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O^6-Alkylguanine-DNA Alkyltransferase Inactivation by Ester Prodrugs of O^6-Benzylguanine Derivatives and their Rate of Hydrolysis by Cellular Esterases

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ABSTRACT. To modulate the bioavailability and perhaps improve the tumor cell selectivity of O^6-alkylguanine-DNA alkyltransferase (AGT) inactivators, pivaloyloxymethyl ester derivatives of O^6-benzylguanine (BG) were synthesized and tested as AGT inactivators and as substrates for cellular esterases. The potential prodrugs examined were the 7- and 9-pivaloyloxymethyl derivatives of O^6-benzylguanine (7- and 9-esterBG), and of 8-aza-O^6-benzylguanine (8-aza-7-esterBG and 8-aza-9-esterBG) and the 9-pivaloyloxymethyl derivative of 8-bromo-O^6-benzylguanine (8-bromo-9-esterBG). The benzylated purines were all potent inactivators of the pure AGT and of the AGT activity in HT29 cells and cell extracts. Each ester was at least 75 times less potent than the corresponding benzylated purine against the pure human AGT. In contrast, the activities of esters and their respective benzylated purine were similar in crude cell extracts and in intact cells. The increase in potency of esters in cellular extracts could be explained by a conversion of the respective prodrug to the more potent benzylated purine in the presence of cellular esterases. The apparent catalytic activity (V_{max}/K_m) of liver microsomal esterase for 8-azaBG ester prodrugs was 70–130 times greater than for BG prodrugs and 10–20 times greater than for 8-bromo-9-esterBG. Tumor cell hydrolysis of the esters varied considerably as a function of cell type and prodrug structure. These data suggest that these or related prodrugs may be advantageous for selective AGT inactivation in certain tumor types. BIOCHEM PHARMACOL **55**;10:1701–1709, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. O^6-benzylguanine; alkyltransferase; ester prodrug; esterase; tumor

BG‖ is one of the most effective inactivators of the DNA repair protein AGT [1–3]. Inactivation of this protein leads to an enhancement in the cytotoxic effect of chloroethylnitrosoureas (e.g. BCNU) and methylating agents (e.g. DTIC) in cell culture [2–4]. More importantly, BG pretreatment results in a significant growth inhibition of human brain and colon tumor xenografts, subsequently treated with BCNU [5–9]. BG in combination with BCNU is currently in human clinical trials. Efforts to develop more potent and perhaps tumor-specific inhibitors of the alkyltransferase have been underway in several laboratories [10–17]. These studies have provided a number of inhibitors as potent or less potent than BG. Recently, however, BGs bearing electron withdrawing groups at the 8-position, such as 8-aza-BG and 8-bromo-BG, were determined to be more potent than BG *in vitro* [14].

Enhanced bone marrow toxicity of BCNU has been noted in both animal studies [18–20] and human clinical trials (Dolan ME, Ratain MJ and Schilsky RL, unpublished observations) of the combination of BG and BCNU. This toxicity could be an important limitation to therapy with the drug combination. The properties of BG may not be optimal for its selective delivery to tumor targets while avoiding tissues of known sensitivity to alkylating agent damage such as bone marrow. It is well known that the prodrug approach is one useful method for improving selectivity for tumor targets [21]. A prodrug is a pharmacologically inactive derivative of a parent drug molecule that requires spontaneous or enzymatic transformation *in vivo* in order to release the active drug [22]. Prodrugs that are substrates for esterases include numerous chemicals with

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‖ *Abbreviations:* BG, O^6-benzylguanine; 8-aza-BG, 8-aza-O^6-benzylguanine; 8-bromo-BG, O^6-benzyl-8-bromoguanine; AGT, O^6-alkylguanine-DNA alkyltransferase; DMF, dimethylformamide; DTIC, 5-(3,3-dimethyl-1-triazenyl)imidazole-4-carboxamide; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; 8-aza-7-esterBG, 8-aza-O^6-benzyl-7-(pivaloyloxymethyl)guanine; 8-aza-9-esterBG, 8-aza-O^6-benzyl-9-(pivaloyloxymethyl)guanine; 8-bromo-9-esterBG, O^6-benzyl-8-bromo-9-(pivaloyloxymethyl)guanine; 9-esterBG, O^6-benzyl-9-(pivaloyloxymethyl)guanine; and 7-esterBG, O^6-benzyl-7-(pivaloyloxymethyl)guanine.

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diverse structures [22–24]. Carboxylesterases play an important role in the activation of prodrugs [25, **]. This family of hydrolytic enzymes is widely distributed in mammalian tissues, with the liver, the gastrointestinal tract, and the blood having the highest proportion of the total ester hydrolytic capacity in any mammal [21, 26].

In efforts to improve the tumor cell selectivity of AGT inactivation, ester prodrugs of BG, 8-bromo-BG, and 8-aza-BG were synthesized and tested as alkyltransferase inactivators and as substrates for tumor esterases. The rate of hydrolysis of these compounds in various cells and tissue extracts was evaluated. In addition, a comparison of the AGT-inactivating potency of these derivatives with their rate of hydrolysis is presented.

MATERIALS AND METHODS

9-EsterBG was prepared by the method of Chae *et al.* [14].

