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ORIGINAL ARTICLE

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Inactivation of O^6-alkylguanine-DNA alkyltransferase by 8-substituted O^6-benzylguanine analogs in mice

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Abstract *Purpose*: The purpose of this study was to determine the usefulness of various 8-substituted O^6-benzylguanine (BG) analogs as modulators of the DNA repair protein, O^6-alkylguanine-DNA alkyltransferase (AGT). More specifically, the degree of inactivation of AGT in mouse brain, liver, kidney and tumor by O^6-benzyl-8-oxoguanine (8-oxoBG), 8-aza-O^6-benzylguanine (8-azaBG), O^6-benzyl-8-bromoguanine (8-bromoBG) and O^6-benzyl-8-trifluoromethylguanine (8-tfmBG) was compared to inactivation by BG, a modulator in phase II clinical trials. BG is converted rapidly to 8-oxoBG in rodents, monkeys and humans. It was reasoned that 8-substituted analogs of BG would exhibit different pharmacological properties compared to BG which could influence tissue bioavailability and, thus, the extent of AGT inactivation in vivo. We compared the tissue distribution of these agents and AGT activity following administration of the 8-substituted analogs. *Materials and methods*: At various time points up to 24 h after i.p. administration of the BG analogs, tissues (i.e. brain, liver, kidney), A549 lung tumor xenografts (i.p.) or D456 brain tumor xenografts (i.c.) were harvested from athymic nude mice for AGT analysis. AGT activity was quantified in tissue extracts using a biochemical assay with [^{3}H]methylated DNA as a substrate. In addition, concentrations of BG and 8-oxoBG were determined by HPLC with fluorescence detection in mouse tissues following administration of drug. *Results*: Each of the 8-substituted analogs of BG demonstrated variable AGT inactivation capabilities that were comparable to or better than those of BG especially in kidney and brain tissues. There was a more pronounced depletion of AGT inactivation in brain and D456 brain tumor xenografts following administration of BG compared to 8-oxoBG that could be explained by a much greater concentration of AGT-inactivating drug (BG plus the metabolite 8-oxoBG for mice treated with BG versus 8-oxoBG for mice treated with 8-oxoBG) present in these tissues. The AUCs for brain, kidney and liver were 3.2, 6.9 and 11.8 times greater for BG than for 8-oxoBG. *Conclusions*: 8-substituted analogs of BG possess unique AGT-inactivation profiles in vivo that are different from that of BG. The AGT-inhibitory activities of BG and its major metabolite, 8-oxoBG, are related to tissue disposition of both drugs.

Key words Alkyltransferase · O^6-Benzylguanine · Mice · Pharmacokinetic · O^6-Benzyl-8-oxoguanine

Abbreviations *AGT:* O^6-alkylguanine-DNA alkyltransferase *BCNU:* 1,3-bis(2-chloroethyl)-1-nitrosourea *BG:* O^6-benzylguanine*8-aza BG:* 8-aza-O^6-benzylguanine*8-bromo BG:* O^6-benzyl-8-bromoguanine*8-oxo BG:* O^6-benzyl-8-oxoguanine*8-tfm BG:* O^6-benzyl-8-trifluoromethylguanine *CCNU:* 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea *DTIC:* 5-(3,3-dimethyl-1-triazenyl)imidazole-4-carboxamide

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Introduction

A major mechanism of resistance to anticancer alkylating agents, specifically chloroethylnitrosoureas (BCNU, CCNU) and methylating agents (temozolomide, pro-

carbazine, DTIC) is the removal of O^6-alkylguanine-DNA adducts by the DNA repair protein, AGT (EC 2.1.1.63) [19]. The number of lesions repaired is limited by the number of molecules of the AGT protein available in the cell. There is strong evidence that the absence or depletion of the AGT protein results in a higher number of interstrand crosslinks and greater toxicity following exposure to chloroethylnitrosoureas [19]. Preclinical studies in tumor cells in culture and animal xenograft models have demonstrated that AGT activity is inversely correlated with sensitivity to alkylating agents [10, 19, 23, 24]. Furthermore, retrospective and prospective human studies have shown a correlation between AGT levels in human brain tumor samples and clinical outcome following treatment with BCNU [1, 12, 13]. In a recent study by Jaeckle et al. [13], in the Southwest Oncology Group, low AGT levels in tumor tissue specimens have been shown to be correlated with longer survival of patients with malignant astrocytoma that is independent of other previously described prognostic variables.

