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SHORT COMMUNICATION

Effect of DNA on the Inactivation of O^6-Alkylguanine-DNA Alkyltransferase by 9-Substituted O^6-Benzylguanine Derivatives

Anthony E. Pegg,† Linda Chung* and Robert C. Moschel‡*

*DEPARTMENTS OF CELLULAR AND MOLECULAR PHYSIOLOGY AND OF PHARMACOLOGY, PENNSYLVANIA STATE UNIVERSITY COLLEGE OF MEDICINE, THE MILTON S. HERSHEY MEDICAL CENTER, HERSHEY, PA 17033; AND ‡CARCINOGEN-MODIFIED NUCLEIC ACID CHEMISTRY, CHEMISTRY OF CARCINOGENESIS LABORATORY, ABL-BASIC RESEARCH PROGRAM, NATIONAL CANCER INSTITUTE-FREDERICK CANCER RESEARCH AND DEVELOPMENT CENTER, FREDERICK, MD 21702, U.S.A.

ABSTRACT. Studies were carried out on the inactivation of pure human O^6-alkylguanine-DNA alkyltransferase by 9-substituted O^6-benzylguanine derivatives in the presence and absence of DNA. The addition of DNA increased the rate of inactivation of the alkyltransferase by O^6-benzylguanine and its 9-methyl derivative but had little effect on the rate of inactivation by the 9-cyanomethyl derivative. In contrast, when O^6-benzylguanine derivatives with larger 9-substituents such as ribose, 2′-deoxyribose, dihydrotestosterone, or 2-hydroxy-3-(isopropoxy)propyl were used, the addition of DNA was strongly inhibitory to the inactivation. In the case of O^6-benzylguanine, O^6-benzylguanosine, and O^6-benzyl-2′-deoxyguanosine, these results were confirmed by directly measuring the rate of formation by the alkyltransferase of guanine, guanosine, or 2′-deoxyguanosine, respectively. The data indicated that the presence of DNA activated the alkyltransferase, rendering it more reactive with O^6-benzylguanine or O^6-benzyl-9-methylguanine, but that DNA interferes with the binding of inhibitors with larger 9-substituents, presumably by competing for the same binding site. Since these inactivators readily inactivate alkyltransferase in cells, the amount of cellular alkyltransferase bound to DNA must be small or readily exchangeable with the free form. BIOCHEM PHARMACOL 53;10:1559–1564, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. O^6-alkylguanine-DNA alkyltransferase; O^6-benzylguanine; chloroethylating agents; methylating agents; chemotherapy

AGT§ is a DNA repair protein that removes adducts from the O^6-position of DNA guanine residues and the O^4-position of DNA thymine residues in a single step by transferring them to a cysteine acceptor site located within the protein sequence [1–4]. The cysteine converted to alkylcysteine in this reaction is not regenerated, and each molecule of AGT can therefore act only once. Alkyl groups at the O^6-position of guanine are removed much more rapidly than alkyl groups at the O^4-position of thymine. There is no doubt that repair of O^6-alkylguanine by AGT protects mammalian cells from the toxic and mutagenic effects of alkylating agents, but the importance of the slow repair of O^4-alkylthymine remains to be established [5].

Mammalian AGTs can be inactivated by O^6-benzylguanine [3, 6, 7]. This inactivation is due to the ability of O^6-benzylguanine to act as a substrate for the protein forming S-benzylcysteine at the active site. Depletion of AGT by O^6-benzylguanine has been shown to facilitate the killing of tumor cells by chloroethylating and methylating agents [3, 8–11]. Although O^6-benzylguanine has now entered clinical trials, further development of this concept may require the synthesis of additional AGT inhibitors with greater potency, solubility, or specificity towards neoplastic cells. As part of a program to develop such inhibitors, a considerable number of analogs and derivatives of O^6-benzylguanine have been synthesized and tested as inactivators of human AGT contained in crude HT29 cell extracts and in intact cultured cells [11–14]. Some of these compounds were quite active as AGT inhibitors and, in a few cases, were more potent than O^6-benzylguanine itself. Studies of the effects of substitution on O^6-benzylguanine have led to the conclusion that the presence of substituents at the 9-position may reduce their ability to interact with the AGT but they do not necessarily have a major deleterious effect on the inhibitory activity, whereas substituents on the 7-position are not well tolerated.

