Journal of Medicinal Chemistry

ARTICLE

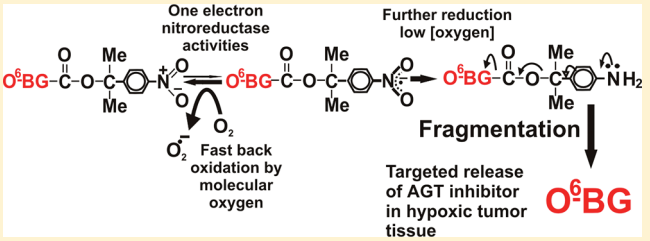
pubs.acs.org/jmc

4-Nitrobenzyloxycarbonyl Derivatives of O^6-Benzylguanine as Hypoxia-Activated Prodrug Inhibitors of O^6-Alkylguanine-DNA Alkyltransferase (AGT), Which Produces Resistance to Agents Targeting the *O*-6 Position of DNA Guanine

Rui Zhu, Mao-Chin Liu, Mei-Zhen Luo, Philip G. Penketh,* Raymond P. Baumann, Krishnamurthy Shyam, and Alan C. Sartorelli

Department of Pharmacology and Developmental Therapeutics Program, Cancer Center, Yale University School of Medicine, 333 Cedar Street, New Haven, Connecticut 06520-8066, United States

ABSTRACT: A series of 4-nitrobenzyloxycarbonyl prodrug derivatives of O^6-benzylguanine (O^6-BG), conceived as prodrugs of O^6-BG, an inhibitor of the resistance protein O^6-alkylguanine-DNA alkyltransferase (AGT), were synthesized and evaluated for their ability to undergo bioreductive activation by reductase enzymes under oxygen deficiency. Three agents of this class, 4-nitrobenzyl (6-(benzyloxy)-9*H*-purin-2-yl)carbamate (**1**) and its monomethyl (**2**) and gem-dimethyl analogues (**3**), were tested for activation by reductase enzyme systems under oxygen deficient conditions. Compound **3**, the most water-soluble of these agents, gave the highest yield of O^6-BG following reduction of the nitro group trigger. Compound **3** was also evaluated for its ability to sensitize 1,2-bis(methylsulfonyl)-1-(2-chloroethyl)-2-[(methylamino)carbonyl]hydrazine (laromustine)-resistant DU145 human prostate carcinoma cells, which express high levels of AGT, to the cytotoxic effects of this agent under normoxic and oxygen deficient conditions. While **3** had little or no effect on laromustine cytotoxicity under aerobic conditions, significant enhancement occurred under oxygen deficiency, providing evidence for the preferential release of the AGT inhibitor O^6-BG under hypoxia.



INTRODUCTION

Alkylating agents are among the most potent and widely used antineoplastic agents. A particularly important class of alkylating agents consists of compounds that methylate and chloroethylate the *O*-6 position of DNA guanine. This class includes as chloroethylating agents, the chloroethylnitrosoureas (e.g., carmustine or BCNU and lomustine or CCNU) and the sulfonylhydrazine prodrugs [e.g., 1,2-bis(methylsulfonyl)-1-(2-chloroethyl)-2-[(methylamino)carbonyl]hydrazine (laromustine, onrigin, cloretazine, VNP40101M, 101M)[1−6] and 1,2-bis(methylsulfonyl)-1-(2-chloroethyl)-2[[1-(4-nitrophenyl)ethoxy]carbonyl]hydrazine (KS119)][7,8] synthesized in our laboratory, and the methylating agents temozolomide, procarbazine, dacarbazine, and streptozotocin. O^6-Alkylation of guanine residues in DNA is repaired by the protein O^6-alkylguanine-DNA alkyltransferase (AGT), which restores the *O*-6 position of guanine in DNA to its native state by transferring the O^6-methyl or O^6-(2-chloroethyl) group to a cysteine residue in the active site of the protein.[9,10] There is no known biological acceptor that removes the alkyl moiety from this cysteine residue, so one AGT molecule can only repair a single alkyl lesion. The presence of increasing levels of AGT appears to correlate relatively well with elevated tumor and host tissue resistance to guanine O^6-methylating and chloroethylating agents.[11−13]

O^6-Benzylguanine (O^6-BG) is among the most effective known inhibitors of AGT;[14] this agent reacts with AGT to form *S*-benzylcysteine in the active site of the protein by acting as a substrate, depleting AGT and increasing the sensitivity of both tumor and host cells to agents that chloroethylate and methylate the *O*-6 position of guanine in DNA.[15−17] Relatively nontoxic levels of O^6-BG have been shown experimentally in both cell and animal tumor systems, as well as in patients, to deplete the AGT content of tumors. This action sensitizes cell systems in vitro and experimental tumors in vivo to BCNU; however, since AGT levels are also depleted by O^6-BG in normal cells, a concomitant sensitization of host tissues occurs, requiring a considerable reduction in the dosage of BCNU. Thus, in cancer patients pretreated with O^6-BG, the resulting myelosuppression requires an 80% decrease in BCNU dosage, resulting in an ineffective therapeutic level of the nitrosourea.[18,19] These findings imply that methodology producing a selective or preferential depletion of AGT in tumor tissue relative to normal tissue is required to circumvent AGT induced tumor resistance to guanine *O*-6 alkylating agents.

