Inactivation of Human O\textsuperscript{6}-Alkylguanine-DNA Alkyltransferase by Modified Oligodeoxyribonucleotides Containing O\textsuperscript{6}-Benzylguanine

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ABSTRACT

Inactivation of the DNA repair protein O\textsuperscript{6}-alkylguanine-DNA alkyltransferase (AGT) enhances tumor cell killing by therapeutic alkylating agents. O\textsuperscript{6}-Benzylguanine (b\textsuperscript{6}G) can inactivate AGT and is currently in clinical trials to enhance therapy. Short oligodeoxyribonucleotides containing b\textsuperscript{6}G are much more effective inactivators, but their use for therapeutic purposes is likely to be compromised by metabolic instability. We have therefore examined the ability to inactivate AGT of an 11-mer oligodeoxyribonucleotide containing b\textsuperscript{6}G (11-mpBG) when modified with terminal methylphosphonate linkages to protect it from nucleases. This modification did not reduce the ability to serve as a substrate/inactivator for AGT, and 11-mpBG had an ED\textsubscript{50} value of 1.3 nM, more than 300-fold lower than that for b\textsuperscript{6}G. A similar oligodeoxyribonucleotide containing O\textsuperscript{6}-methylguanine (m\textsuperscript{6}G) was also found to be a good substrate (ED\textsubscript{50} value of 10 nM), but the benzyalted form was repaired more rapidly and preferentially. When added to HT29 cell cultures, 5 μM 11-mpBG was able to cause a prolonged inactivation of cellular AGT for at least 72 h and to greatly sensitize the cells to killing by 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU). The 11-mpMG was ineffective at up to 20 μM, suggesting that the benzyl group allows better uptake into the cell. However, even with 11-mpBG, the 1000-fold decrease in potency toward AGT in HT29 cells compared to that toward the protein in vitro suggests that uptake may be a limiting factor. These results suggest that oligodeoxyribonucleotides such as 11-mpBG may prove to be useful drugs for potentiation of alkylating agent chemotherapy if uptake can be improved.

AGT is a DNA repair protein that acts on a variety of adducts at the O\textsuperscript{6}-position of guanine including methyl-, ethyl-, benzyl-, 2-chloroethyl-, and pyridyloxobutyl- (Mitra and Kaina, 1993; Pegg et al., 1995a; Pegg, 2000). Repair is brought about in a direct single reaction in which the adduct is transferred to a cysteine acceptor site. The S-alkylcysteine is not regenerated, and the alkylated form of the protein is ubiquitinated and rapidly degraded. Thus, substrates of the AGT protein are also inactivators.

Repair of either O\textsuperscript{6}-methylguanine (m\textsuperscript{6}G) or O\textsuperscript{6}-(2-chloroethyl)guanine by AGT protects cells from the cytotoxicity of methylating and chloroethy1ating agents, respectively (Erickson et al., 1980; Margison et al., 1996; Ludlum, 1997; Pegg et al., 2000). Therefore, attempts are being made to use inactivators of the AGT protein to increase the sensitivity of tumor cells to these agents. The most widely studied compound that is being used for this purpose is O\textsuperscript{6}-benzy1guanine (b\textsuperscript{6}G) (Dolan et al., 1990; Dolan and Pegg, 1997; Kreklau et al., 1999; Pegg et al., 2000), although other agents that act similarly, such as O\textsuperscript{6}-benzyl-2′-deoxyguanosine, O\textsuperscript{6}-benzyl-N\textsuperscript{2}-acytethylguanosine, and O\textsuperscript{6}-(5-bromoethyl)guanine, are also being pursued (Marathli et al., 1994; Kokkinakis et al., 2000; Middleton et al., 2000; Pegg et al., 2000). b\textsuperscript{6}G is currently undergoing clinical trials, but its use may be limited by its poor solubility, relatively low potency, and inability to inactivate mutant forms of AGT that contain single point mutations rendering a high level of resistance to it (Pegg et al., 2000; Xu-Welliver and Pegg, 2000).