In the present work, we examined some of the 9-substituted O^6-benzylguanine derivatives (Fig. 1) with respect to

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† Corresponding author: Dr. Anthony E. Pegg, Department of Cellular and Molecular Physiology, Pennsylvania State University College of Medicine, Milton S. Hershey Medical Center, P.O. Box 850, 500 University Drive, Hershey, PA 17033. Tel. (717) 531-8152; FAX (717) 531-5157.

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§ *Abbreviations:* AGT, O^6-alkylguanine-DNA alkyltransferase (EC 2.1.1. 63); and EC$_{50}$, concentration of AGT inactivator needed to reduce the AGT activity by 50% in a 30-min incubation at 37°.

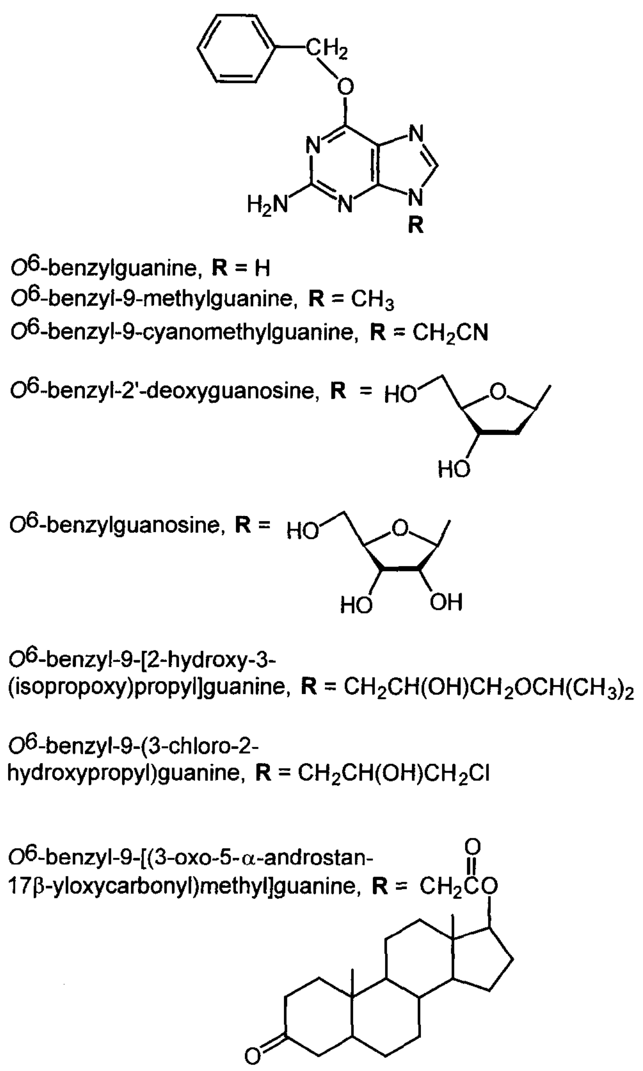

FIG. 1. Structures of AGT inactivators.

their ability to inactivate homogeneous preparations of purified human AGT in the presence and absence of DNA. These studies revealed striking differences in the effect of DNA on the inactivation according to the 9-substituent.