The oxygen deficient tumor cell fractions present in solid tumors, which we believe are major sites of tumor vulnerability, can be used to selectively activate prodrugs susceptible to reductive fragmentation to generate a potent inhibitor of AGT (Figure 1).[20,21] To this end, we have synthesized a series of

Received: August 19, 2011
Published: September 28, 2011

dx.doi.org/10.1021/jm201115f | *J. Med. Chem.* 2011, 54, 7720–7728

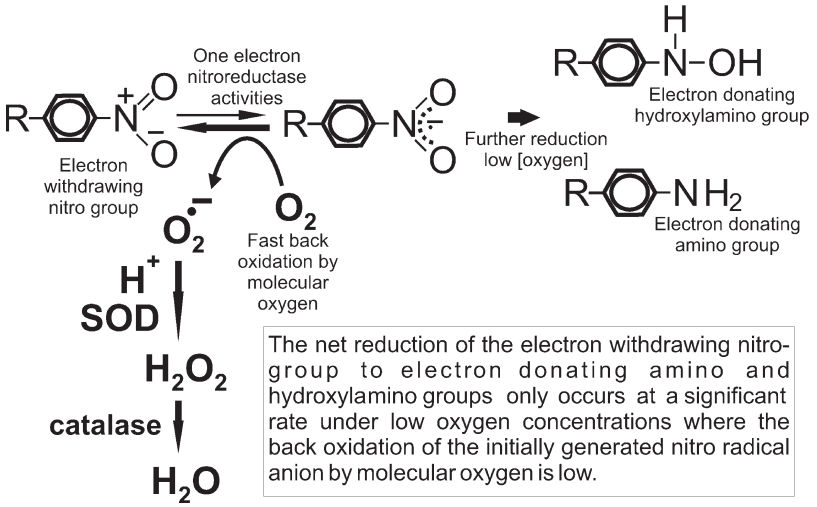
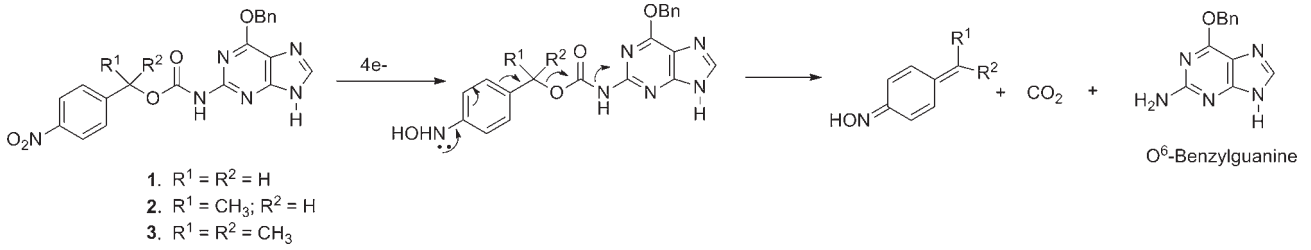


Figure 1. Influence of oxygen concentration on nitro reduction. Shown is the chemical mechanism underlying the inverse oxygen concentration dependency of net prodrug reduction that leads to molecular fragmentation and the release of the AGT inhibitor O^6-BG.

Scheme 1



agents containing a 4-nitrobenzyloxycarbonyl trigger/linker and have presented evidence to show selective/preferential activation under oxygen deficiency to generate the AGT inhibitor O^6-BG. These agents contain the nitrobenzyloxycarbonyl moiety in place of one of the protons in the 2-amino group of O^6-BG. Since a free amino group in O^6-BG is essential for potent AGT inhibitory activity, the proposed prodrug should be much less active than the parent compound (i.e., O^6-BG) until unmasking of the 2-amino group occurs following initial one electron nitro reduction in the oxygen deficient cellular fractions.[22,23] In the past, we have used the α-methyl-4-nitrobenzyloxycarbonyl moiety as a masking group in KS119, a highly selective sulfonylhydrazine prodrug that displayed excellent activity against oxygen deficient EMT6 murine mammary carcinoma cells with little or no activity against their normoxic counterparts.[7,8] The proposed activation mechanism is depicted in Scheme 1.