To overcome resistance resulting from AGT expression in tumors, BG, a direct-acting substrate of the repair protein has been developed and is presently in clinical testing [6, 7, 9, 11, 25]. It has been noted in preclinical and clinical studies that BG is converted to an equally potent AGT inactivator, 8-oxoBG, by human P450-mediated reactions (1A2 and 3A4) and cytosolic aldehyde oxidases [21, 22]. Thus, the observed AGT inactivation occurring in humans after administration of BG is at least partly attributed to its equipotent metabolite, 8-oxoBG [9]. Pharmacokinetic analysis has revealed that 8-oxoBG has a much longer half-life and is present in plasma at a much higher concentration (AUC 17-fold higher) compared to BG [9]. Furthermore, since BG is converted to 8-oxoBG by human cytochrome P450 1A2, an enzyme with significant interindividual variability, it was reasoned that 8-oxoBG or perhaps other 8-substituted BG analogs that are not metabolized by this isoform may be effective AGT inactivators worth developing further.

Several O^6-benzylated guanine derivatives have been synthesized and many have been shown to effectively inactivate the AGT protein in vitro and in cells [4, 5, 18–20]. BG analogs bearing electron-withdrawing groups at the 8-position (e.g. 8-azaBG, 8-bromoBG) are more potent than BG or 8-oxoBG [5]. On the other hand, 8-tfmBG is only slightly less active than BG. These compounds would be expected to be metabolized quite differently from BG and may offer some advantages depending on their tissue bioavailability and metabolic conversions. We speculated that structural differences among these compounds could produce differences in biodistribution, as well as metabolism, and thereby show differences in their ability to inactivate AGT. Consequently, we compared the in vivo AGT inhibitory activity of 8-substituted derivatives of BG with that of BG in tissues and transplanted tumor xenografts harvested from mice after i.p. administration of drug. We also present a comparison of tissue distribution and drug concentration of BG and 8-oxoBG in mouse tissue and in D456 brain tumor in the intracranial region of mice and in A549 lung tumor xenograft grown in the flank region.

Materials and methods

Drugs

BG, 8-oxoBG, 8-azaBG, 8-bromoBG and 8-tfmBG were synthesized as previously described [5, 7, 8]. PEG-400 was obtained from Spectrum Chemicals (Gardena, Calif.).

Animals

Female NIH athymic nude mice, aged 3–5 weeks (Taconic Farms, Germantown, N.Y.), were used for all studies. The mice were housed in an environmentally controlled room (12 h light/12 h dark cycle), and were provided with food and water ad libitum. Experimental procedures followed the guidelines of the University of Chicago Manual on Laboratory Animals prepared by the Animal Care Committee.

Tumor inoculation

For the A549 human lung carcinoma model, subcutaneous xenograft transplantation was performed by inoculation of 7×10^6 cells in a 200 μl volume into the right rear flank region of each animal. Tumors were allowed to grow to an average of 150 mm^3 at which time animals were treated with drug. For the intracranial brain tumor model, 5 μl of tumor homogenate from D456 human glioma cells were inoculated into the brain of nu/nu BALB/C mice.

Treatment regimens

Stock solutions containing molar equivalents of BG (2 mg/ml), 8-oxoBG (2.01 mg/ml), 8-azaBG (2.01 mg/ml), 8-bromoBG (2.65 mg/ml) and 8-tfmBG (2.56 mg/ml) were prepared in PEG-400/saline (40:60). Drugs were administered at a final dose of 41 μmol/kg body weight (i.e. BG 10 mg/kg or 30 mg/m^2) as an i.p. injection. Following administration of BG, or the 8-substituted analogs, triplicate animals were killed at various time points. For drug quantitation studies, liver, brain, and kidneys were harvested from animals at 0, 0.5, 1, 2, 4, 6, 12, and 24 h after i.p. drug administration, snap-frozen in liquid nitrogen and stored at −70 °C until analysis. For determination of AGT activity, two separate triplicate sets of animals were treated with drugs as mentioned above, and liver, brain, kidneys and tumor xenografts were harvested at 0, 6, 13, and 24 h after i.p. drug administration.