MATERIALS AND METHODS

Materials

Most biochemical reagents including hemocyanin (from *Limulus polyphemus*) and calf thymus DNA were obtained from the Sigma Chemical Co. (St. Louis, MO). *N*-[^{3}H]-Methyl-*N*-nitrosourea (5.9 mCi/mmol) was obtained from the Amersham Corp. (Arlington Heights, IL). The synthesis of all of the AGT inactivators used has been described previously [12–14]. Labeled O^6-benzylguanine, O^6-benzylguanosine, and O^6-benzyl-2′-deoxyguanosine were prepared as previously described by catalytic [^{3}H] exchange [15]. This procedure places the [^{3}H] label at the 8-position of the guanine residue.

Isolation of Human AGT

Homogeneous preparations of recombinant human AGT were obtained by expressing the protein from the pINAGT plasmid in GWR109 cells and purifying it by ammonium sulfate precipitation and mono S chromatography as previously described [6]. The pINAGT plasmid contains the human AGT sequence in the *Escherichia coli* expression vector pINIII-A3(lpp^{P-5}).

Assay of Inactivation of AGT

The purified AGT protein (60 ng) was incubated with O^6-benzylguanine or other inhibitors in 0.5 mL of 50 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 5.0 mM dithiothreitol in the presence of either 50 μg of calf thymus DNA or 50 μg of hemocyanin for 30 min at 37°. Under these conditions, the AGT activity was completely stable with less than 5% loss of activity in the 30-min incubation in the absence of an AGT inhibitor. In some experiments, the purified AGT was replaced with crude HT29 cell extract prepared as previously described [7, 12–14].

After the initial 30-min incubation in the presence of the inhibitor, the residual AGT activity was determined by a 30-min incubation with a ^{3}H-methylated DNA substrate that had been methylated by reaction with *N*-[^{3}H]methyl-*N*-nitrosourea essentially as described [7, 9]. The results were expressed as the percentage of the AGT activity remaining. The graphs of activity remaining against inhibitor concentration were used to calculate an EC$_{50}$ value representing the amount of inhibitor needed to produce a 50% loss of activity.

Assay of Guanine, Guanosine and 2′-Deoxyguanosine Production by AGT

The purified AGT protein (10–50 μg) was incubated with the [8-^{3}H]-labeled O^6-benzylguanine, O^6-benzylguanosine, or O^6-benzyl-2′-deoxyguanosine substrates for 30 min at 37° in the presence or absence of 50 μg/mL of DNA in 0.5 mL of 50 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 5.0 mM dithiothreitol; the amount of [8-^{3}H]-labeled guanine, guanosine, or 2′-deoxyguanosine formed was determined by HPLC as previously described [15]. The results were expressed as counts per minute of product formed per microgram of protein from the average of assays using different amounts of protein where the production of the labeled product was proportional to both the time of incubation and to the amount of protein added.

RESULTS AND DISCUSSION

The compounds (Fig. 1) were assayed for their ability to inactivate pure AGT by incubating them for 30 min at 37° with the AGT protein. Even though the assay buffer contained 5 mM dithiothreitol, the AGT protein was not stable at the very high dilutions (0.12 μg/mL) of pure AGT