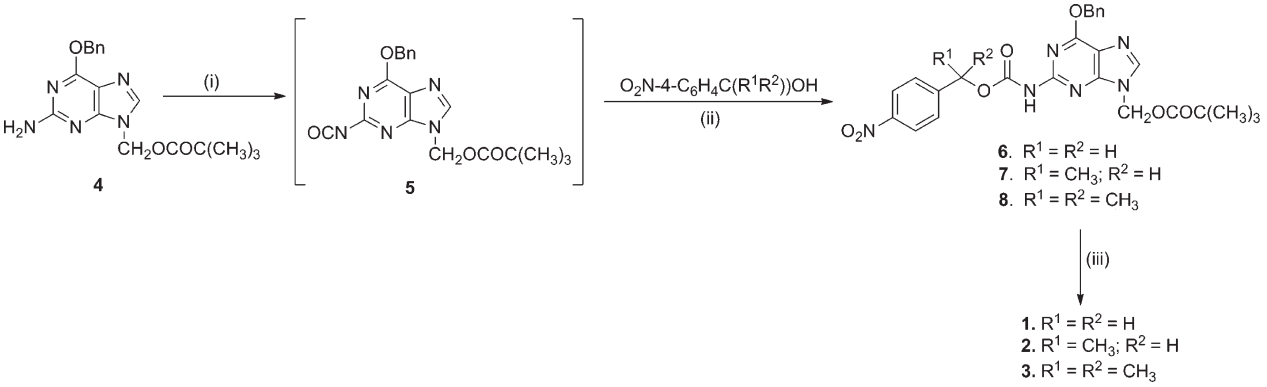
O^6-BG, once released, would be expected to enhance the kill of oxygen deficient tumor cells treated with agents such as laromustine, carmustine, and KS119; in addition, it is probable that the activated compounds will diffuse from the oxygen deficient fractions into adjacent oxygenated tumor cells. These phenomena should permit selective depletion of solid tumor AGT, thereby allowing a nearly full dosage of guanine O^6-methylating and chloroethylating agents to be used in the treatment of cancer patients, resulting in increases in the therapeutic efficacy of prodrugs containing agents targeting the *O*-6 position of DNA guanine in tumors that exhibit resistance to these compounds by virtue of the presence of relatively high levels of AGT.

CHEMISTRY

We have synthesized three 4-nitrobenzyloxycarbonyl derivatives of O^6-BG, i.e., the desmethyl (**1**), the monomethyl (**2**), and the gem-dimethyl (**3**) analogues, as shown in Scheme 2. These compounds differ structurally only in the degree of methylation of the benzylic carbon in the linker region. Thus, treatment of (2-amino-6-(benzyloxy)-9*H*-purin-9-yl)methyl pivalate (**4**)[22] with phosgene in the presence of pyridine in anhydrous dichloromethane gave the corresponding 2-isocyanatopurine (**5**) which, when reacted with the appropriate benzyl alcohol, gave the corresponding carbamate (**6**, **7**, or **8**).[24] The reaction time and yield of the carbamate depended greatly upon the structure of the benzyl alcohol. Thus, intermediate **5**, when reacted with 4-nitrobenzyl alcohol, α-methyl-4-nitrobenzyl alcohol, or α,α-dimethyl-4-nitrobenzyl alcohol for 2 h, gave carbamate yields of 89% (**6**), 63% (**7**), and 14% (**8**), respectively. However, with prolonged reaction times, compounds **7** and **8** were produced in yields of 81% (6 h) and 68% (40 h), respectively. It is obvious that steric hindrance to nucleophilic addition produced by the α-methyl and the α,α-dimethyl groups on the benzylic carbon is responsible for the observed decrease in reaction rates, with the more hindered α,α-dimethyl alcohol being the least reactive. Two methods were employed for the deprotection of the pivaloyloxymethyl group in compounds **6**, **7**, and **8**. Treatment of **7** and **8** with ethanolic sodium hydroxide, followed by neutralization with 10% acetic acid, gave **2** and **3** in 60% and 63% yields, respectively; however, this procedure resulted in the cleavage of the carbamate linkage in **6** to give the original starting

dx.doi.org/10.1021/jm201115f |*J. Med. Chem.* 2011, 54, 7720–7728

Scheme 2[a]



[a] Reagents and conditions: (i) phosgene CH_2Cl_2/pyridine, 0 °C to room temperature, 20 h; (ii) room temperature, 2−40 h; (iii) EtOH/1 M NaOH (6:1), 0 °C, 30 min, followed by neutralization with 10% acetic acid, or 0.1−1 M NH_3 in CH_3OH, 20−40 h.