AGT determination

Samples of harvested organs/tumor xenografts were weighed, and extracts were prepared by adding 2 ml 50 m*M* Tris (pH 7.5), 0.1 m*M* EDTA, and 5 m*M* dithiothreitol buffer per gram of tissue sample. Samples were homogenized for 1 min, sonicated for 1 min, and centrifuged at 14,000 *g* for 30 min. The assay for AGT activity was performed as previously described by Domoradzki et al. [10]. Briefly, AGT activity was measured as the removal of O^6-[^{3}H]methylguanine from a [^{3}H]-methylated DNA substrate (5.8 Ci/mmol) following incubation with tissue extract at 37 °C for 30 min. The DNA was precipitated by adding ice-cold perchloric acid (0.25 N), and hydrolyzed by the addition of 0.1 N HCl at 70 °C for 30 min. Following filtration using a microfilter apparatus, the modified bases were separated by reverse-phase HPLC with 0.05 *M*

ammonium formate, pH 4.5, containing 10% methanol. Protein was determined by the method of Bradford [3] and the amount of O^6-methylguanine released from the DNA substrate per milligram of protein was calculated.

Quantitation of BG and 8-oxoBG

Tissue homogenates were prepared by adding two volumes of 10 m*M* potassium phosphate (pH 7.5) buffer per gram wet weight of tissue and homogenized for 30 s. Internal standard (75 µl O^6-(*p*-fluorobenzyl)guanine in methanol) was added to 150 µl of the homogenate and 5 ml ethyl acetate was added to the mixture. Samples were vortexed for 10 s, and centrifuged at 2500 rpm at room temperature for 20 min. The organic layer was transferred into a clean disposable culture tube, evaporated under a stream of nitrogen at 37 °C to dryness and reconstituted with 200 µl mobile phase (10 m*M* potassium phosphate, pH 7.5/methanol, 72:28). The reconstituted sample (100 µl) was injected onto a Waters Nova Pak Phenyl 60 Å 4-µm reverse-phase column (3.9 cm × 150 mm). The resolved peaks were monitored with an ultraviolet detector at a wavelength of 280 nm. BG and 8-oxoBG concentrations in tissue homogenate were determined in terms of the ratios to the internal standard and quantified by a standard curve generated from known concentrations of the compounds spiked in tissue homogenates of untreated mice. The observed coefficient of variation between days for the standard curves was less than 10%. The limits of detection for BG and 8-oxoBG were 30 ng/ml and 20 ng/ml, respectively.

Pharmacokinetic analysis

AGT activity in harvested tissue of treated animals was estimated and expressed as a percent of AGT activity in similarly harvested tissues of mice treated with vehicle alone (control). The pharmacokinetics of BG and 8-oxoBG were modeled using WinNonlin (PharSight Corporation, Apex, N.C.). The concentrations of BG and 8-oxoBG in the tissues were normalized to micromoles per gram of organ sample studied. A noncompartmental model, using the linear trapezoidal technique, was used to estimate area under tissue concentration-time curve [$AUC_{(0-4\ h)}$], and to determine the rate of disappearance of each drug from individual organs. C_{last} was the drug concentration in harvested tissue 4 h after drug administration. The 4-h concentration was chosen because in brain, kidney and liver tissues, drug concentrations were below their limit of detection after this time point. The mean of AGT activity in tissue was correlated with tissue pharmacokinetics of BG, and 8-oxo-BG when required. Student's *t*-test was used to determine the significance of differences between the estimated disposition parameters in the different tissues. *P*-values ≤ 0.05 were considered significant.

Results

Figure 1 illustrates BG and 8-substituted BG analogs evaluated in this study. BG is converted to 8-oxoBG in rodents [8, 21], nonhuman primates [2] and humans [9, 22]. BG and 8-oxoBG are equipotent inactivators of AGT activity [8]. NIH Swiss mice were treated with 10 mg/kg BG or 8-oxoBG, and liver, kidney and brain tissues were evaluated for AGT activity at 1, 6, 13 and 24 h (Fig. 2). Although both agents depleted AGT activity in all three tissues, the extent of depletion and rate of AGT repletion varied considerably. BG was much more effective than 8-oxoBG at inactivating AGT in liver up to 13 h. Although 8-oxoBG depleted AGT activity to a greater extent than BG at 1 h in kidney, the recovery of AGT activity after 8-oxoBG treatment was much more rapid with 50% activity at 6 h and full recovery of activity at 24 h. In contrast, AGT activity had recovered to 40% of the basal level in kidney at 13 h after injection of BG and remained at this level for 24 h. AGT activity in brain reached a nadir at 13 h after administration of 8-oxoBG, but it was lowest at 24 h following BG administration.