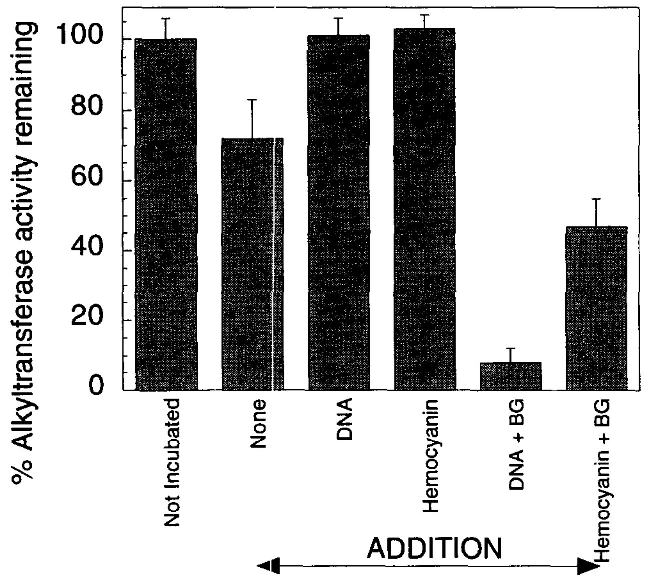

FIG. 2. Stability of AGT. AGT protein (60 ng) was incubated for 60 min at 37° in 0.5 mL of 50 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 5.0 mM dithiothreitol with no addition, 50 μg of calf thymus DNA, 50 μg of hemocyanin, 50 μg of calf thymus DNA plus 0.2 μM O^6-benzylguanine (BG), and 50 μg of hemocyanin plus 0.2 μM O^6-benzylguanine (BG). The remaining AGT activity was then determined and is expressed as the percentage of the activity found without preincubation. See Materials and Methods for experimental details. Results are means ± SD for 4 estimations.

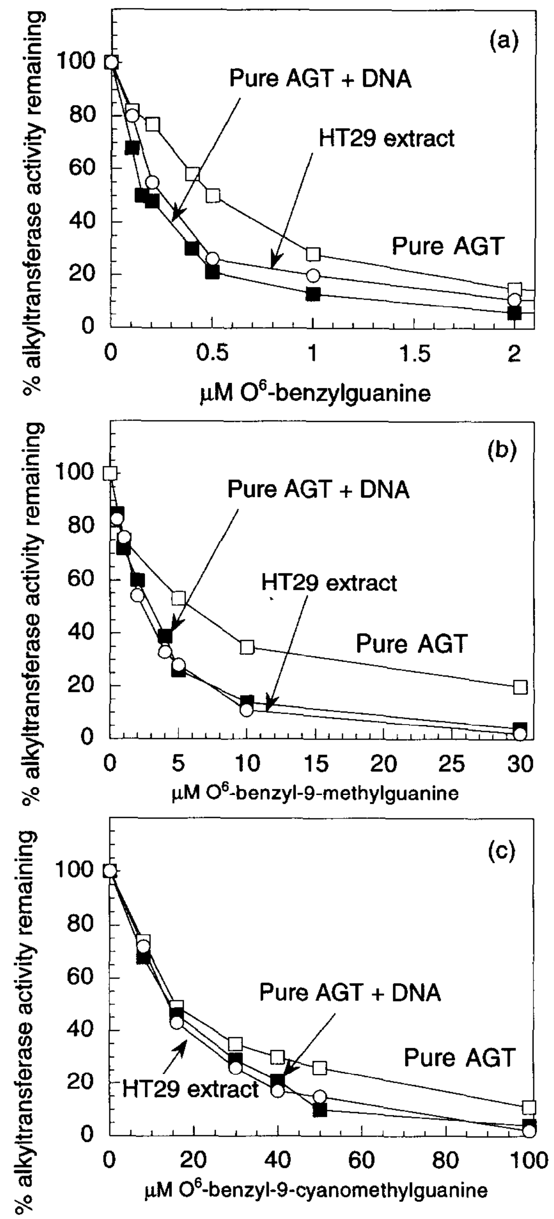

FIG. 3. Effect of O^6-benzylguanine and its 9-methyl and 9-cyanomethyl derivatives on AGT activity. Sixty nanograms of purified AGT protein (squares) or 0.8 mg of an HT29 cell extract (circles) was incubated in 0.5 mL of 50 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 5.0 mM dithiothreitol with the amounts of O^6-benzylguanine or the derivative indicated for 30 min at 37°; then the AGT activity remaining was determined. The incubations with pure AGT contained either 50 μg of DNA (filled symbols) or 50 μg of hemocyanin (open symbols). Those with HT29 extract had no other additions. Results are the means of at least three observations.

protein used in this assay, unless either DNA or another protein was present (Fig. 2). Therefore, either 50 μg of calf thymus DNA or 50 μg of hemocyanin was added. Under these conditions, there was no loss of AGT activity in a 60-min incubation in the absence of O^6-benzylguanine or another inhibitor (Fig. 2).