Table 1. Yields of O^6-BG after Prodrug Reduction by Zn/EDTA[a]

prodrug	% molar O^6-BG yield
1	4.0 ± 1.1
2	35.4 ± 3.6
3	54.8 ± 3.2

[a] A solution of each agent in 50 mM Tris-HCl, 10 mM EDTA, pH 7.0 buffer was shaken with ∼4 mg/mL Zn dust and allowed to settle. It was then centrifuged and the supernatant analyzed by HPLC for O^6-BG. The results represent the mean of three independent experiments ± SEM.

material O^6-BG. The greater stability of the carbamate linkage in **7** and **8** relative to **6** may be not only due to the steric protection afforded by the C-methyl groups in compounds **7** and **8** but also because the electron-releasing inductive effect of the C-methyl groups rendered the carbonyl carbon less prone to attack by the hydroxide ion. Therefore, a milder method was devised for the selective removal of the pivaloyloxymethyl group in compound **6**. Thus, not only did 0.1 M ammonia in methanol afford an excellent yield of **1** from compound **6** but a similar deprotection procedure employing 1 M ammonia in methanol gave better yields of **2** and **3** from compounds **7** and **8**, respectively.

RESULTS

The synthesized agents were evaluated for their ability to generate O^6-BG by chemical (Table 1) and enzymatic activation (Tables 2 and 3), as well as by their ability to inhibit AGT (Table 4). The poor inhibition of AGT by the intact prodrugs **1**, **2**, and **3** compared to that by O^6-BG was essential for the success of this strategy. Therefore, we compared their relative abilities to inhibit AGT using a modification of previously described methodology.[25] This assay is based upon the ability of AGT to repair O^6-(2-chloroethyl)guanine and N^1,O^6-ethanoguanine adducts in DNA and thereby prevent the formation of 1-(N^3-deoxycytidinyl)-2-(N^1-deoxyguanosinyl)ethane cross-links. No significant inhibition of AGT was observed over a 30 min test period at 37 °C with **1**, **2**, and **3** up to the maximum concentration tested (50 μM), while O^6-BG produced about a 50% inhibition at $^1/_{500}$ of this concentration under the same conditions (Table 4).

The ability of the chemical and enzyme systems to reduce the nitro group was also evaluated using nitrobenzene as a model nitroaromatic. *N*-Phenylhydroxylamine and aniline, which are commercially available, were used as standards for quantifying the product yields by HPLC under the various reducing conditions employed. The chemical reduction of nitrobenzene (Table 5), as well as that of the three O^6-BG prodrugs, was carried out using zinc/EDTA. This methodology, which afforded the complete reduction of the nitro group in nitrobenzene to give aniline, resulted in the formation of O^6-BG from each of the three synthesized prodrugs, the rank order being **3** ≥ **2** > **1** (Table 1). Enzymatic reduction of nitrobenzene with xanthine/xanthine oxidase, as well as with NADPH:cytochrome P450 reductase, gave a mixture of partially reduced (*N*-phenylhydroxylamine) and fully reduced (aniline) products. Xanthine/xanthine oxidase gave a much higher yield of aniline on a molar basis than NADPH:cytochrome P450 reductase, which gave *N*-phenylhydroxylamine as the main product of nitroreduction (Table 5). The relative yields of reduction products had a bearing on the yield of O^6-BG from each prodrug. Thus, enzymatic reduction with xanthine/xanthine oxidase resulted in the formation of O^6-BG in every case when the three O^6-BG prodrugs were employed as substrates. The rank order of O^6-BG formation was unchanged from the chemical reduction, i.e., **3** ≥ **2** > **1** (Table 2). Enzymatic reduction by NADPH:cytochrome P450 reductase gave slightly different results. While all three parental prodrugs (∼20 μM) were reduced by this enzyme, only **3** gave a significant yield of O^6-BG (Table 3). In addition, a greater proportion of **3** was also reduced, implying that it was a better substrate than either **1** or **2** for NADPH:cytochrome P450 reductase (Table 3). As observed earlier, nitroreduction by this enzyme gave the hydroxylamine as the principal product, as evidenced by the observation that approximately 8 times as much *N*-phenylhydroxylamine as aniline was formed when nitrobenzene was employed as the substrate (Table 5). Compound **3** also generated very small quantities of O^6-BG under aerobic conditions. From these results we can infer that (a) the amino derivatives of **1**, **2**, and **3** readily fragment to form O^6-BG, with **3** giving the best yield, (b) the hydroxylamino derivative of **3** generates O^6-BG, while the hydroxylamino derivatives of **1** and **2** do not readily fragment to give O^6-BG, and (c) **3** has a greater tendency to form O^6-BG under aerobic conditions than **1** and **2**, possibly because of a low

Table 2. Production of O^6-BG from 1, 2, and 3 by Xanthine (X)/Xanthine Oxidase (XO) under Oxygen Deficient and Normoxic Conditions[a]

X/XO activation	**1**, normoxic	**1**, oxygen deficient	**2**, normoxic	**2**, oxygen deficient	**3**, normoxic	**3**, oxygen deficient
[prodrug] μM, T = 0	15.4 ± 0.4	18.8 ± 0.7	19.0 ± 0.2	21.4 ± 1.7	17.7 ± 0.6	20.8 ± 0.9
[O^6-BG] μM, T = 0	BLD	BLD	BLD	BLD	BLD	BLD
[prodrug] μM, T = 1	11.7 ± 2.1	13.4 ± 0.3	12.3 ± 2.7	8.0 ± 1.5	11.4 ± 2.7	4.0 ± 1.1
[O^6-BG] μM, T = 1	BLD	2.8 ± 0.5	BLD	11.6 ± 0.7	BLD	14.8 ± 0.3

[a] Production of O^6-BG from ~20 μM **1**, **2**, and **3** by 1 mM xanthine (X) and 0.16 units/mL xanthine oxidase (XO) was determined under oxygen deficient and normoxic conditions over a 1 h incubation period at 37 °C. The concentrations are given with ±SEM (N = 3) and were determined by HPLC. BLD = below the limit of detection (<0.05 μM).