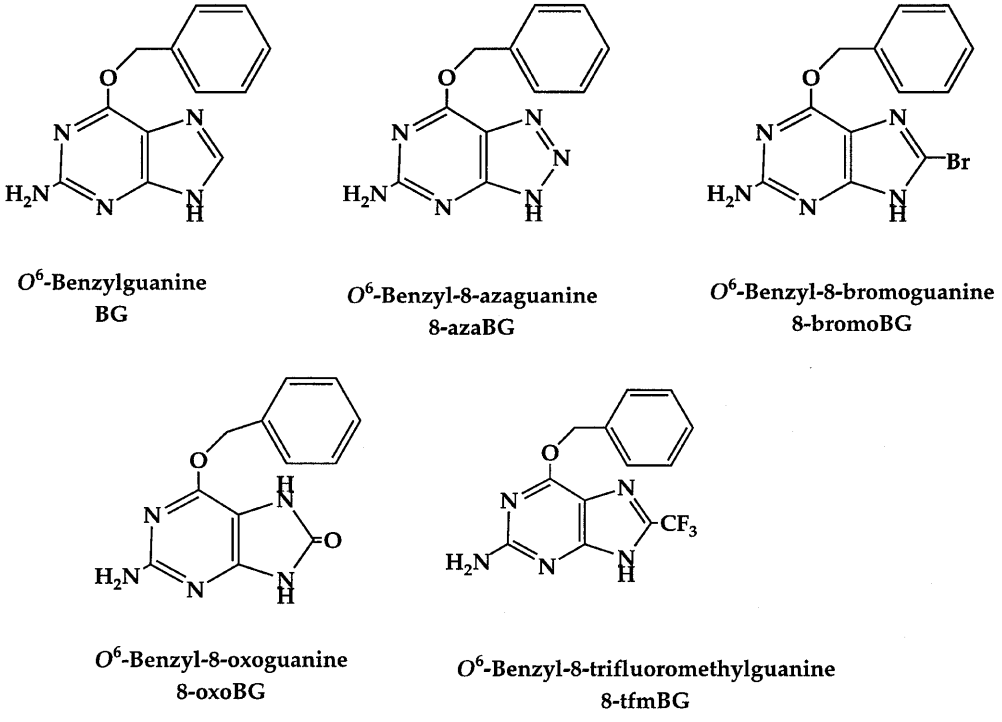

Fig. 1 Structures of BG and BG analogs

The tissue drug concentration versus time profiles of BG and 8-oxoBG are shown in Fig. 3. There was a rapid uptake of BG and 8-oxoBG in all tissues with high concentrations achieved within 30 min and levels below the limit of detection 6 h after injection. The AUC of 8-oxoBG after i.p. administration of BG was higher than that achievable after i.p. administration of 8-oxoBG alone, implying a greater bioavailability of AGT-inactivating drug following administration of BG (Table 1). The calculated areas under the tissue concentration-time curves after BG administration (BG plus metabolite, 8-oxoBG) were 3.2, 6.9 and 11.8 times higher than the values calculated after 8-oxoBG administration in brain, kidney and liver, respectively (Table 1).

To ascertain the extent of AGT depletion following treatment with BG and 8-oxoBG in human tumors, we measured AGT activity in D456 glioma tumor xenografts implanted in the intracranial region and A549 lung tumor xenografts implanted in the flank region (Fig. 4). Depletion of AGT activity following BG administration was significantly greater than that observed following 8-oxoBG administration in D456 gliomas and A549 lung tumor xenografts. As a result of BG administration, AGT activity in the D456 glioma was reduced to less than 50% of control activity at 1 h and maintained AGT activity below 80% up to 24 h. Similar to the results obtained in normal brain, the greatest depletion of AGT activity was observed 24 h after injection. The degree of depletion was much greater in A549 lung tumor than D456 glioma even though A549 basal activity (535 fmol/mg protein) is much higher than basal activity in D456 gliomas (25.5 fmol/mg protein).