As previously reported [15], the rate of reaction of AGT with O^6-benzylguanine increased in the presence of DNA. O^6-Benzylguanine was, therefore, a more potent inactivator when DNA was present. This is shown in Fig. 2 by comparing the loss of activity in the presence of DNA with the loss of activity in the presence of hemocyanin. A complete curve of inhibition of AGT at various concentrations of O^6-benzylguanine (Fig. 3a) indicated that the EC_{50} value was reduced from 0.6 to 0.15 μM when DNA was present (Table 1). Inactivation of AGT in a crude HT29 cell extract was intermediate between these values with an EC_{50} of 0.25 μM.

Although the 9-methyl derivative of O^6-benzylguanine is a weaker inhibitor than the parent compound, essentially similar results were seen when it was used as the inactivator (Fig. 3b). In this case, the increase in potency upon adding DNA was only 2-fold with the EC_{50} value being reduced from 6 to 3 μM (Fig. 3b and Table 1). When the slightly larger and even less active 9-cyanomethyl derivative of O^6-benzylguanine was used, there was little if any stimulatory effect of DNA on the reaction (Fig. 3c).

When O^6-benzylguanine derivatives with larger substituents such as ribose (Fig. 4a), 2′-deoxyribose (Fig. 4b), dihydrotestosterone (Fig. 4c), or 2-hydroxy-3-(isopropoxy)-propyl (Fig. 4d) were tested, not only was there no stimulatory effect of DNA on the reaction, but inactivation of the AGT was, in fact, greatly reduced. In the presence of DNA, the EC_{50} values for these compounds were 40 μM or greater, whereas inactivation in the absence of DNA required only 0.8 μM to 14 μM, depending on the

TABLE 1. Effect of DNA on inactivation of AGT

	EC$_{50}$ (μM)		
Compound	Pure protein + DNA	Pure protein + hemocyanin	Crude HT29 cell extract*
O^6-Benzylguanine	0.15	0.6	0.25
O^6-Benzyl-9-methylguanine	3.0	6.0	2.6
O^6-Benzyl-9-cyanomethylguanine	16	18	13
O^6-Benzyl-2′-deoxyguanosine	40	2.0	2.0
O^6-Benzylguanosine	60	14	11
O^6-Benzyl-9-[(3-oxo-5-α-androstan-17β-yloxycarbonyl)methyl]guanine	40	0.8	4.0
O^6-Benzyl-9-[2-hydroxy-3-(isopropoxy)propyl]guanine	>50	4.0	7.0
O^6-Benzyl-9-(3-chloro-2-hydroxypropyl)guanine	>50	9.0	18.0

* The EC$_{50}$ values shown for crude HT29 cell extracts have been published previously [12–14]. Similar results were obtained from experiments shown in Figs. 2 and 3, which were carried out at the same time as those with the pure AGT protein. The EC$_{50}$ values for the pure AGT protein were derived from these experiments.

substituent (Table 1). The effect was particularly striking for O^6-benzyl-9-[(3-oxo-5-α-androstan-17β-yloxycarbonyl)-methyl]guanine (Fig. 4c), where the EC$_{50}$ value was increased from 0.8 to 40 μM, and for O^6-benzyl-2′-deoxyguanosine (Fig. 4b), where there was a 20-fold increase in EC$_{50}$ (from 2 to 40 μM) (Table 1). Inactivation by O^6-benzyl-9-[2-hydroxy-3-(isopropoxy)propyl]guanine in the presence of DNA was so weak that the EC$_{50}$ value was more than 50 μM (Fig. 4d) and could not be determined accurately. In contrast, this inhibitor was quite potent in