Table 3. Production of O^6-BG from 1, 2, and 3 by NADPH:Cytochrome P450 Reductase under Oxygen Deficient and Normoxic Conditions[a]

P450 reductase activation	**1**, normoxic	**1**, oxygen deficient	**2**, normoxic	**2**, oxygen deficient	**3**, normoxic	**3**, oxygen deficient
[prodrug] μM, T = 0	18.2 ± 0.9	17.6 ± 0.5	19.8 ± 2.1	18.0 ± 1.1	20.3 ± 1.2	19.3 ± 0.9
[O^6-BG] μM, T = 0	BLD	BLD	BLD	BLD	BLD	BLD
[prodrug] μM, T = 1	15.5 ± 2.8	13.8 ± 2.6	18.8 ± 2.0	17.3 ± 1.6	18.2 ± 2.8	4.2 ± 1.0
[O^6-BG] μM, T = 1	BLD	BLD	BLD	0.1 ± 0.1	0.1 ± 0.1	13.9 ± 0.2

[a] Production of O^6-BG from ~20 μM **1**, **2**, and **3** by 1 mM NADPH and 1.04 units/mL NADPH:cytochrome P450 reductase was determined under oxygen deficient and normoxic conditions over a 1 h incubation period at 37 °C. The concentrations are given with ±SEM (N = 3) and were determined by HPLC. BLD = below the limit of detection (<0.05 μM).

Table 4. AGT Inactivation by Various Agents[a]

agent	percent AGT inactivation
O^6-BG	47 ± 3
1	<1
2	<1
3	<1

[a] Comparison of the levels of purified recombinant human AGT inactivation by 50 μM **1**, **2**, or **3** and 0.1 μM O^6-BG after a 30 min incubation at 37 °C by assaying the ability of each agent to repair substrate DNA containing O^6-(2-chloroethyl)guanine and N^1,O^6-ethanoguanine lesions relative to AGT incubated in the absence of agent. Values are the mean of three determinations (±SEM where applicable).

Table 5. Nitrobenzene Reducing Agent Dependent Aniline:Phenylhydroxylamine Reduction Product Ratios[a]

Zn/EDTA	xanthine/xanthine oxidase	NADPH:P450 reductase
1:0.0	1:0.7 ± 0.2	1:7.8 ± 1.9

[a] The influence of different reducing agents on the nitrobenzene reduction product ratio of aniline:N-phenylhydroxylamine. Nitrobenzene was reduced with either Zn dust/EDTA, xanthine/xanthine oxidase, or NADPH:cytochrome P450 reductase as described in the Experimental Section, and the reduction products were analyzed by HPLC. The concentration ratios are the mean of three deteriminations ± SEM.

level of reduction despite the presence of oxygen or because of the relief of steric strain or because of other possible routes of activation. However, this background rate was always relatively small when observed. Loss of initial material was observed with all three prodrugs under normoxic conditions with both enzyme systems, but this loss of parental agent was not associated with any significant O^6-BG production.

Reductive activation of the synthesized prodrugs was also studied using two tumor cell lines, the murine EMT6 mammary carcinoma and the human DU145 prostate carcinoma (Figure 2). While only **3** generated measurable quantities of O^6-BG in the case of the DU145 under conditions of oxygen deficiency, all three compounds generated O^6-BG in the rank order **3** > **2** > **1** in the case of the EMT6 carcinoma. Little or no aerobic activation was seen with these agents in both the enzymatic and cellular studies. As with the enzymatic studies, some loss of parental agent was observed with both of these cell lines under normoxic conditions. This normoxic loss was most marked with **1**; however, this loss did not result in the formation of O^6-BG. These findings collectively indicate that the linker used in **3** is superior to those employed in **1** and **2**. Furthermore, clonogenic assays using **3** at 40 μM sensitized DU145 prostate carcinoma cells to various concentrations of laromustine (50−200 μM) under conditions of oxygen deficiency but not under aerobic conditions (Figure 3).