Synthesis of 8-Aza-9-esterBG and 8-Aza-7-esterBG

8-Aza-BG [14] (0.484 g, 2.0 mmol) was mixed with 4 mL 0.5 M of sodium ethoxide in ethanol and stirred for 30 min. The ethanol was evaporated under reduced pressure. The residue was dissolved in anhydrous DMF (6 mL), and chloromethylpivalate (0.3 mL, 2.1 mmol) was added. The clear solution was stirred for 8 hr at room temperature. DMF was evaporated under reduced pressure to give a brown solid. The solid was dissolved in chloroform and loaded onto a silica gel column (Davisil grade 633, 200–435 mesh, 60 Å). The 9-isomer was eluted from the column with $CHCl_3$:hexane (4:1), while the 7-isomer was subsequently eluted with $CHCl_3$. 8-Aza-9-esterBG: yield, 405 mg (57%); m.p. 119–120°; UV: (pH 1) λ_{max} 246 nm ($\varepsilon = 0.494 \times 10^4$), 286 ($0.878 \times 10^4$); (pH 6.9) 247 ($0.472 \times 10^4$), 288 ($0.819 \times 10^4$); (pH 13) (decomposes to 8-aza-BG); ^{1}H NMR: d 1.10 (s, 9 H, $C(CH_3)_3$), 5.50 (s, 2 H, $ArCH_2$), 6.31 (s, 2 H, CH_2), 7.38 (s, 2 H, NH_2, exchange with D_2O), 7.40–7.54 (m, 5 H, ArH); MS (El): calcd *m/z* for $C_{17}H_{20}N_6O_3$ 356.1596, found 356.1578; Anal. ($C_{17}H_{20}N_6O_3 \cdot 1/5H_2O$) C, H, N. 8-Aza-7-esterBG: yield, 103 mg (15%); m.p. 153–154°; UV: (pH 1) λ_{max} 244 nm ($\varepsilon = 0.820 \times 10^4$), 294 ($1.249 \times 10^4$); (pH 6.9) 250 (sh) ($0.296 \times 10^4$), 313 ($0.503 \times 10^4$); (pH 13) (decomposes to 8-aza-BG); ^{1}H NMR: d 1.12 (s, 9 H, $C(CH_3)_3$), 5.56 (s, 2 H, $ArCH_2$), 6.40 (s, 2 H, CH_2), 7.04 (s, 2 H, NH_2, exchange with D_2O), 7.4–7.58 (m, 5 H, ArH); MS (El): calcd *m/z* for $C_{17}H_{20}N_6O_3$ 356.1596, found 356.1602; Anal. ($C_{17}H_{20}N_6O_3$).

Synthesis of 8-Bromo-9-esterBG

8-Bromo-BG [14] (0.48 g, 1.5 mmol) was mixed with 1.5 mL of a 1.0-M solution of sodium ethoxide in ethanol and was stirred for 20 min. The ethanol was removed under reduced pressure, and the solid residue was dissolved in DMF (5 mL). Chloromethylpivalate (0.24 mL, 1.65 mmol) was then added, and the solution was stirred overnight. The DMF was removed under reduced pressure. The residue was dissolved in chloroform and was loaded onto a silica gel column (Davisil grade 633, 200–425 mesh, 60 Å) eluted with chloroform. The 9-isomer eluted with chloroform was recovered in pure form under these conditions. 8-Bromo-9-esterBG yield: 150 mg (23%); m.p. 217–218°; UV: (pH 1) λ_{max} 250 nm ($\varepsilon = 0.944 \times 10^4$), 291 ($1.166 \times 10^4$); (pH 6.9) 266 ($0.916 \times 10^4$), 295 ($0.916 \times 10^4$); (pH 13) decomposes to 8-bromo-BG; ^{1}H NMR: d 1.13 (s, 9 H, $C(CH_3)_3$), 5.48 (s, 2 H, $ArCH_2$), 5.93 (S, 2 H, CH_2), 6.80 (s, 2 H, NH_2, exchange with D_2O), 7.35–7.52 (m, 5 H, ArH), MS (El): calcd *m/z* for $C_{18}H_{20}N_5O_3$ ^{79}Br 435.0750, found 433.0725; calcd *m/z* for $C_{18}H_{20}N_5O_3$ ^{81}Br 433.0729, found 435.0672; Anal. ($C_{18}H_{20}N_5O_3$ Br) C, H, N, Br.

Synthesis of 7-esterBG

BG (2.41 g, 10 mmol) was mixed with 10 mL of a 1.0-M solution of sodium ethoxide in ethanol and was stirred for 30 min. The ethanol was evaporated under reduced pressure. The residue was dissolved in anhydrous DMF (30 mL), and chloromethylpivalate (1.5 mL, 10.4 mmol) was added. The clear solution was stirred overnight at room temperature. DMF was evaporated under reduced pressure to give a pale peach-colored solid. The solid was dissolved in chloroform:ethanol (9:1) and was loaded onto a silica gel column (Davisil grade 633, 200–245 mesh, 60 Å). The column was eluted with chloroform:ethanol (9:1) to elute the 9-isomer [13] followed by the 7-isomer. The 7-isomer was further purified by silica gel column chromatography (Davisil grade 633, 200–245 mesh, 60 Å) using chloroform:ethanol (98:2) as eluent: yield 36 mg (1%); m.p. 166–168° dec; UV: (pH 1) λ_{max} 240 nm (sh) ($\varepsilon = 0.656 \times 10^4$), 290 ($1.164 \times 10^4$); (pH 6.9) 240 (sh) ($0.635 \times 10^4$), 293 ($0.528 \times 10^4$); (pH 13) decomposes to BG; ^{1}H NMR: d 0.98 (s, 9 H, $C(CH_3)_3$), 5.51 (s, 2 H, $ArCH_2$), 6.07 (S, 2 H, CH_2), 6.32 (s, 2 H, NH_2, exchange with D_2O), 7.36–7.58 (m, 5 H, ArH), 8.25 (s, 1 H, H-8); MS (El): calcd *m/z* for $C_{18}H_{21}N_5O_3$ 355.1644, found 355.1626.