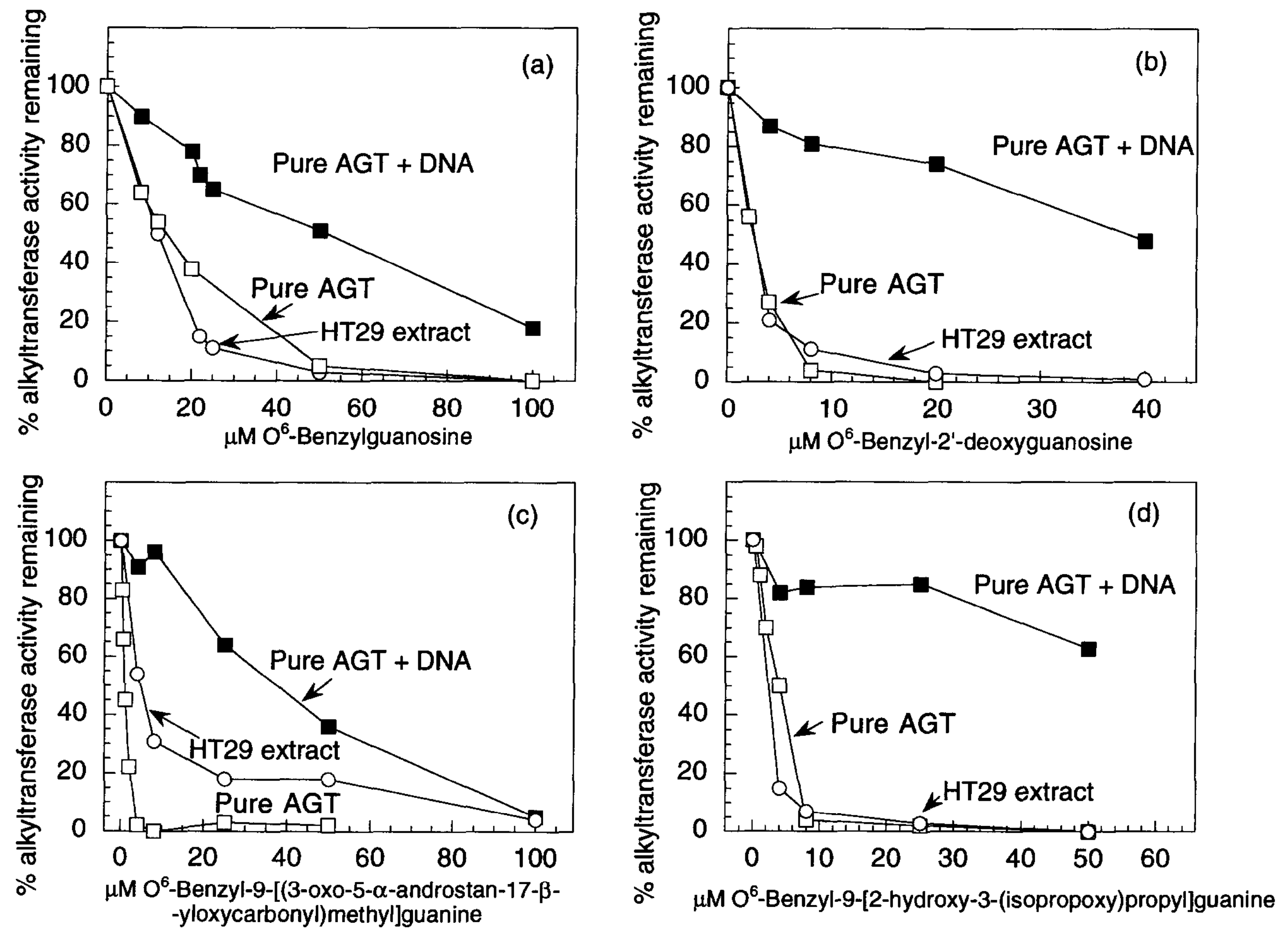

FIG. 4. Effect of bulky-9-substituted O^6-benzylguanine derivatives on AGT activity. The experiments were carried out as described in the legend to Fig. 3. Results are the means of at least three observations.