b\textsuperscript{6}G is recognized as a substrate by AGT and is acted upon by its formation of free guanine and S-benzy1cysteine at the acceptor site of the protein leading to irreversible inactivation of the AGT (Pegg et al., 1995a; Pegg, 2000). The reaction of AGT with b\textsuperscript{6}G is much slower than the reaction with

**ABBREVIATIONS:** AGT, human O\textsuperscript{6}-alkylguanine-DNA alkyltransferase; b\textsuperscript{6}G, O\textsuperscript{6}-benzy1guanine; m\textsuperscript{6}G, O\textsuperscript{6}-methylguanine; 11-mpBG, 5′-d(TmpGTGAb\textsuperscript{6}GCTGTmpG)-3′; 11-mpMG, 5′-d(TmpGTGAm\textsuperscript{6}GCTGTmpG)-3′; 11-MG, 5′-d(TGTGAm\textsuperscript{6}GCTGTG)-3′; ED\textsubscript{50}, the amount of inhibitor needed to produce a 50% loss of activity; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; DMT, 4,4′-dimethoxytrityl; HPLC, high performance liquid chromatography.
methylated DNA. This is likely to be due to the weak binding of b6G to the active site of the AGT protein since it lacks the ability to interact with the DNA binding domain of AGT. We have shown earlier that relatively short oligodeoxyribonucleotides that contain a b6G residue are much better substrates and effective inhibitors of both wild-type and the mutant human AGT proteins than the b6G free base (Goodtzova et al., 1997; Pegg et al., 1998). This is consistent with the concept that the interactions with the other components of the oligodeoxyribonucleotide lead to a more efficient transfer of the benzyl group to the AGT protein.

Previously undescribed oligodeoxyribonucleotides were synthesized on a 10-μmol scale using an Applied Biosystems, Inc. (Foster City, CA) model 394 DNA/RNA synthesizer. The O9-substituted guanine 2'-deoxyribonucleoside phosphoramidites were prepared as previously described (Pauly et al., 1988). All other DNA synthesis reagents were from Glenn Research (Sterling, VA). The standard Applied Biosystems, Inc. 10-μmol synthesis cycle was used except that methylphosphonates were allowed to couple for an additional 6 min, and O9-substituted guanine-containing phosphoramidites were allowed to couple for an additional 15 min. At the end of synthesis, the 4,4'-dimethoxytrityl (DMT) protecting group was not removed from the 5'-end of the oligodeoxyribonucleotides. Oligodeoxyribonucleotides were cleaved from the solid support by standard ammonium hydroxide treatment followed by the immediate removal of ammonium hydroxide under vacuum. Removal of the protecting groups was accomplished using a modification of a published method (Polushin et al., 1994). Briefly, oligodeoxyribonucleotides were exposed to 3.3 ml of a solution of hydrazine, ethanolamine, and methanol (1:5:5, v/v/v) for 1.5 h at room temperature. The solution was then treated with 1.18 ml of glacial acetic acid in 5 ml of water until the pH of the aqueous solution reached 7.5. At this point the solution became cloudy and it was stored at −20°C overnight. Centrifugation of the cold suspension produced a pellet. The supernatant was decanted, and the pellet was redissolved in 5 ml of 0.1 M triethylammonium acetate, pH 7, containing 10% acetonitrile by volume. The 5'-DMT-containing oligodeoxyribonucleotides were purified by HPLC on a 10 mm × 25 cm Luna column (Phenomenex, Torrance, CA). The solvents were 0.1 M triethylammonium acetate, pH 7 (A), and acetonitrile (B). Column elution was carried out using a linear gradient of 10 to 40% B over 60 min at a flow rate of 3 ml/min. UV absorbance was monitored at 270 nm. The O9-G-containing oligodeoxyribonucleotide methylphosphonates were recovered in two peaks at 46 and 50 min that were designated DMT-11-mpMG-1 and DMT-11-mpMG-2, respectively. The O9-benzylguanine-containing oligodeoxyribonucleotide methylphosphonates chromatographed as two peaks at 48 and 52 min, and these were designated DMT-11-mpBG-1 and DMT-11-mpBG-2, respectively. The resolution of these oligodeoxyribonucleotides into two peaks was a consequence of the proximity of the bulky DMT group to the chiral methylphosphonate linkage at the 5'-end of each oligodeoxyribonucleotide. The solutions for these various samples were processed separately. Each was evaporated to dryness, and the resulting oligodeoxyribonucleotides were detritylated by treatment with acetic acid/water (8:2, v/v) for 15 min followed by coevaporation with excess ethanol under vacuum. The detritylated oligodeoxyribonucleotides were purified by HPLC using a gradient of 5 to 40% B over 60 min at 3 ml/min. The resulting 11-mpMG-1 and 11-mpBG-2 eluted at 22 min. Oligodeoxyribonucleotides 11-mpBG-1 and 11-mpBG-2 were eluted at 25 min. After removal of the 5'-DMT group, the stereoisomeric methylphosphonate-containing oligodeoxyribonucleotides were not resolved under these preparative chromatographic conditions. However, many were resolved under the analytical chromatographic conditions described below.