Cell Culture

The human colon carcinoma cell lines HT29 and BE were gifts from Dr. L. C. Erickson, Loyola Medical Center, and were grown in Dulbecco's Modified Eagle's medium supplemented with 10% fetal bovine serum and 2 mM of L-glutamine. The human lung carcinoma cell line A549 was obtained from the American Type Culture Collection, and was grown in Ham's F-12K medium supplemented with 10% fetal bovine serum and 2 mM of L-glutamine. MCF7 and MCF7 ADR cells were obtained from the laboratory of Dr. K. Cowan, NCI. Cells were grown in Richter's Improved Modified Eagle's medium supplemented with 10% fetal bovine serum and 2.2 g/L of sodium bicarbonate. U87

** Hosokawa M and Satoh T, Molecular aspects of the inter-species variation in carboxylesterase. *7th North American ISSX Meeting*, Vol. 10, p. 7, 1996.

MG cells were grown in Dulbecco's Modified Eagle's Medium with 10% fetal bovine serum, nonessential amino acids (100 μM), sodium pyruvate (1 mM), and HEPES buffer (10 mM). The androgen-independent prostate cancer cell line Du145 was maintained in RPMI 1640 and 10% fetal bovine serum. The metastatic rat prostate cell line AT6.1 was a gift from Dr. C. W. Rinker-Schaeffer, Department of Surgery, University of Chicago, and was grown in RPMI medium supplemented with 8% fetal bovine serum. All cells were grown in 100 units/mL of penicillin and 100 μg/mL of streptomycin, except for HT29, which utilized 50 μg/mL of gentamycin. All cell cultures were maintained at 37° in 5% CO_2/95% humidified air. D341 and D456 human tumor xenografts were provided by Dr. Henry Friedman, Department of Pediatrics, Duke University Medical Center.

Preparation of Mononuclear Cells from Human Bone Marrow

Approximately 20–30 mL of bone marrow was drawn into heparinized tubes from the posterior iliac crest of normal volunteers. The bone marrow was mixed 1:1 with RPMI 1640 cell culture medium, layered 2:1 onto 15 mL of Histopaque 1077, and centrifuged at 400 *g* for 30 min at 25°. The mononuclear cell layer was carefully removed, washed twice with phosphate-buffered saline, resuspended in 0.5 mL of 50 mM of Tris, pH 7.5, 0.1 mM of EDTA, and 5 mM of dithiothreitol buffer, and frozen at −70° until analyzed.

Assay of Alkyltransferase Activity

Prior to assaying for AGT activity, increasing concentrations of drug were incubated in 50 mM of Tris, pH 7.5, 0.1 mM of EDTA, and 5 mM of dithiothreitol buffer with either crude cell extracts (HT29, MCF7, MCF7 ADR, A549, bone marrow) for 30 min or HT29 cells for 4 hr at 37° or pure alkyltransferase protein (0.06 μg) and calf-thymus DNA (50 μg) or hemocyanin (50 μg) for 30 min at 37°. DNA or hemocyanin was added to assays containing the pure AGT to stabilize the protein. Under these conditions, the pure AGT protein was completely stable with less than 5% loss of activity in the 30-min incubation in the absence of AGT inhibitor. The assay for alkyltransferase activity was performed as previously described [27, 28]. Briefly, alkyltransferase activity was measured as removal of O^6-[^{3}H]methylguanine from a ^{3}H-methylated DNA substrate (5.9 Ci/mmol) following incubation with extract or pure protein at 37° for 30 min. The DNA was precipitated by adding ice-cold perchloric acid (0.25 M), and hydrolyzed by the addition of 0.1 M of HCl at 70° for 30 min. The modified bases were separated by reversed-phase HPLC with 0.05 M of ammonium formate, pH 4.5, containing 7.5% methanol. Protein was determined by the method of Bradford [29], and the results are expressed as femtomoles of O^6-methylguanine released from the DNA substrate per milligram of protein.

Rate of Ester Hydrolysis

Tumor cell pellets suspended in 50 mM of Tris, pH 7.5, 0.1 mM of EDTA, and 5 mM of dithiothreitol buffer were sonicated with a cell disruptor prior to centrifugation at 14,000 *g* for 30 min at 4°. Protein determination was performed on the supernatant. Increasing amounts of tumor cell protein were incubated with 200 μM of ester prodrug at 37° for 1 hr. Following incubation, the tubes were immediately placed on ice and extracted with 2 vol. of ethyl acetate. An internal standard of O^6-(*p*-chlorobenzyl)guanine [12] was added, and the mixtures were centrifuged for 15 min at 250 *g*. The ethyl acetate layer (500 μL) was dried under N_2, and the residue was brought up in mobile phase. Separation of products was by HPLC on a 25 cm × 4.6 mm Beckman Ultrasphere reversed-phase column eluted with 50% methanol in 0.05 M of ammonium formate, pH 4.5, for 5 min followed by a linear gradient of increasing methanol up to 85% over 10 min at 35° at a flow rate of 1 mL/min. Products were detected by UV absorption at 286 and 280 nm, using a diode array detector.