TABLE 2. Inhibitory effect of DNA on inactivation of pure AGT by O^6-benzyl-2′-deoxyguanosine and O^6-benzyl-9-[(3-oxo-5-α-androstan-17β-yloxycarbonyl)methyl]guanine

	% AGT activity remaining*	
Addition	Hemocyanin	Assayed + Hemocyanin + DNA
None	100	100
O^6-Benzyl-2′-deoxyguanosine (20 μM)	13	79
O^6-Benzyl-2′-deoxyguanosine (40 μM)	1	48
O^6-Benzyl-9-[(3-oxo-5-α-androstan-17β-yloxycarbonyl)methyl]guanine (20 μM)	20	89

See Materials and Methods for experimental details.

* Results shown are the means of at least three observations.

the absence of DNA with an EC_{50} value of 4 μM. A related compound, O^6-benzyl-9-(3-chloro-2-hydroxypropyl)guanine, showed a similar effect with little inactivation of AGT at up to 50 μM in the presence of DNA and an EC_{50} value of 9 μM in the absence of DNA (Table 1).

These results imply strongly that the presence of DNA interferes with the ability of O^6-benzylguanine derivatives with bulky 9-substituents to react with AGT, but a second less probable explanation would be that the hemocyanin (or some contaminant) enhances the inhibitory activity. This possibility was ruled out by adding DNA to the assays that also contained hemocyanin. The results show clearly that inactivation by O^6-benzyl-2′-deoxyguanosine or O^6-benzyl-9-[(3-oxo-5-α-androstan-17β-yloxycarbonyl)-methyl]guanine was actually prevented by the addition of DNA (Table 2).

The mechanism by which O^6-benzylguanine inactivates AGT is well understood. The O^6-benzylguanine is recognized as a substrate by the protein and converted to guanine in parallel to the inactivation of the AGT by the formation of S-benzylcysteine at the acceptor site [6, 16]. The rate of reaction of O^6-benzylguanine-derived inhibitors with the protein can, therefore, be measured by comparing the formation of guanine derivative under conditions where the fraction of the protein inactivated is small and the reaction is proportional to time. The influence of DNA on this reaction was studied with O^6-benzylguanine, O^6-benzylguanosine, and O^6-benzyl-2′-deoxyguanosine, which were all available in a labeled form (Fig. 5). The results showed clearly that the rate of reaction with the free base was stimulated by DNA, whereas the rate of reaction with the 9-substituted ribonucleoside and 2′-deoxyribonucleoside was inhibited substantially by DNA. These results confirm those from the inactivation curves shown in Figs. 3 and 4.

The crystal structure of the Ada-C alkyltransferase from *E. coli*, which has substantial similarity in amino acid sequence to the human AGT, indicates that the protein must undergo a conformational change upon binding DNA [17, 18]. It has been postulated that this conformational change leads to the disruption of a hydrogen bonding network involving several key amino acid residues including Glu^{172} and His^{146} resulting in the activation of the Cys^{145} acceptor site of the protein [17]. The increased rate of reaction of the AGT with O^6-benzylguanine in the presence of DNA, which is also seen with a number of 8-substituted O^6-benzylguanines including the 8-bromo-, 8-oxo-, and 8-aza-derivatives (results not shown), is consistent with this activation.

Our results show that the addition of groups larger than methyl to the 9-position abolishes this stimulation and, with all of the relatively bulky groups tested, actually reverses the situation such that inactivation is reduced greatly by DNA. The most likely explanation of this effect is that when bound at the active site of the AGT, the 9-substituent extends into the space occupied by DNA. Thus, binding of DNA, which is much stronger than the binding of the inhibitors, interferes with the reaction with the inhibitors.