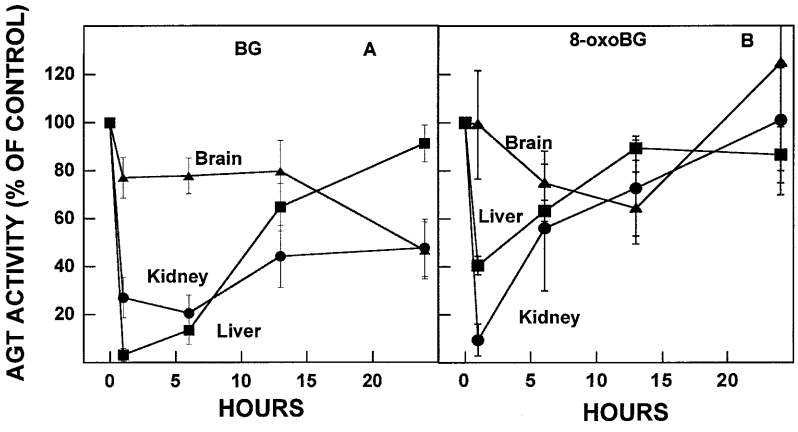

Fig. 2A, B AGT inactivation in mouse tissues by BG and 8-oxoBG. NIH Swiss mice received 10 mg/kg BG (**A**) or 8-oxoBG (**B**) prepared in PEG-400/saline (40:60). The mice were killed at various times after injection as indicated, and brain (▲), kidney (●) and liver (■) were removed for determination of AGT activity. The absolute values for AGT activity (fmol/mg protein) were: liver 41, kidney 21, and brain 16. The means ± SD are shown for determinations of AGT activity in at least six mice which were each assayed in duplicate

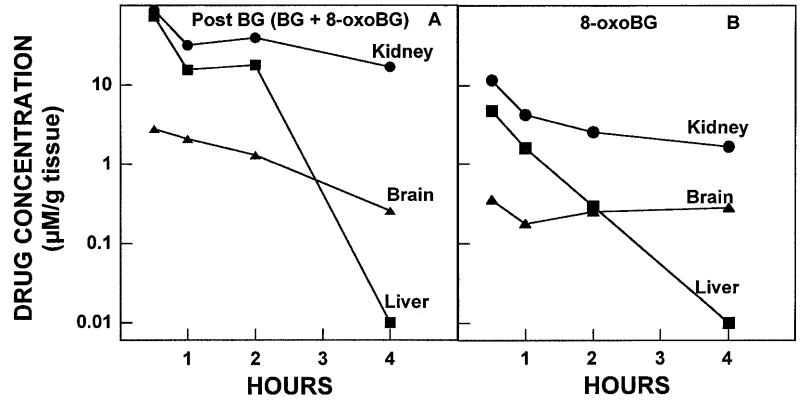

Fig. 3A, B Tissue drug concentration vs time profile after injection of BG or 8-oxoBG. Mice received 14 mg/kg BG (**A**) or 8-oxoBG (**B**). The mice were killed at various times following drug injection, and brain (▲), kidney (●) and liver (■) were removed for measurement of drug concentration. Each point for drug concentration post BG (**A**) represents the total micromolar concentration of BG plus 8-oxoBG present in harvested tissue

Table 1 Estimated pharmacokinetic parameters of BG and 8-oxoBG in tissues of mice (*ND* not done)

		Brain	Kidney	Liver
BG	$T_{1/2}$ (h)	0.9	1.7	0.8
	AUC ($\mu M \cdot$ h/g tissue)	2.2	43.1	28.3
8-oxoBG (after i.p. BG)	AUC ($\mu M \cdot$ h/g tissue)	0.9	57.9	19.2
Total AUC ($\mu M \cdot$ h/g tissue)		3.1	101.0	47.5
8-oxoBG (after i.p. 8-oxoBG)	$T_{1/2}$ (h)	ND	2.4	0.4
	AUC ($\mu M \cdot$ h/g tissue)	0.97	14.6	4.0
Ratio of AGT inhibitory activity as determined from total AUCs of BG relative to AUC of 8-oxo-BG		3.2	6.9	11.8