Determination of Kinetic Parameters

Pig liver carboxylesterase was obtained from the Sigma Chemical Co. Human liver was obtained from the Cooperative Human Tissue Network. Human microsomes and cytosol were prepared as described [30]. Human liver microsomes (10 μg for 8-aza esters, 50 μg for BG esters, 500 μg for 8-bromo-9-esterBG) were incubated with 8-aza-9- or -7-esterBG (0.5 to 150 μM), 9-esterBG (0.5 to 50 μM), 7-esterBG (50 to 200 μM) or 8-bromo-9-esterBG (0.5 to 20 μM), for 15 min at 37°. To determine the kinetic parameters for porcine liver carboxylesterase, the enzyme (0.002 U for 8-aza esters, 0.1 U for BG esters, 2 U for 8-bromo-9-esterBG) was incubated with a 0.5- to 20-μM concentration of each ester. All reaction mixtures were incubated for 15 min at 37° in a buffer consisting of 100 mM of potassium phosphate, pH 7.4. Protein content was determined by the method of Bradford [29].

RESULTS

AGT Inactivation Potency of Prodrugs and Purine Derivatives

Structures of the pivaloyloxymethyl derivatives of the AGT inactivators used in these studies are illustrated in Fig. 1. The AGT inactivation profile of these compounds was compared with the activity of their respective parent drugs against pure alkyltransferase protein to determine if the ester prodrugs were pharmacologically inactive derivatives of their respective parent drug (Fig. 2 and Table 1).

Figure 2 shows the inactivation of the pure AGT protein in the presence of DNA, and Table 1 shows the EC_{50} values for these inhibition curves. Both the 9- and 7-esterBG were inactive (EC_{50} > 100 μM) against pure AGT protein, whereas the parent drug, BG, was highly potent (EC_{50} =

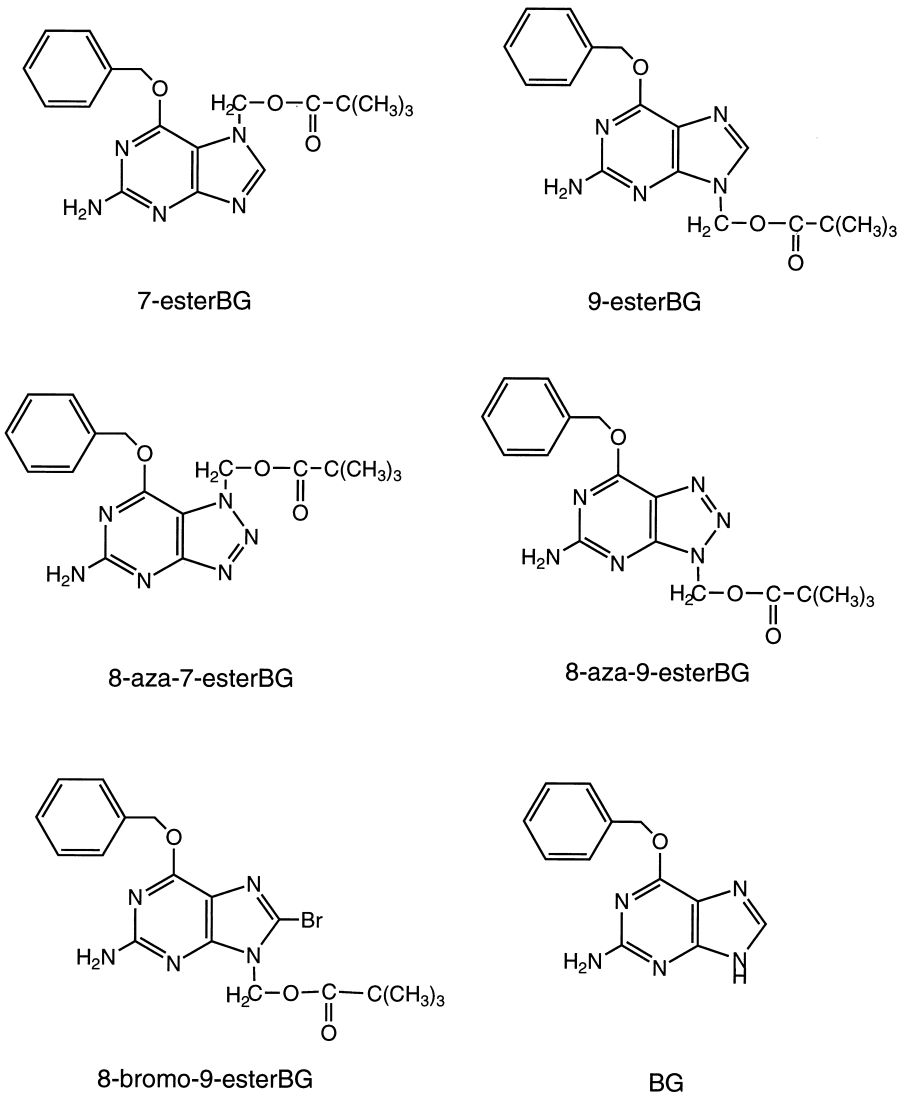

FIG. 1. Structures of ester prodrugs and BG.

0.15 μM). Furthermore, the 8-bromo-9-esterBG was 440 times less effective than 8-bromo-BG, and 8-aza-7- and -9-esterBG were, respectively, 75- and 375-fold less potent than 8-aza-BG against the pure AGT protein. The presence of DNA (which was added to stabilize the pure AGT protein) may contribute to these differences since previous studies have shown that the binding of DNA to AGT increases its rate of reaction with BG but strongly inhibits the reaction with a variety of derivatives having substitutions on the 9-position [31]. Evidence supporting this interpretation was obtained for the esters of 8-aza-BG and 8-bromo-BG since they were much more strongly inhibitory towards the pure AGT when hemocyanin rather than DNA was used to stabilize the protein (Table 1). However, as shown in Table 1, the lack of effectiveness of the 9- and 7-esters of BG itself was maintained even when hemocyanin was used in place of DNA.