DISCUSSION AND CONCLUSIONS

The three factors that would be expected to have an impact on the activation of the synthesized O^6-BG prodrugs are (a) the ease and extent of reduction of the nitro group, (b) the relative position of the nitro group with respect to the side chain, and (c) the ease of fragmentation of the C−O bond, where C is the benzylic carbon, once the nitro group is converted to the hydroxylamino or amino function. Although all three prodrugs have similar half-wave reduction potentials, as measured by differential pulse polarography (Table 6), **3** is a much better substrate for NADPH:cytochrome P450 reductase than **2** and **1**. The relative position of the nitro group with respect to the side chain is identical for all three prodrugs and therefore cannot explain the differences in the yields of O^6-BG generated from these agents. However, any factor that stabilizes the partial positive charge on the benzylic carbon in the transition state of

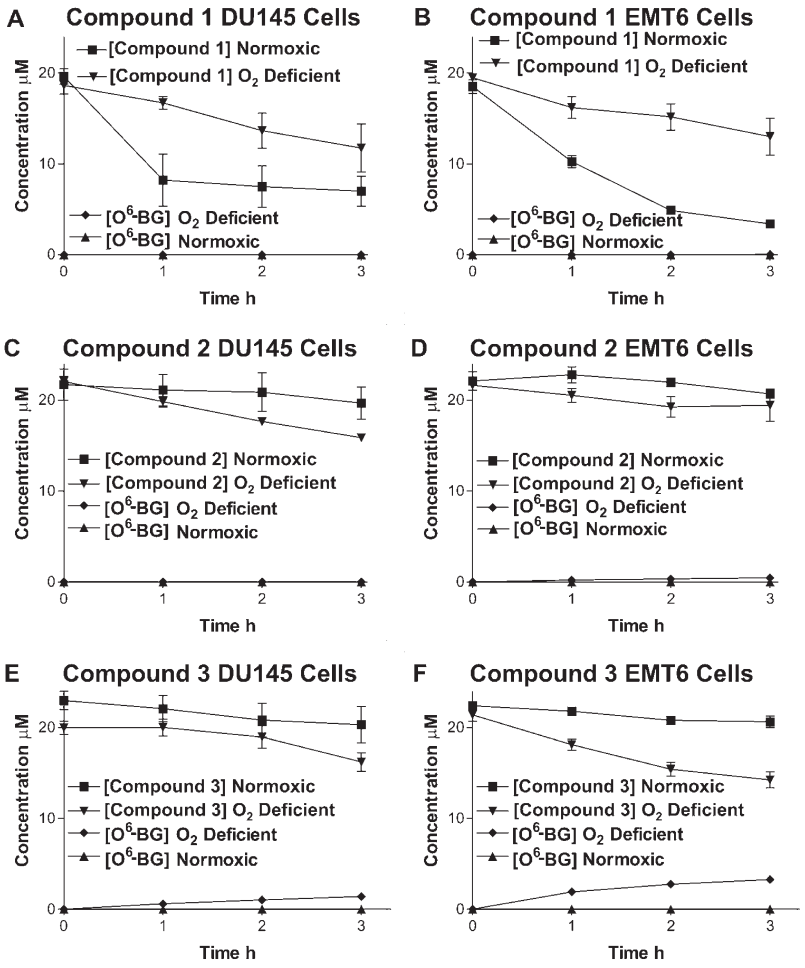


Figure 2. Loss of parental prodrugs (**1**, upper row; **2**, middle row; **3**, lower row) and the subsequent generation of O^6-BG over a 3 h 37 °C incubation period in medium in the presence of either 10^7/mL DU145 cells (left-hand column) or 10^7/mL EMT6 cells (right-hand column). In all panels, ■ represents the concentration of parental prodrug under normoxic conditions, ▼ represents the concentration of parental prodrug under oxygen deficient conditions, ▲ represents the concentration of generated O^6-BG under normoxic conditions, and ◆ represents the concentration of generated O^6-BG under oxygen deficient conditions. The plotted values represent the mean of three separate experiments ± SEM.

the fragmentation step, following reduction of the nitro group to a hydroxylamino or amino function, will facilitate the release of O^6-BG. In the reduction product of **3** the charge is stabilized not only by the electron-releasing mesomeric (resonance) effect of the hydroxylamino or amino group in the para position but also by the electron-releasing inductive effect of not one but two methyl groups. However, in the absence of the benzylic methyl group(s), a complete reduction of the nitro group to the highly electron-releasing amino function, relative to the more weakly electron releasing hydroxylamino group, appears to be needed for the release of O^6-BG from the compounds under study. Thus, **3** is not only more easily reduced by a key nitroreductase, i.e., NADPH:cytochrome P450 reductase, than **2** and **1** but also has the structural features necessary for a more facile fragmentation once the nitro group is reduced.

Thomson et al.[26] recently reported the evaluation of 4-nitrobenzyl and gem-dimethyl substituted 4-nitrobenzyl derivatives of 6-thioguanine as hypoxia-selective agents. While reduction of the nitro group did not result in the release of 6-thioguanine in the case of the desmethyl analogue, hypoxia-selective release of 6-thioguanine was indeed observed with the gem-disubstituted analogue. It is conceivable that factors similar to those described for the 6-thioguanine prodrugs are operative in this case.