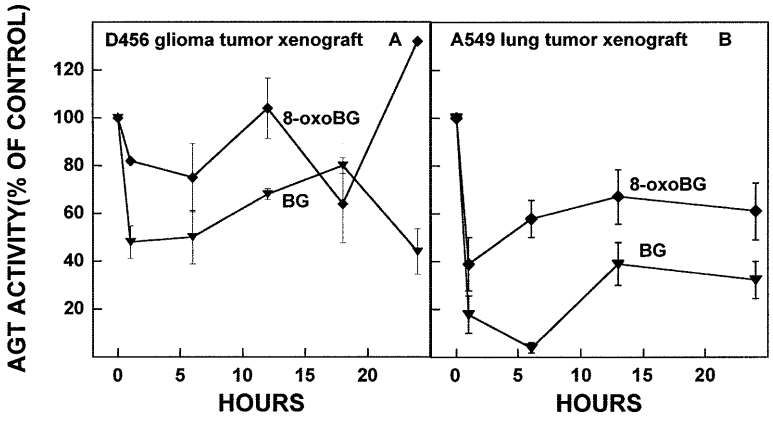

Fig. 4A, B AGT activity depletion in D456 intracranial glioma tumor, and A549 lung tumor carried in nude mice. Mice received 10 mg/kg BG (▼) or 8-oxoBG (◆) prepared in PEG-400/saline (40:60). D456 glioma xenografts (**A**) grown in the intracranial region or A549 lung tumor xenografts (**B**) in mice were removed at various times as shown following injection of drug and assayed for AGT activity. Each point represents the mean ± SD ($n=6$ for lung, $n=3$ for glioma)

The AGT inactivation profile of three 8-substituted BGs, 8-azaBG, 8-bromoBG and 8-tfmBG in mouse brain, liver, kidney and A549 lung tumor xenograft, were compared to that of BG (Fig. 5). In brain, all three analogs were superior to BG at inactivating AGT activity up to 13 h. In liver and A549 lung tumor xenografts, BG depleted AGT to a greater extent than 8-azaBG, 8-bromoBG and 8-tfmBG. Interestingly, AGT activity recovered fastest in the liver, possibly due to distribution differences between the tissues of the various drugs and their metabolism in the liver.

Discussion

Our findings indicate differences in AGT inactivation profiles following administration of BG, 8-oxoBG, 8-azaBG, 8-bromoBG and 8-tfmBG. BG was superior to all 8-substituted BG analogs at depleting AGT activity in human lung tumor xenografts. 8-AzaBG and 8-bromoBG displayed superior AGT-depleting activity in brain and kidney tissues, respectively, suggesting that these analogs might have better penetration into these organs, and that they might be more effective for AGT modulation in tumors located in these organs. BG was clearly superior to 8-oxoBG as evidenced by more pronounced AGT inactivation profiles in mouse tissues and human tumors concomitantly with a higher concentration of AGT-inactivating drug (BG plus 8-oxoBG) in tissues following BG administration.

There are differences between the dose, schedule and route of administration described in this study and those used in human clinical trials with BG. BG is administered at a dose of 120 mg/m^2 over a 1 h by i.v. infusion in humans compared to a bolus i.p. dose of 30 mg/m^2 administered to mice. It is, therefore, difficult to conclude that the pharmacokinetic-pharmacodynamic relationships observed in mice would be reflective of outcome(s) in a clinical setting. Furthermore, the extent of metabolism of BG to 8-oxoBG is much greater in

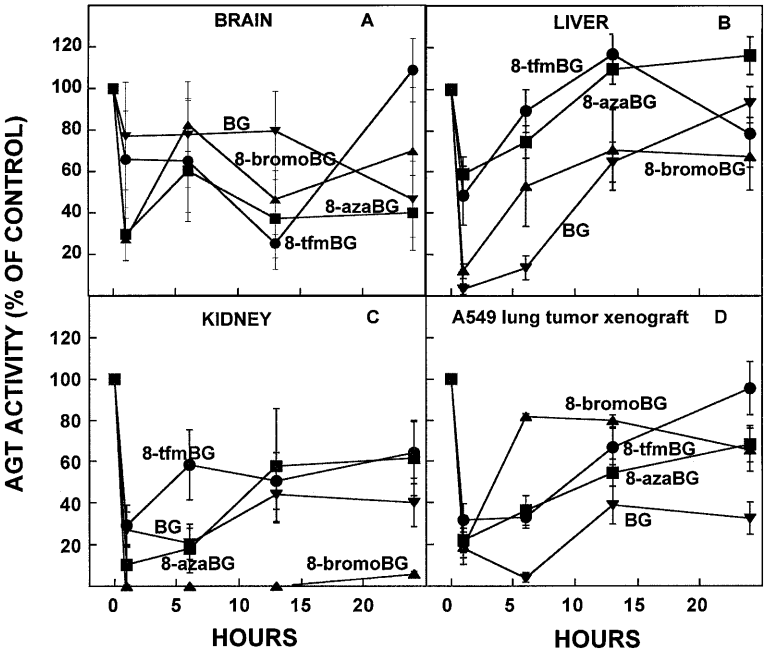

Fig. 5A–D AGT inactivation in mouse tissues and A549 xenograft tumors following administration of BG, 8-azaBG, 8-bromoBG and 8-tfmBG. NIH Swiss mice were injected with 10 mg/kg BG (▼), 8-azaBG (■), 8-bromoBG (▲), 8-tfmBG (●). The mice were killed at various times after injection as indicated and brain (**A**), liver (**B**), kidney (**C**) and A549 lung tumor (**D**) were removed for determination of AGT activity. Each point represents the mean ± SD ($n=6$–9, except 8-bromoBG $n=3$)