To determine if the esters were transformed in cellular systems to release a more active drug, the prodrugs were evaluated in crude cell extracts from HT29 cells and in HT29 cell cultures (Table 1). The purine derivatives, 8-aza-BG, 8-bromo-BG, and BG were similarly effective in reducing AGT activity in intact HT29 cells, extracts prepared from these cells, or pure AGT. However, the potency of the esterified derivatives was found to be significantly greater against AGT in cells and extracts than against the purified AGT protein. This difference was most marked for the esters of BG. Although there was a large difference in the EC_{50} values for inactivation of AGT for the esters of 8-aza-BG and 8-bromo-BG when crude HT29 cell extracts were compared with pure AGT incubated in the presence of DNA, the difference was much less when the pure AGT was incubated without DNA in the presence of hemocyanin (Table 1). These results suggest that the esters of BG were enzymatically transformed in cells and crude cellular extracts to significantly more active compounds.

Conversion of Ester Prodrugs to Purine Derivatives

This conclusion was supported by the finding that both human liver microsomes and purified porcine carboxyles-

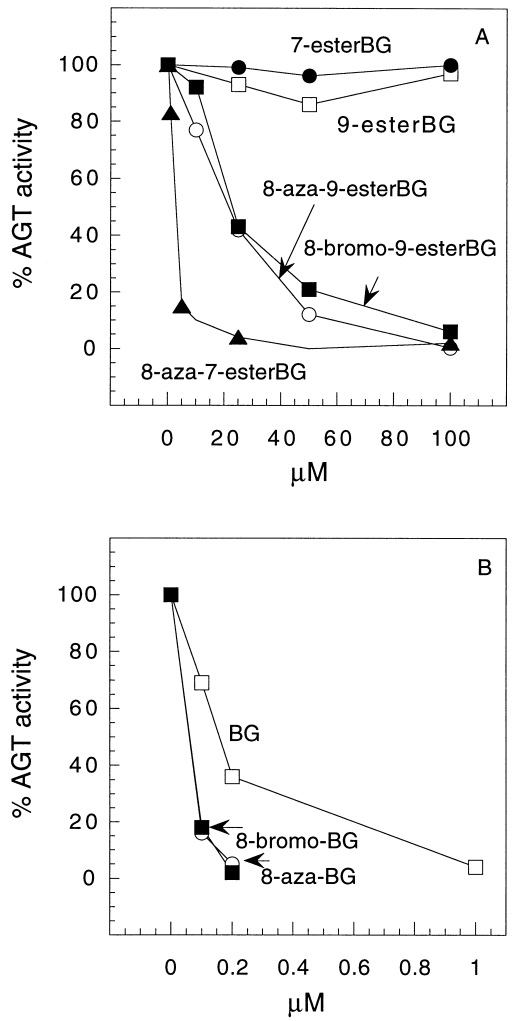

FIG. 2. Inactivation of purified alkyltransferase in the presence of DNA by pivaloyloxymethyl esters of BG derivatives. Percent AGT activity refers to alkyltransferase activity without the addition of drug (equivalent to transfer of 16,000 dpm from a ^{3}H-methylated DNA substrate). (A) Alkyltransferase activity remaining 30 min following incubation of purified protein with increasing concentrations of 9-esterBG (□), 7-esterBG (●), 8-aza-9-esterBG (○), 8-bromo-9-esterBG (■), and 8-aza-7-esterBG (▲). (B) Alkyltransferase activity remaining 30 min following incubation of purified protein with increasing concentrations of BG (□), 8-aza-BG (○), and 8-bromo-BG (■). The values shown are the means of at least three estimations, which agreed within ±10%. The whole experiment was repeated at least twice with similar results.

terase were able to act *in vitro* on the esters of BG, 8-aza-BG, and 8-bromo-BG to form the parent drug (Table 2). Table 2 lists the K_m, V_{max}, and V_{max}/K_m (relative catalytic activity) for ester hydrolysis. The relative catalytic activity of human liver microsomes towards esters of 8-aza-BG was at least 6-fold higher than 9-esterBG, 70-fold higher than 8-bromo-9-esterBG, and 130-fold higher than 7-esterBG in human liver microsomes. We observed more than a 40- to 70-fold greater catalytic activity of porcine liver carboxylesterase towards the 8-aza-7- and -9-esterBG when compared with esters of BG, and 8-bromo-9-esterBG was a very poor substrate for this enzyme (Table 2).

TABLE 1. Effects of ester prodrugs on AGT activity

	EC_{50}* (μM)			
	HT29		Pure AGT	
Compound	Extract	Cells	+ DNA	+ Hemocyanin
8-aza-BG	0.07	0.06	0.04	0.1
8-aza-7-esterBG	0.11	0.16	3	0.1
8-aza-9-esterBG	0.28	0.23	15	0.4
8-bromo-BG	0.08	0.05	0.05	0.15
8-bromo-9-esterBG	0.5	0.08	22	2.0
BG	0.2	0.05	0.15	0.5
7-esterBG	9	0.3	>100	>100
9-esterBG	3.1	0.3	>100	50

*Concentration required to produce 50% AGT inactivation following incubation of drug in cell-free extracts for 30 min, in cells for 4 hr, or with pure AGT protein for 30 min. The data for AGT inactivation in HT29 extracts and cells for BG were from Ref. 12 and that for 8-bromo-BG and 8-aza-BG were from Ref. 14.

