM BG, is present in 0 of 56 donors, (95% confidence interval, 0–6%), suggesting that polymorphisms producing AGT-resistant BG are unusual in humans.

INTRODUCTION

The DNA repair protein AGT[3] is the key protein responsible for repair of chemotherapy and carcinogen-induced DNA adducts at the O^6 position of guanine (1). High levels of AGT correlate with resistance to a number of chemotherapy agents, including BCNU, dacarbazine, procarbazine, streptozotocin, and temozolomide (1–3), protect against the carcinogenic effects of methylating agents in animal models (4). Low levels of AGT predict sensitivity to these agents (5). Recently, a new therapeutic inhibitor of AGT, BG, has been described. BG sensitizes AGT expressing, BCNU-resistant tumors both *in vitro* and in xenograft models and is nontoxic at therapeutic concentrations (6, 7). Ongoing clinical trials have shown that it is possible to completely inhibit human AGT in patient peripheral blood mononuclear cells (8) and in tumors (9). We have documented, using sequential deep tissue tumor biopsies, 100% depletion of human tumor AGT at doses of 120 mg/m^2 BG, which produced maximum serum concentrations of 15 μM BG.

The wild-type AGT is remarkably sensitive to inhibition by BG, with an ED_{50} of about 0.5 μM in cell extracts (10). However, mutations in certain regions of AGT confer resistance to BG. For instance, the bacterial AGT protein derived from the *ada* gene (11), is remarkably resistant to BG but has similar capacity to remove O^6-mG adducts compared with that of human AGT (12). By point-directed mutagenesis, a number of amino acids that differ between *ada* and the human AGT gene, *MGMT*, when introduced into MGMT result in resistance to BG (13–15). Some mutant AGTs have reduced reactivity with O^6-mG or are unstable (15). The recent description of a polymorphism in the Japanese population at codon 160 of MGMT, a glycine to arginine mutation, gly160arg, (16), and the verification that this polymorphism resulted in a 20-fold increase in the ED_{50} for BG (17) raised concerns that polymorphisms in MGMT may be common and if present, could result in partial resistance to BG, which could impact significantly on clinical efficacy of BG as a modulator of AGT.

MATERIALS AND METHODS

Heparinized blood was collected after informed consent from normal volunteer Caucasian and Japanese donors. The mononuclear layer from 40 ml of blood was obtained after Ficoll-Hypaque isopyknic gradient and processed for alkyltransferase (AGT) activity as described previously (18, 19).

This laboratory has reported lymphocyte AGT from normal donors since 1985 and has developed a method to standardize activity measurements over many years that allows us to relate measurements performed in 1985 (18) with those performed in 1998 (20–22). Key components of this standardization procedure include a single method of measurement using direct assay of residual O^6-mG adducts on a substrate DNA, standardization of the substrate made from [^{3}H]MNU-treated calf thymus DNA
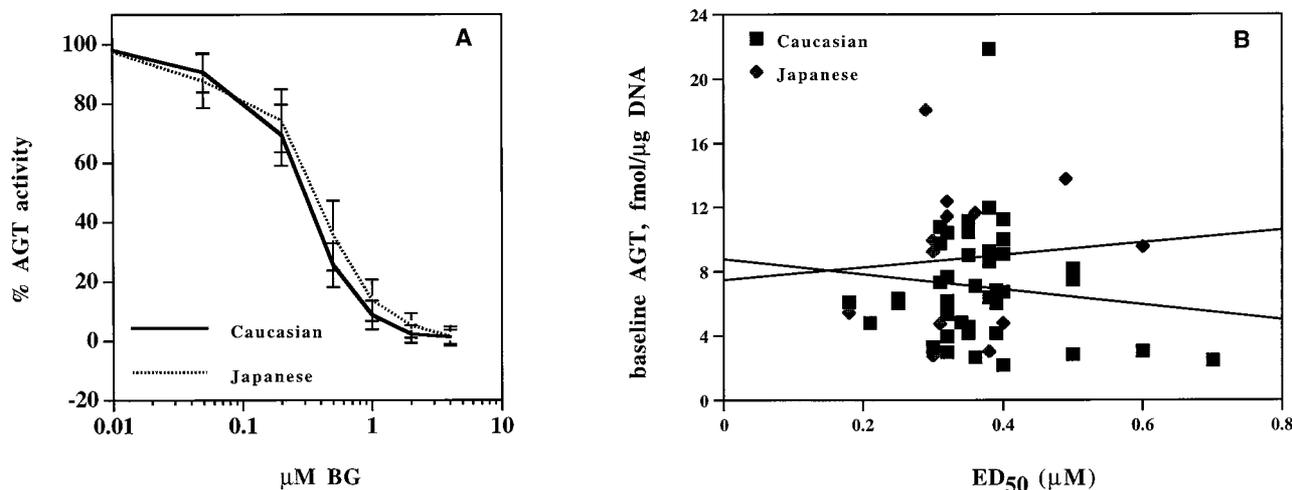
Fig. 1 Inactivation of human blood mononuclear cell AGT by O^6-benzylguanine. *A,* blood mononuclear cell extract was treated with increasing concentrations of BG for 30 min before the addition of [*methyl*-^{3}H]DNA substrate containing O^6-mg. Residual AGT was determined and plotted against the concentration of BG. The mean inactivation curves are shown for Caucasian and Japanese donors. The means for Caucasian and Japanese donors are shown; *bars,* SD. *B,* the distribution of ED50s for BG inactivation are plotted relative to the baseline AGT activity for each donor. ■, Caucasian donors; ◆, Japanese donors.