Samples of the oligodeoxyribonucleotides were digested to nucleosides with snake venom phosphodiesterase and alkaline phosphatase, and the nucleoside composition was determined as described previously (Pauly et al., 1988). In addition to peaks corresponding to the expected nucleosides, two additional peaks were observed in each sample. These two peaks coeluted with the two peaks produced in equal intensity when 5'-TmpG (where mp indicates a chiral methylphosphonate linkage) was chromatographed under the same HPLC conditions. However, in the case of digests of 11-mpMG-1 and 11-mpBG-1 the two peaks were produced in a ratio of 1:3, while in digests of 11-mpMG-2 and 11-mpBG-2 they were produced in a ratio of 3:1 (data not shown). These data are consistent with the conclusion that the oligodeoxyribonucleotide pairs, i.e., 11-mpMG-1 and 11-mpBG-2 or 11-mpBG-1 and 11-mpMG-2, differ in the stereochemistry of their methylphosphonate linkages at the 5'-end of the oligodeoxyribonucleotide.
godeoxyribonucleotide. The normal nucleoside composition of these oligodeoxyribonucleotides is presented in Table 1.

Construction of Plasmid and Expression of b6G-Resistant Mutant Alkyltransferases. The wild-type human AGT and O6-benzylguanine-resistant mutants (P140A, P140K, G156A, Y158H, G160R, K165A) were made by inserting the relevant cDNA into the pQE30 vector from Qiagen (Chatsworth, CA) for the expression of the recombinant protein and purified to homogeneity by immobilized metal affinity chromatography as described earlier (Xu-Welliver et al., 1998, 1999, 2000).

Inactivation of AGT in Vitro. The purified wild-type or mutant AGT proteins were incubated with different concentrations of the potential inhibitors in 0.1 ml of reaction buffer (50 mM Tris-HCl, pH 7.6, 0.1 mM EDTA, 0.5 mM dithiothreitol) in the presence of 10 μM of hemocyanin for 30 min at 37°C. The remaining AGT activity was determined after incubation with [3H]methylated calf thymus DNA substrate for 30 min at 37°C as described previously (Xu-Welliver et al., 1998). The concentration of inhibitor which led to a 50% loss of AGT activity (ED50) was calculated from graphs where percentage of remaining AGT activity was plotted against inhibitor concentration.

Separation of Oligodeoxyribonucleotides by HPLC. The 11-mer oligodeoxyribonucleotides containing O6-adducts were incubated with different amounts of wild-type AGT in 50 mM Tris-HCl, pH 7.6, 0.1 mM EDTA, and 0.5 mM dithiothreitol for 10 min at 37°C. The reaction was then stopped by the addition of 1% (final concentration) sodium dodecyl sulfate, and the mixture of oligodeoxyribonucleotides was separated on a reverse-phase C18 5-μm Ultrasphere ODS column (Beckman, Palo Alto, CA), and oligonucleotides were detected by UV at 254 nm as described earlier (Goodtzova et al., 1997; Pegg et al., 1995b). Separation was carried out at 45°C using a linear gradient of 14 to 50% methanol in 50 mM sodium phosphate buffer (pH 6.3) over 60 min with a flow rate of 2 ml/min. In most cases, the oligodeoxyribonucleotides containing methylphosphonate linkages could be resolved into multiple peaks corresponding to the respective stereoisomers. The exact starting point of the gradient was varied slightly according to the experiment. This accounts for the slightly different retention times in Figs. 1 to 3 and 5, but in all cases all samples from a particular experiment were run under identical conditions.