Since the catalytic activity of the human microsome esterases towards 8-bromo-9-esterBG and 7-esterBG was much lower than the activity against the other esters, only the 9-esterBG and the 8-aza-7-esterBG and 8-aza-9-esterBG were tested further using extracts from tissues, tumor cells, and tumor xenografts (Tables 3 and 4 and Figs. 3–5).

Hydrolysis of Prodrugs in Cell and Tissue Extract

Figure 3 illustrates the percent conversion by extracts from HT29 cells of three esterified derivatives to the corresponding purine: 9-esterBG to BG and 8-aza-9-esterBG and 8-aza-7-esterBG to 8-aza-BG. The extent of conversion of prodrug (200 μM) to more active parent drug increased linearly with increasing concentrations of protein following a 1-hr incubation. The rate of 9-esterBG hydrolysis to BG was 26 times slower than the rate of conversion of the esters of 8-aza-BG, indicating that prodrug structure influences the rate of hydrolysis. Therefore, the increased potency of the esterified derivatives in cells and extracts as AGT

TABLE 2. Kinetic parameters for the hydrolysis of esterified prodrugs of benzylated purines in human liver microsomes and purified porcine carboxylesterase

Substrate	Amount of protein	K_m (μM)	V_{max} (nmol/min/mg)	V_{max}/K_m
Human microsomes				
8-aza-7-esterBG	10 μg	50	332	6.6
8-aza-9-esterBG	10 μg	100	638	6.4
8-bromo-9-esterBG	500 μg	2.1	0.19	0.09
7-esterBG	50 μg	465	24	0.05
9-esterBG	50 μg	32	32	1.0
Porcine carboxylesterase				
8-aza-7-esterBG	0.002 U	7.7	96	12
8-aza-9-esterBG	0.002 U	8.6	135	16
8-bromo-9-esterBG	2 U	4.7	0.05	0.01
7-esterBG	0.1 U	14	3.8	0.27
9-esterBG	0.1 U	25	6.0	0.24

TABLE 3. Hydrolysis of ester prodrugs by human tissue extracts

Extract	Rate of conversion to 8-aza-BG or BG* (%/min/mg protein)		
	8-aza-7-esterBG	8-aza-9-esterBG	9-esterBG
Buffer	0	0.2	0.01
Human bone marrow	0.44	0.056	ND†
Human lung microsomes	5.7	13	0.07
Human liver microsomes	101	111	0.82
Human liver cytosol	15	12	0.7
Human plasma	0.05	0.05	0.0007
Rat plasma	0.9	1.2	0.06

*Prodrug (200 μM) was incubated with cellular extract for 1 hr at 37°. The amount of prodrug and benzylated purine was quantified using HPLC.
†Not determined.

inactivators seen in Table 1 is likely to have resulted from hydrolysis of the ester derivative to the respective benzylated purine.

The prodrugs were then evaluated against extracts prepared from a variety of cell lines including A549 human lung, MCF7 human breast, HT29 and BE human colon, U87 human brain, DU145 human prostate, and AT6.1 rat prostatic cell lines (Fig. 4) and human brain tumor xenografts (D341 and D456) (Fig. 5). The percent conversion varied considerably as a function of cell or tumor type and was most pronounced in the D341 tumor extract and least pronounced in BE colon cellular extract. The percent conversion of 8-aza-7-esterBG to 8-aza-BG was 3.1, 0.32, and 0.14%/min/mg protein in extracts from A549 human lung tumor cells, MCF7 human breast tumor cells, and BE human colon tumor cells, respectively. The rate of conversion for 9-esterBG to BG was considerably slower (reduced by 6- to 180-fold) than the rate of conversion of either of the esters of 8-aza-BG upon incubation with tumor cell and xenograft extracts. These data indicate that the rate of conversion is also a function of cell type.

The rates of conversion of 8-aza-7- and -9-esterBG and 9-esterBG were evaluated in normal human tissues including liver, lung, plasma, and bone marrow and in rat plasma (Table 3). Under the conditions tested, human liver microsomal fraction hydrolyzed the esters most efficiently, whereas plasma had the lowest hydrolytic activity. Also, in each preparation examined, esters of 8-aza-BG were better substrates compared with 9-esterBG. Interestingly, the 8-aza-9-esterBG was converted to 8-aza-BG at a 25-fold lower rate in human bone marrow than in A549 lung tumor cell extract.

TABLE 4. Comparison of the AGT-inactivation potency of 8-aza-9-esterBG and percent of 8-aza-BG formed in cell extract

Tumor	% of 8-aza-BG	EC_{50} (μM)
A549	92	0.06
HT29	22	0.28
MCF7	20	0.33
MCF7 ADR	24	0.20
Buffer alone	0.11	

Crude extract (1 mg total protein) was incubated for 30 min with 200 μM of 8-aza-9-esterBG in 50 mM of Tris, pH 7.5, 0.1 mM of EDTA, and 5 mM of dithiothreitol buffer, pH 7.5. The percent of 8-aza-BG formed was determined by HPLC analysis. The concentration of 8-aza-9-esterBG to inactivate 50% AGT activity (EC_{50}) was determined following a 30-min incubation of increasing concentrations of 8-aza-9-esterBG with 1 mg crude cellular extract.