In the enzymatic and cellular studies, loss of parental prodrug was also seen to various degrees under normoxic conditions; this occurred without the concomitant generation of O^6-BG. This loss could be due to the reaction of the prodrugs with reactive oxygen species generated as a consequence of prodrug redox cycling under normoxic conditions and in the case of xanthine oxidase directly from the oxidation of xanthine with oxygen. In the presence of cells, additional metabolic routes not resulting in O^6-BG formation may contribute to this normoxic loss. The cellular sensitization studies which indicated pronounced potentiation of the toxicity of laromustine only under oxygen deficient conditions strongly support the lack of formation of AGT inhibitory agents of any type under normoxic conditions.

Surprisingly, the rank order of water solubility for the three prodrugs is **3** > **2** > **1**. The absence of a chiral center also makes **3** a more attractive candidate for further possible development as a pharmaceutical than **2**. Thus, **3**, conceived as a hypoxia-activated prodrug of O^6-BG, appears to be a promising new agent with the following desirable properties:

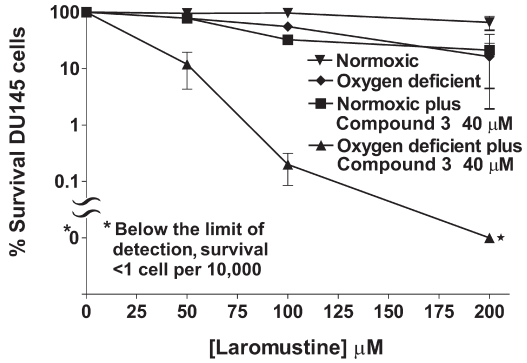


Figure 3. Sensitization of DU145 human prostate carcinoma cells to laromustine by **3** induced inhibition of AGT in clonogenic experiments. DU145 AGT containing cells in confluent monolayers were exposed to 40 μM **3** for 6 h and then treated with graded concentrations of laromustine for a total of 24 h under conditions of normoxia (■) or oxygen deficiency (▲). The cytotoxicity of laromustine alone under conditions of normoxia (▼) or oxygen deficiency (◆) is also displayed. After treatment, cells were detached by trypsinization and survival was quantified using clonogenicity. The *Y* axis indicates the percent survival and the *X* axis the concentration of laromustine employed. Oxygen was removed from test samples enzymatically in the presence of 2 units/mL glucose oxidase, 120 units/mL catalase, and 25 mM glucose during incubation and plating of cells to measure cloning efficiency. All points are the result of at least three independent experimental determinations ± SEM.

Table 6. Polarographic Half-Wave Reduction Potentials of Synthesized Prodrugs

prodrug	1	2	3
polarographic half-wave reduction potential, pH 7.0	−463 mV[a]	−447 mV[a]	−449 mV[a]

[a] The supporting electrolyte contained 10% DMSO in all cases for consistency. The DMSO was required to aid in the dissolution of **1**. The presence of 10% DMSO resulted in the measured polarographic half-wave reduction potentials being shifted approximately 50 mV more negative than in its absence.

(a) The intact **3** prodrug is >500-fold less effective as an AGT inhibitor than O^6-BG.
(b) Reduction of **3** by common nitroreductases such as NADPH:cytochrome P450 reductase and xanthine/xanthine oxidase under oxygen-deficient conditions results in the generation of O^6-BG.
(c) The activation rate for **3** under normoxic conditions is low or below the limit of detection under the conditions and incubation times examined to date.
(d) Once O^6-BG is released in the oxygen deficient compartment, it is conceivable that the inhibitor will diffuse into the surrounding oxygenated tumor cells and sensitize them to the cytodestructive effects of guanine O-6 alkylating agents (i.e., will exert a bystander effect). However, very extensive release and equilibration around host tissues could be problematic, since it would attenuate the targeting effect.
(e) The trigger/linker employed (i.e., the gem-dimethyl 4-nitrobenzyloxycarbonyl moiety) ensures that release of O^6-BG in normal tissues will be relatively low relative to oxygen deficient areas of solid tumors. This should minimize the undesirable effects that result from treatment with a guanine *O*-6 alkylating agent following nonselective (global) ablation of AGT.

EXPERIMENTAL SECTION

All chemicals, solvents, reagents, and enzymes were purchased from Sigma-Aldrich (St. Louis, MO) and used without further purification with the exceptions of laromustine which was provided by Vion Pharmaceuticals Inc. (New Haven, CT), the fluorescent dye Hoechst 33258 which was purchased from Molecular Probes, Inc. (Eugene, OR.), and purified recombinant human AGT which was a kind gift from Dr. Joann Sweasy (Yale Medical School, New Haven, CT).