with direct comparison between old and new substrates, and use of AGT enzyme standards with each assay that have been from the same source over the entire time period.

RESULTS

In this study population of 56 donors, baseline peripheral blood mononuclear cell AGT varied 10-fold, from 2.7 to 21.9 fmol/μg DNA. The activity was not significantly related to age or gender. The AGT activity was similar in the Caucasian (7.1 ± 3.6 fmol/μg DNA, n = 42) and Japanese (8.9 ± 4.4 fmol/μg DNA, n = 14) groups. The overall range of activity was similar to that which we have reported previously in peripheral blood mononuclear cell AGT for >13 years in >300 samples (9, 19–22) and is the same range of peripheral blood mononuclear cell AGT activity seen in over 130 additional patient and normal donor samples analyzed in our laboratory that remain unreported. In none of these samples have we observed AGT of <1.5 or >32 fmol/μg DNA. Most importantly, we have never observed a human mononuclear sample with no AGT activity (limit of detection, 0.05 fmol/μg DNA, corresponding to ~180 molecules/cell). As we have reported previously, AGT measurement on the basis of cellular DNA is preferred because it allows better comparison to other tissues that have different relative amounts of DNA and protein (19), and because in peripheral blood mononuclear cell preparations, red cell contamination may sometimes increase the amount of apparent protein that is not derived from mononuclear cells. Because red cells contain negligible amounts of AGT, this can lead to a falsely low estimate of AGT. Although we have data (which is available) for all samples in units of activity/mg cellular protein, we will not report it here.

To measure BG inhibition of AGT activity, an amount of cellular extract representing a fixed amount of enzyme activity, as determined by baseline enzyme assay, was incubated with BG in a concentration range of 0, 0.05, 0.2, 0.5, 1, 2, and 4 μM before adding [*methyl*-^{3}H]calf thymus DNA. The amount of cellular protein used was 50 fmol (0, 0.05, and 0.2 μM BG) or 200 fmol (0.5, 1, 2, and 4 μM BG) of activity, depending upon the concentration of BG used. One BG stock solution in DMSO was used for all reaction concentrations, keeping final DMSO concentrations to 1% or less in the reactions. The reaction was preincubated for 30 min at 37°C, after which residual AGT activity was determined by adding [*methyl*-^{3}H]calf thymus DNA substrate. AGT inactivation at each concentration was determined as the percentage of baseline activity. ED$_{50}$ and ED$_{90}$ values were obtained for each donor sample. AGT depletion by 30 min exposure to BG of extracts of these blood mononuclear cells was consistent in all samples. Fig. 1*A* indicates that a similar pattern of AGT inactivation was observed in samples from Caucasian and Japanese groups. As shown in Table 1, the mean ED$_{50}$ for BG in the Caucasian donors was 0.37 ± 0.09 μM, n = 42, and among Japanese donors was 0.36 ± 0.11 μM, n = 14. Mean ED$_{90}$ for BG in the Caucasian donors was lower, 1.06 ± 0.29 μM, n = 42, than in Japanese donors, 1.52 ± 0.51 μM, n = 14, P = 0.002. The distribution of ED$_{50}$s is shown in Fig. 1*B* and is independent of baseline AGT. Similar data were obtained for the ED$_{90}$s. No outliers were evident, and all have ED$_{50}$s were <0.8 μM BG.