Cell Culture, Inactivation of Cellular AGT, and Cytotoxicity Assays. HT29 cells were grown in Dulbecco’s modified Eagle’s medium containing 36 mM NaHCO3 supplemented with 10% fetal bovine serum plus 3% glutamine and 50 μg/ml gentamycin (Pegg et al., 1995b; Loktionova et al., 1999). The cells were plated at densities of 0.25 × 106 in 35-mm dishes, and the medium was changed 36 h later. Cells were incubated with different concentrations of oligodeoxyribonucleotides for 14 h in 1 ml of medium and then exposed to freshly prepared solutions of 40 μM BCNU for 2 h. (The BCNU was first dissolved in absolute ethanol at a concentration of 8 mM, then diluted with the same volume of phosphate-buffered saline, and immediately applied to the cells.) The medium was replaced with fresh medium containing oligodeoxyribonucleotides where indicated, and the cells were left to grow for an additional 16 to 18 h. (The AGT inhibitor was added to the medium in which the cells were incubated for 16 to 18 h after treatment with BCNU to ensure that the inhibitor was present during the entire period in which DNA adducts formed by BCNU at the O6-position of guanine exist in the cell.) The cells were then replated at densities of 100 to 2000 cells/25-cm2 flask and grown for 8 days until discrete colonies could be stained and counted. The colonies were washed with 0.9% saline solution, stained with 0.5% crystal violet in ethanol, and counted. The plating efficiency for HT29 cells not treated with drugs was about 50%. In experiments to assess the effect of AGT inactivation by b6G on sensitivity to killing by BCNU, cells were treated with b6G 2 h before exposure to 40 μM BCNU. The medium was then replaced with fresh medium containing b6G where indicated.

Results

Ability of Wild-Type AGT to React with Oligodeoxyribonucleotides Modified with Terminal Methylphosphonate Linkages. Since introduction of a substitu-
ent on the phosphate linkages generates a new center of chirality, the 11-mers containing a m6G (11-mpMG) or a b6G (11-mpBG) each exist as four stereoisomers. Purification after synthesis of the 5′-O-DMT-containing oligodeoxyribonucleotides was able to separate both 11-mpBG and 11-mpMG into two mixtures of stereoisomers that are arbitrarily designated 1 and 2. This separation was a consequence of the proximity of the DMT group to the chiral methylphosphonate linkage at the 5′-end of each oligodeoxyribonucleotide.

The ability of these 11-mers containing either b6G or m6G to serve as substrates for wild-type AGT was examined by using reverse-phase HPLC to separate the alkylated and nonalkylated forms. Different amounts of wild-type AGT protein were incubated with these potential substrates for 10 min at 37°C and then the formation of the dealkylated products was examined. Wild-type AGT was able to repair the benzyl adduct in both 11-mpBG-1 (Fig. 1, left panels) and 11-mpBG-2 (Fig. 1, right panels). In both cases, the product peak of 11-mpG could actually be resolved into the two expected isomers that are arbitrarily designated 11-mpG-1a and 11-mpG-1b derived from 11-mpBG-1 (Fig. 1, left panels) and 11-mpG-2a and 11-mpG-2b derived from 11-mpBG-2 (Fig. 1, right panels). There was no obvious difference in the extent of repair of the four potential substrates since the four product peaks were formed in similar amounts.

As shown in Fig. 2, the two samples of 11-mpMG obtained after synthesis could both be partially resolved into two peaks with the HPLC system used showing clearly that, as expected, there are four isomers. Each sample consists of two isomers arbitrarily designated as 11-mpMG-1a and 11-mpMG-1b (Fig. 2, left panels) and 11-mpMG-2a and 11-mpMG-2b (Fig. 2, right panels). In both cases, wild-type AGT was able to repair both peaks, and as with 11-mpBG there was no difference in the relative repair of the four potential substrates.