It can be seen from Table 1 that the results obtained using pure AGT in the absence of DNA more closely relate

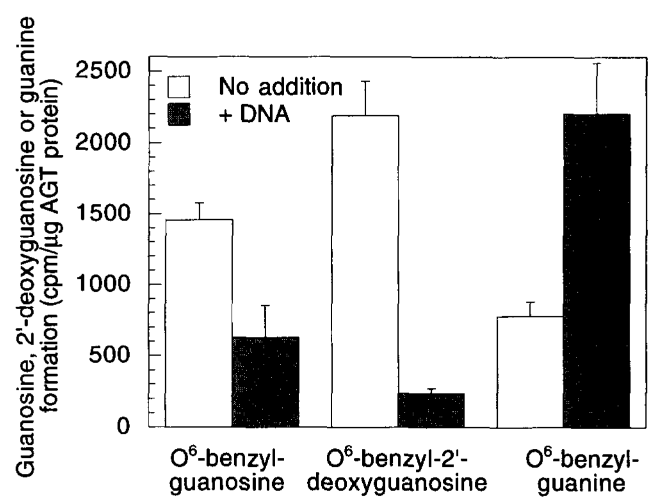

FIG. 5. Effect of DNA on formation of guanosine from O^6-benzylguanosine, 2′-deoxyguanosine from O^6-benzyl-2′-deoxyguanosine, and guanine from O^6-benzylguanine. The AGT protein was incubated with the [^{3}H]-labeled substrate, and the amount of [^{3}H]product formed in the presence and absence of DNA was determined. The experiment was carried out with 0.05 μM O^6-benzyl[8-^{3}H]guanine (500 mCi/mmol), 0.5 μM O^6-benzyl-2′-deoxy[8-^{3}H]guanosine (500 mCi/mmol) and 1 μM O^6-benzyl[8-^{3}H]guanosine (1 Ci/mmol). Results are means ± SD for 4 estimations.

to those in which crude HT29 cell extracts were used as a source of AGT than the assays in the presence of DNA. The crude HT29 cell extracts do contain some DNA but clearly not enough to bind all of the AGT. The amount of DNA present in these extracts was less than 5 μg per assay and was also likely to be fragmented since the extracts are sonicated to release AGT from nuclear pellets. It is also possible that the residual DNA in these extracts is bound to other proteins that have a higher affinity for it than AGT. The addition of 50 μg of calf thymus DNA to the assays with crude HT29 extracts did increase the EC_{50} values for O^6-benzyl-9-[(3-oxo-5-α-androstan-17β-yloxycarbonyl)-methyl]guanine (to about 25 μM) and O^6-benzyl-2′-deoxyguanosine (to about 20 μM) (results not shown). The difference between these values and those found for the pure AGT protein assayed plus DNA may be due to the presence of other proteins in the extracts capable of binding to either AGT or DNA.

It appears that a significant fraction of AGT in the cell is at least transiently in the free form since there is a relatively good correlation between the EC_{50} values for assays of AGT inactivation *in vivo* in HT29 cell cultures and those with crude HT29 cell extracts [12–14]. The inhibitors with bulky adducts at the 9-position presumably inactivate the free form of the AGT protein and subsequently inactivate the DNA bound form when it dissociates from the DNA. The 9-position of O^6-benzylguanine is, therefore, a potentially valuable site for derivatives in the design of AGT inhibitors with improved properties with regard to tumor selectivity, pharmacokinetics, and formulation.

Our results suggest that the preferred screening method for new AGT inhibitors *in vitro* is to test them with pure AGT plus a carrier protein to ensure AGT stability. The ability of these compounds to react with AGT *in vivo* may also be influenced by metabolism (which could act to either increase or decrease potency) and by the ability to pass through the cell membrane and be accumulated in the cell. Follow-up *in vivo* assays can then be carried out to examine the contribution of these factors.

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