humans than in rodents [9]. Caution should be exercised in extrapolating the current data to predict efficacy in humans. Notwithstanding, the finding that BG is more effective as an AGT inactivator in vivo as a result of metabolism to 8-oxoBG resulting in higher levels of BG plus 8-oxoBG compared to 8-oxoBG alone in tissue is most likely unaffected by dose or route of administration.

Our data demonstrate superiority of BG in depletion of AGT in human tumor xenografts and lack of superiority over other analogs in mouse kidney and brain. Although this may be explained by distribution differences between the tissues of the various drugs and their metabolites, it is also possible that there are differences in the sensitivity of mouse and human AGT to these analogs. Mouse AGT is much more resistant to inactivation by BG than human AGT [17]. The lower sensitivity is due to a mutation of amino acid residue Leu180 in the mouse protein associated with BG resistance. Whether this mutation confers resistance to inactivation by 8-substituted BG analogs to the same degree as that of BG is not known. Sensitivity to inactivation may contribute at least in part to differences in depletion/repletion kinetics following treatment with structurally different compounds.

Our data show that AGT activity in brain tissue and tumor was low at 14 to 16 h after injection of BG and 8-oxoBG. Coincident was a slow decline in the brain tissue drug concentration following administration of BG or 8-oxoBG. There was a slight increase in 8-oxoBG concentration in brain tissue from 1 to 4 h. Kokkinakis et al. [14–16] found that O^6-benzyl-2′-deoxyguanosine demonstrates a greater tumor growth inhibition and more potent inhibition of AGT in vivo despite its demonstrated lower activity in vitro compared to BG. In vivo O^6-benzyl-2′-deoxyguanosine is converted to BG and 8-oxoBG. This analog produces prolonged suppression of AGT activity in brain after complete disappearance of the parent drug from tissue. The authors noted BG and 8-oxoBG exhibit longer half-lives in brain tissues than parent drug and that 8-oxoBG appears to accumulate in brain. Our data showing an increase in 8-oxoBG concentration in the brain following treatment with 8-oxoBG is consistent with the findings of Kokkinakis et al. [14]. It is possible that recirculation or metabolism accounts for a greater depletion at 6 h than at 13 h for the 8-substituted analogs.

The rate of repletion of AGT activity is much slower in tissues and tumors of mice after treatment with BG than after treatment with 8-oxoBG. The observed differences in tissue AGT repletion might be due to the fact that after BG administration, more active drug (AUC of BG plus 8-oxo-BG) is available for AGT inactivation, compared to after 8-oxo-BG administration (AUC of oxo-BG alone). Previous studies in rats have shown that after a single i.v. administration of BG or 8-oxoBG, up to 8% of BG and 62% of 8-oxoBG are excreted unchanged in the urine [21]. Renal clearance of BG has been reported to be 16 times slower for BG than for 8-oxoBG in rats [21]. A much slower rate of renal clearance in rodents may contribute to the greater bioavailability of BG than of 8-oxoBG.

The location of a tumor might influence the dose of BG or its analogs, as well as the choice of analog(s) that will be required for optimal AGT inactivation. Although there are limitations to using animal studies to predict the fate of a particular drug in humans, our data suggest that BG is more effective than its 8-substituted analogs at depleting AGT activity in liver and tumors. This may be due to conversion of BG to 8-oxoBG, and both agents being equally potent, resulting in additive AGT inactivation in tissues.

In summary, AGT inactivation after i.p. administration of BG, 8-oxoBG, and other 8-substituted analogs is organ-specific. This is probably related to differences in tissue penetration based on the structure of the 8-substituted analogs. BG is superior to 8-oxoBG at modulating AGT activity in a mouse model. Future studies evaluating the prodrugs that may generate BG or 8-oxoBG are warranted.

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