The relative preference of wild-type AGT for repair of either 11-mpBG or 11-mpMG was determined by mixing equal parts of 11-mpBG-1 and 11-mpMG-1 and incubating for 10 min at 37°C with various amounts of AGT protein and then examining the formation of the dealkylated products by HPLC as described above. The AGT protein showed a clear preference for the repair of 11-mpBG (Fig. 3), which is consistent with our previous observations using unmodified 16-mers containing O6-alkylguanines indicating that b6G is preferentially repaired to m6G (Goodtzova et al., 1997; Pegg et al., 1998). A time course of repair of the mixture of 11-mpBG-1 and 11-mpMG-1 also confirmed that the benzylated 11-mpBG-1 was repaired faster than methylated 11-mpMG-1 (Fig. 4).

All these results suggest that the presence of the methylphosphonate linkages at the terminal nucleosides of the 11-mers does not reduce the ability to inactivate alkyltransferase protein. This was examined directly by mixing 11-MG with an equal amount of either 11-mpMG-1 or 11-mpMG-2 and examining the formation of products after incubating for 10 min at 37°C with various amounts of AGT protein (Fig. 5). There was no difference in the relative appearance of 11-mpG-1a and 11-mpG-1b (which are derived from 11-mpMG-1) and the appearance of the unmodified 11-mer, which is derived from 11-MG (Fig. 5, left panels). A similar result was obtained when 11-MG was mixed with 11-mpMG-2 (Fig. 5, right panels).

The inactivation of purified wild-type AGT by 11-mers containing either a m6G or a b6G residue was determined by incubating the protein with various concentrations of the potential inhibitor for 30 min and then calculating an ED50 value as described under Experimental Procedures. Both 11-mpBG-1 and 11-mpMG-2 were excellent inactivators with ED50 values of about 1.3 nM (Table 2). This value is similar to values for a 16-mer, 5′-(d(AACAGCCCATATb6GGCCC)-3′) (Table 2), and for the 11-mer 11-BG (Pegg et al., 1998) that do not contain terminal methylphosphonate linkages and confirms that these linkages do not interfere with the ability of short oligodeoxyribonucleotides to interact with wild-type AGT. The ED50 values are more than 2 orders of magnitude lower than the ED50 value for the free base b6G, which was 400 nM when tested under the same conditions (Table 2). This shows the advantage in interaction with the protein that is obtained by incorporation of the potential substrate into an oligodeoxyribonucleotide. The presence of b6G rather than m6G decreased the ED50 value by almost an order of magnitude since the ED50 value for 11-mpMG-1 was 10 nM (Table 2).
Inactivation of b6G-Resistant Mutant AGTs. A number of point mutations in the AGT sequence have been identified that lead to resistance to b6G (Xu-Welliver et al., 1998, 1999, 2000). A series of such mutant proteins were examined for their ability to be inactivated by either 11-mpBG-1 or the 16-mer 5’-d(AACAGCCATAtb6GGCCC)-3’ (Table 3). There was very little difference in the ED50 values for these two oligodeoxyribonucleotides with only mutants Y158H and P140K showing a slight preference for inactivation by the

Fig. 3. Competition for repair by wild-type AGT of m6G and b6G present in 11-mer methylphosphonates. Various amounts of the AGT protein as indicated were incubated with an equimolar mixture (1.4 nmol) of 11-mpBG-1 and 11-mpMG-1 for 10 min at 37°C, and the formation of 11-mpG was measured by HPLC as in Fig. 1. As in Figs. 1 and 2, 11-mpMG-1 and 11-mpG could be resolved into two peaks, which are designated as a and b. The substrate and product peaks are labeled as in Figs. 1 and 2.