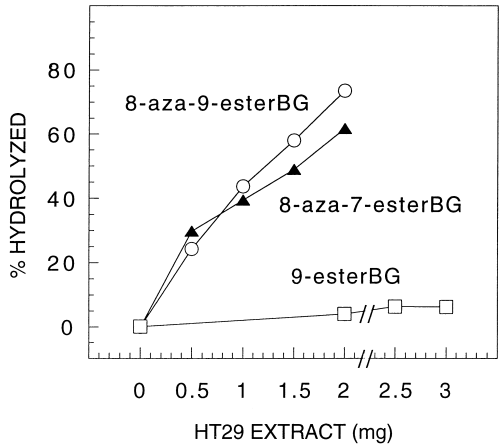

FIG. 3. Rates of conversion of ester prodrugs to benzylated purines by HT29 cell extracts. Ester prodrugs (200 μM): 8-aza-9-esterBG (○), 8-aza-7-esterBG (▲), and 9-esterBG (□) were incubated with increasing concentrations of HT29 cellular extract for 1 hr. Reaction products were analyzed by HPLC. The rate of hydrolysis in buffer alone was 0.19 ± 0.21%, 0.21 ± 0.22%, and 0.01 ± 0.03% for 8-aza-9-esterBG, 8-aza-7-esterBG, and 9-esterBG, respectively. The values shown are representative of an experiment run in duplicate.

The potency of 8-aza-9-esterBG in cellular extract correlates with the degree of conversion of 8-aza-9-esterBG to 8-aza-BG (Table 4). The conversion in HT29, MCF7, and MCF7ADR cells was approximately 20–24%, while the EC_{50} values were similar ranging from 0.2 to 0.33 μM. The percent conversion to 8-aza-BG was much greater (92%) in A549 cells, and the concentration required to inactivate AGT was correspondingly much lower (0.06 μM).

DISCUSSION

Pivoloyloxymethyl prodrugs of BG, 8-bromo-BG, and 8-aza-BG were synthesized and found to be considerably more potent against AGT in tumor cells and tumor cell extracts than against the pure AGT protein. The increase in potency was coincident with an increase in the extent of hydrolysis of ester prodrug to active parent drug *in vivo*. The ester prodrugs were substrates for purified porcine liver carboxylesterase. The degree to which active benzylated purine was formed was a function of structure of ester prodrug and cell type. Differences in the rate of hydrolysis resulted in differences in their AGT inactivation potency.

Previous studies [31] have shown that, although the reaction of BG with AGT is enhanced in the presence of

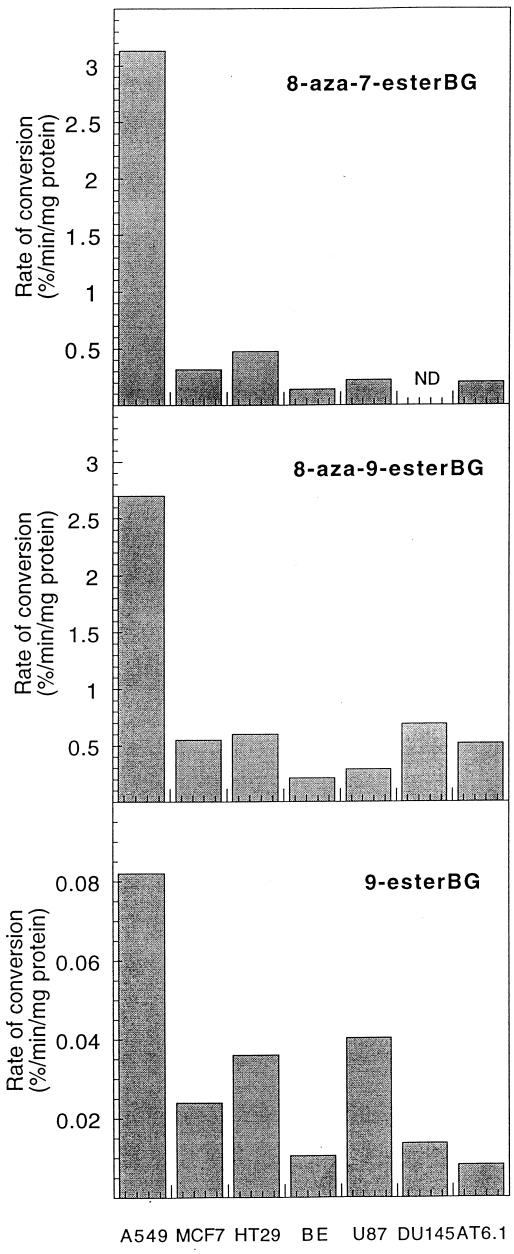

FIG. 4. Rates of conversion of ester prodrugs to benzylated purines by cellular extracts. Ester prodrugs (200 μM) were incubated with extracts from human lung (A549), human breast (MCF7), human colon (HT29 and BE), human brain (U87), human prostate (Du145), and rat prostate (AT6.1) for 1 hr at 37°. Reaction products were analyzed by HPLC as described. The rate of conversion was determined by plotting the percent conversion against the amount of protein extract. At least four different reaction mixtures were analyzed per cell line.