To estimate the situation in which a donor mononuclear cell preparation expressed AGT from two different alleles, both wild-type AGT protein and AGT gly160arg protein carrying the Japanese polymorphism at position 160, we mixed either wild-type purified protein or an activity-equivalent amount of human mononuclear cell extract with purified *Escherichia coli* transformant-derived AGT gly160arg protein (17). The aliquots of equal activity of AGTs (50 fmol activity for the purified wild-type or gly160arg mutant AGT, or a mixture of 25 fmol of wild-type with 25 fmol of gly160arg or mononuclear cell ex-

Table 1 Baseline AGT, ED_{50}, and ED_{90} values between Caucasian and Japanese lymphocyte donors

	Caucasian $n = 42$	Japanese $n = 14$	P^a
Mean AGT baseline value, fmol/µg DNA	7.05 ± 3.59	8.9 ± 4.42	0.12
ED_{50}, µM O^6-BG	0.37 ± 0.09	0.36 ± 0.11	0.77
ED_{90}, µM O^6-BG	1.14 ± 0.34	1.52 ± 0.51	0.002

^{a} Comparisons between Caucasian and Japanese samples.

tracts) were pre-incubated with different concentrations of BG at 37°C for 30 min in assay buffer containing 50 µg of calf thymus DNA. The residual AGT activity was determined by incubating the samples with [^{3}H] methylated DNA for 30 min at 37°C. The ED_{50}s reflected the presence of the BG-sensitive wild-type protein (ED_{50}, 1.3 µM) and the BG-resistant gly160arg AGT (ED_{50}, 24 µM). In the mixture of purified proteins, the ED_{50} was 17 µM. For the mixture containing mononuclear cell extract and wild-type purified AGT, the ED_{90} was 1.8 µM, and with BG-resistant mutant AGT, the ED_{50} was 9 µM. These values were much higher than the ED_{50} and ED_{90} values we observed in any of the donor samples, indicating that there was no sample with a high degree of BG resistance.

DISCUSSION

This study indicates that inactivation of AGT by BG is very uniform in a sample of 56 normal donors in the United States, including 42 Caucasians and 14 Japanese. No outliers with increased sensitivity or resistance to BG were seen, and there was no correlation between baseline AGT and BG inactivation in individual donors. These studies were undertaken because of a number of reports about the presence of polymorphisms in AGT, which could render the protein resistant to BG. Such polymorphisms, if prevalent in the population, could impact on the therapeutic efficacy of BG. Imai (16) reported that a polymorphism at position Gly160Arg was present in 10–15% of the Japanese population. Edara *et al.* (17) and Dolan (23) reported recently that this mutation renders the human AGT 20-fold resistant to inactivation of BG and its active metabolite, 8-oxo-benzoguanine, suggesting that 15% of the Japanese population might be clinically resistant to BG. However, in a recent publication, Dolan (28) failed to find the gly160arg mutation in >200 DNA samples from American patients (95% confidence interval, 0–1.6%). Nonetheless, if the gly160arg mutation is present in human tumors, the two active compounds present in the plasma after BG exposure, using doses used in current clinical trials of between 80 and 120 mg/m^2 (8, 9), would not be effective.

More recently, other AGT polymorphisms have been reported. These include Ile143Val, Lys178Arg (24); Leu84Phe and Trp65Cys (25); and other isolated mutations in cancer patients (26). Whether these result in a BG-resistant AGT remains to be determined. On the other hand, some of the described MGMT mutants produce an AGT protein, which have less activity against O^6-mG yet are BG resistant, suggesting that some of the possible mutation sites may be selected against because they have less ability to protect against naturally occurring O^6-mG DNA adducts.

The tact we took, analysis of BG sensitivity, is quite different than a search for polymorphisms in the *MGMT* gene. We wanted to determine whether there was a functional difference in AGT among individuals, *i.e.,* whether we could detect differences in AGT based on analysis of AGT activity in lymphocytes. From this we would infer that these BG-resistant AGT reflected germ-line mutations in the *MGMT* gene. We observed no such functional polymorphism among 56 donors and conclude that ED_{50} values of >1 µM BG are uncommon (95% confidence interval, 0–6%).

A final issue that remains in the assessment of altered AGT sensitivity to BG is whether AGT mutations that are BG resistant are present in patient tumors, although they may not be germ line. These could arise either during the transformation process, perhaps due to genomic instability, or be acquired during drug treatment. We have reported in preliminary form the acquisition of resistance to BG and BCNU in MCF-7 breast cancer cells (27) and colon cancer HCT116 cells (28) after repeated exposure to these agents. The MCF-7 cells resistant to BG and BCNU have normal AGT activity and normal AGT sensitivity to BG, suggesting that in these cells, resistance is not due to an alteration in AGT. In HCT116, however, data suggest that the cell has a mutated AGT with less reactivity toward O^6-mG and markedly increased resistance to BG.[4] This suggests that tumors might acquire resistance to BG and BCNU through mutation in the *MGMT* gene. Whether this occurs clinically and is responsible for acquired resistance to BG and BCNU remains to be evaluated.

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