Inactivation of b6G-Resistant Mutant AGTs. A number of point mutations in the AGT sequence have been identified that lead to resistance to b6G (Xu-Welliver et al., 1998, 1999, 2000). A series of such mutant proteins were examined for their ability to be inactivated by either 11-mpBG-1 or the

Fig. 4. The time course of repair of 11-mpMG-1 and 11-mpBG-1 by AGT. The AGT protein was incubated with either 11-mpMG-1 or 11-mpBG-1 as shown, and the extent of repair was determined by HPLC as in Figs. 1 and 2.

Fig. 5. Competition for repair by wild-type AGT of m6G present in 11-mers with and without terminal methylphosphonate linkages. Various amounts of the AGT protein as indicated were incubated with an equimolar mixture (1.4 nmol) of 11-mpMG-1 and 11-MG (left panels) or with an equimolar mixture (1.4 nmol) of 11-mpMG-2 and 11-MG (right panels) for 10 min at 37°C, and the formation of 11-mpG was measured by HPLC as in Fig. 2. The peak marked X represents an impurity in the 11-MG preparation that was not affected by AGT. As in Fig. 2, 11-mpMG-1, 11-mpMG-2, 11-mpG-1, and 11-mpG-2 were partially separated by the HPLC into the two isomers, which are designated as a and b, respectively. The substrate and product peaks are labeled as in Figs. 1 and 2.
16-mer. More importantly, both of these oligodeoxyribonucleotides were much more potent inactivators of the mutant forms of AGT than was b6G itself by factors that ranged from 900 to 80,000. The effect was greatest with the most b6G-resistant mutants, Y158H and P140K, where the advantage of incorporation of b6G into 11-mpBG-1 was more than 10,000-fold (Table 3). Even with mutant P140K, which is totally impervious to free base b6G (Xu-Welliver et al., 1998), the ED50 value for 11-mpBG-1 was only 0.15 μM.

**Inactivation of AGT in HT29 Cells.** The addition of 11-mpBG-1 to the culture medium of HT29 human colon carcinoma cells led to a loss of AGT activity in a dose- and time-dependent manner (Fig. 6). Approximately 7 μM 11-mpBG-1 was needed to achieve 50% inhibition in 4 h (Fig. 6A), but maximal inhibition was not reached until more than 12 h after exposure to 5 μM 11-mpBG-1 and complete inactivation was maintained for at least a further 60 h (Fig. 6B). These results suggest that uptake of the 11-mpBG-1 does occur but is relatively slow. Similar results (not shown) were obtained with 11-mpBG-2. No inactivation of AGT in HT29 cells was achieved with either the oligodeoxyribonucleotides 11-mpMG-1 (Fig. 6A) and 11-mpMG-2 (not shown), which contain m6G even at levels of 20 μM.

**Ability of 11-mpBG to Sensitize HT29 Cells to Killing by BCNU.** To test whether pretreatment with 11-mpBG-2 is able to enhance the cytotoxicity of BCNU, HT29 cells were preincubated with different concentrations of 11-mpBG-2, 11-mpMG-2, or 11-BG for 14 h. The cells were then treated with 40 μM BCNU for 2 h. This dose of BCNU alone did not alter survival of HT29 cells. As shown in Fig. 7, 11-mpBG-2 increased killing by BCNU with more than a 500-fold increase at 5 μM. In these experiments the medium that was added to the cells after the 2-h exposure to BCNU also contained the respective oligodeoxyribonucleotide, but this second addition was probably not necessary since a repetition of the study carried out at 5 μM 11-mpBG-2 without a second addition gave a similar increase in the cytotoxicity of BCNU (Fig. 7). Exposure to 11-mpBG-1 had a similar effect to that observed with 11-mpBG-2. Both 11-mpMG-2 and 11-BG had much less of an effect on BCNU toxicity with at most a 2-fold reduction in survival after 10 μM (results not shown).