DNA, the ability of BG derivatives with bulky adducts on the 9-position to inactivate AGT is greatly decreased by DNA. This decrease is probably due to the DNA interfering with the binding of these derivatives to the AGT active site. There was a much better correlation between the EC_{50} values for inactivation of AGT in HT29 cells and the EC_{50} values for inactivation of the AGT protein when hemocyanin was used as a means to stabilize the AGT than when

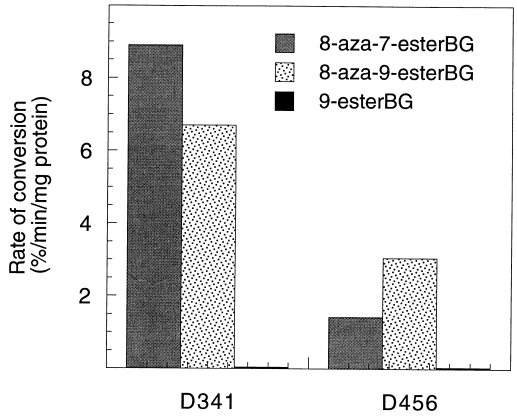

FIG. 5. Rates of conversion of ester prodrugs to benzylated purines by human tumor xenografts. Ester prodrugs (200 μM) were incubated with extracts prepared from human brain tumor (D341 and D456) xenografts for 1 hr at 37°. Reaction products were analyzed by HPLC as described. The rate of conversion was determined by plotting the percent conversion against the amount of protein extract. At least four different reaction mixtures were analyzed per cell line.

DNA was used. This suggests that a major fraction of the cellular AGT is in the free form and/or it can readily dissociate from the nuclear DNA. The results in Table 1 with pure AGT protein indicate that the 7- and 9-esters of BG are inactive as direct inactivators of AGT and that the loss of AGT activity in HT29 cells treated with these agents is due to their conversion to BG itself.

The strong inhibition of pure AGT by the esters of 8-aza- and 8-bromo-benzylguanines indicates that these compounds can act as inactivators if their binding to the protein is not prevented by the presence of DNA. Although the results shown in Table 4 suggest that cellular metabolism of 8-aza-9-esterBG to 8-aza-BG does contribute to the inactivation of AGT in tumor cells, there is only a small (4-fold) enhancement of potency by this conversion. It is therefore possible that the ester substituent does not greatly reduce the rate of reaction with these 8-substituted purines and that much of the inactivation is brought about by the compound itself. This striking contrast with the esters of BG, which have little or no inactivating capacity even in the absence of DNA, raises the possibility that the esters of O^6-benzyl-8-aza- and -8-bromoguanines were actually converted by the AGT protein to the parent drugs and that inactivation was then brought about by the parent drugs. It, therefore, appears that the esters of BG would be the preferred compounds for use as prodrugs with poor AGT-inactivating ability until degraded by esterases.

The observed difference in rate between the 9-esterBG and the 8-aza-7- and -9-esterBG suggests that the susceptibilities to enzymatic hydrolysis of these ester prodrugs are influenced not only by the structure of the promoiety but also by the structure of the parent drug. The rate of conversion of 9-esterBG to BG was 100–200 times slower than the rates of conversion of 8-aza-9-esterBG or 8-aza-7-esterBG to 8-aza-BG. Electronic factors that differ between

BG and 8-aza-BG are at the 8-position, with carbon being electron donating (i.e. BG) and nitrogen being electron withdrawing (i.e. 8-aza-BG). The electron-withdrawing nature of the nitrogen at the 8-position may create a more reactive ester at the 7- or 9-position which is more susceptible to hydrolysis. Electronegative substituents on either side of the ester bond accelerate base-catalyzed hydrolysis [23].

Several factors are important in the design of a prodrug; they include: desired outcome (i.e. targeting), selection of appropriate metabolic or chemical transformation for conversion of the prodrug into the active drug, and the use of appropriate animal species and/or model systems for the evaluation of the prodrug. Ester prodrugs of BG and 8-aza-BG were designed to improve the therapeutic index by targeting tumor esterases, while avoiding tissues of known toxicity to alkylating agents. Prodrugs were more active in tumor tissue than in human bone marrow; however, liver and plasma carboxylesterases would be expected to convert prodrugs to active derivatives, which then may distribute throughout the body. Since esterases present in rat plasma convert these derivatives much more efficiently than human plasma, the rat may not be an appropriate model for *in vivo* studies.

Our results indicate that the highest rate of conversion of esterified BG derivatives occurs with liver microsomes. The hydrolytic potential of the liver is due to its high concentration of carboxylesterases found in liver microsomes where they are loosely bound to the luminal surface of the endoplasmic reticulum [23]. The rates of hydrolysis varied considerably for different tumor cell extracts. These data suggest that these or related prodrugs may be advantageous for selective alkyltransferase inactivation in certain tumor types. The ideal compound would be one with no or little activity against the pure AGT protein and a high rate of conversion in tumor cells with a low rate of conversion in normal tissue. Both the 7- and 9-esters of BG have little or no activity against the pure AGT protein, yet have a low rate of conversion relative to the 8-azaBG ester prodrugs. The 8-azaBG esters have a higher rate of conversion yet exhibit activity against pure AGT protein. 8-Bromo-9-esterBG exhibits a high differential toxicity of the benzylated purine relative to prodrug (440 times) and a low K_m (2.1 μM) for liver carboxylesterase, yet the catalytic activity is low, which will limit the production of active 8-bromo-BG.

Further studies using human tumor xenografts in athymic mice to compare the therapeutic index of AGT modulators (ester prodrug vs benzylated purine) and alkylating agent are warranted. A strategy that could be employed to increase the amount of active drug at the site of the tumor is gene-directed enzyme prodrug therapy. It seems likely that the appropriate human carboxylesterase gene delivered to the tumor will confer a degree of tumor selectivity by increasing the amount of active benzylated purine at the site of the tumor.

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