**Discussion**

Recent studies of the crystal structure of the protein and its benzylated form have provided plausible models for the binding of free base b6G and of alkylated DNA substrates (Daniels et al., 2000; Wibley et al., 2000). These show that b6G is held at the active site largely by hydrophobic interactions with the benzyl group and some interactions with the purine base, whereas the interaction of AGT with alkylated DNA also makes a substantial number of contacts with the surrounding DNA and flips the alkylated base into the binding pocket. It is also probable that there is a small change in the structure of the protein upon binding DNA that facilitates the alkyl transfer reaction. These factors produce a much higher affinity of AGT for the DNA substrate, improve the chances of its binding in a productive manner for reaction, and thus accelerate the reaction. This explains well in molecular terms why short oligodeoxyribonucleotides are much more potent inactivators of AGT than the free base.
structure is very similar to that of helix-turn-helix-containing proteins such as Escherichia coli catabolite gene activator protein and Mu transposase for which definite structures of protein-DNA are available (Daniels et al., 2000; Wibley et al., 2000). The negatively charged DNA phosphodiester backbone matches a complementary positively charged surface on the AGT centered around Arg-128. It was therefore quite possible that the incorporation of methylphosphonate linkages into such short oligodeoxyribonucleotides was sufficient to block or distort binding to AGT. However, our results show clearly that this is not the case and that the ability of human AGT to interact with and repair O\(^6\)-alkylguanine adducts is not affected adversely by the presence of methylphosphonate linkages to the 5' and 3' terminal residues of 11-mers as tested here. It is also apparent from the studies shown in Figs. 1, 2, and 5 that the presence of different stereoisomers in these linkages does not alter the ability to serve as AGT substrates since all four of the possible isomer products are formed to the same extent. Since the AGT reaction is not catalytic and a single repair event irreversibly inactivates the protein, 11-mers such as 11-mpBG are therefore useful potential inactivators of AGT. This fact is supported by the results in Table 2, which shows that 11-mpBG has a much lower ED\(_{50}\) value for the inactivation of AGT than free b\(^6\)G, which is currently undergoing clinical trials that have indicated it is able to reduce AGT levels in tumors (Dolan et al., 1997; Pegg et al., 1998), which are confirmed by the competition experiments shown in Fig. 3, it was found that the presence of an O\(^6\)-benzyl group is to be able to inactivate AGT in HT29 cells (Fig. 6) and to abolish the resistance to BCNU provided by the AGT activity (Fig. 7). In addition to the greater potency for AGT inactivation, the presence of the benzyl group is likely to increase the uptake of the oligodeoxyribonucleotide into the cell. Based on many experiments using oligodeoxyribonucleotides as potential therapeutics, uptake is likely to be the limiting factor in the use of such compounds as drugs (Crooke, 1998; Stein, 1999; Lebedeva et al., 2000). When the results of addition of 11-mpBG to cells are compared with the results with b\(^6\)G as a free base, there is a striking loss of potency of the former, and the time taken to achieve maximal inactivation of AGT is much longer. With b\(^6\)G, which passes very well through mammalian cell membranes, inactivation of AGT in HT29 cells occurs within a few minutes, and the ED\(_{50}\) value for loss of AGT activity at 4 h is similar to the ED\(_{50}\) value for inactivation of AGT in vitro (Dolan et al., 1990, 1991). In contrast, with 11-mpBG the ED\(_{50}\) value for reduction of AGT in HT29 cells is 1000-fold greater than with purified enzyme, and exposure of the cells for at least 12 h was needed to achieve a maximal effect. 11-mpBG-1 and 11-mpBG-2 were equally effective in reducing AGT activity and abolishing BCNU resistance in HT29 cells showing that the different isomers do not have altered uptake.

It is probable that the methylphosphonate modifications do provide significant stabilization of the oligodeoxyribonucleotides by preventing exonuclease digestion since the inactivation of AGT by 11-mpBG in the HT29 cell cultures was maintained over a 72-h period (Fig. 6B) and 11-BG was ineffective in producing sensitization to BCNU. The use of methylphosphonates to provide metabolically stable antisense oligodeoxyribonucleotides with potentially useful pharmacological activity is now well established (Miller et al., 2000), but the mechanisms by which such oligodeoxyribonucleotides are taken up by the cells, interact with plasma components, and accumulate within a cell are poorly under-
Therapeutic Modulation of Alkyltransferase


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