Melting points were determined on a Thomas-Hoover Unimelt melting point apparatus and are uncorrected. NMR spectra were recorded on a Varian EM-390 (90 MHz) or Bruker Avance DRX-400 (400 MHz) NMR spectrometer with tetramethylsilane as an internal standard. Mass spectra were recorded on a VG-ZAB-SE mass spectrometer in the fast atom bombardment mode (glycerol matrix). Column chromatography was conducted with Merck silica gel 60, 230−400 mesh. Thin layer chromatography was performed on EM precoated silica gel sheets containing a fluorescent indicator. High resolution mass spectra were obtained at the W. M. Keck Foundation Biotechnology Resource Laboratory at Yale University. The purity of the tested compounds was determined by analytical HPLC, using a Beckman System Gold HPLC system, comprising a 168 UV/vis detector, a 502 autosampler, and a 127P solvent module monitoring at 280 nm with a 40 nm bandwidth. The purity of the tested compounds was >95% in each and every case.

(6-(Benzyloxy)-2-((((4-nitrobenzyl)oxy)carbonyl)amino)-9*H*-purin-9-yl)methyl Pivalate (6). To a stirred solution of (2-amino-6-(benzyloxy)-9*H*-purin-9-yl)methyl pivalate (**4**) (1.06 g, 3 mmol) and dry pyridine (3 mL) in 70 mL of anhydrous dichloromethane was added a 20% solution of phosgene in toluene (1.5 mL, 3.4 mmol) dropwise at 0−5 °C. The mixture was stirred overnight, while the ice bath was allowed to warm to room temperature. A solution of 4-nitrobenzyl alcohol (0.46 g, 3 mmol) in anhydrous dichloromethane (10 mL) was added, and the reaction mixture was stirred at room temperature for 2 h. It was then evaporated with 5 g of silica gel to dryness in vacuo and the residue was purified by column chromatography (silica gel, dichloromethane−ethanol, 30:1) to give 1.42 g (89%) of the title compound as a white solid: mp 146−148 °C; ^{1}H NMR ($CDCl_3$, 90 MHz) δ 10.20 (s, D_2O exchangeable,1H), 8.24 (d, J = 8.5 Hz, 2H), 8.03 (s, 1H), 7.33−7.61 (m, 7H), 6.08 (s, 2H), 5.62 (s, 2H), 5.37 (s, 2H), 1.16 (s, 9H); MS m/z 535 $[M + H]^+$.

Compounds **7** and **8** were synthesized using procedures analogous to the one described above except that for compound **7**, after the addition of 1-(4-nitrophenyl)ethanol, the reaction mixture was stirred at room temperature for 6 h, and for compound **8**, the reaction mixture was stirred at room temperature for 40 h after the addition of 2-(4-nitrophenyl)-propan-2-ol.

(6-(Benzyloxy)-2-(((1-(4-nitrophenyl)ethoxy)carbonyl)-amino)-9*H*-purin-9-yl)methyl Pivalate (7). Compound **7** was obtained in an 81% yield as a white solid: mp 140−142 °C; ^{1}H NMR ($CDCl_3$, 90 MHz) δ 10.34 (s, D_2O exchangeable, 1H), 8.26 (d, J = 9 Hz, 2H), 8.04 (s, 1H), 7.54 (d, J = 9 Hz, 2H), 7.34−7.41 (m, 5H), 6.07 (s, 2 H), 6.06 (q, J = 6.6 Hz, 1H), 5.61 (s, 2H), 1.65 (d, J = 6.6 Hz, 3H), 1.15 (s, 9H); MS m/z 549 $[M + H]^+$.

(6-(Benzyloxy)-2-((((2-(4-nitrophenyl)propan-2-yl)oxy)-carbonyl)amino)-9*H*-purin-9-yl)methyl Pivalate (8). Compound **8** was obtained in a 68% yield as a white solid: mp 92−94 °C; ^{1}H NMR ($CDCl_3$, 90 MHz) δ 10.20 (s, D_2O exchangeable, 1H), 8.19 (d, J = 9 Hz, 2H), 7.97 (s, 1H), 7.45 (d, J = 9 Hz, 2H), 7.29−7.45 (m, 5H), 6.02 (s, 2 H), 5.58 (s, 2H), 1.87 (s, 6H), 1.13 (s, 9H); MS m/z 563 $[M + H]^+$.

4-Nitrobenzyl (6-(Benzyloxy)-9*H*-purin-2-yl)carbamate (1). A suspension of **6** (0.53 g, 1 mmol) in 0.1 M ammonia in methanol (40 mL) was stirred at room temperature until TLC showed a complete disappearance of the starting material (approximately 40 h). The white solid that was formed was collected by filtration after cooling the reaction mixture in an ice−water bath for 1 h, washed with methanol and ether, and dried to give 0.36 g (85%) of the target molecule: mp 233−235 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 13.24 (s, 1H), 10.51 (s, 1H), 8.29−8.21 (m, 3H), 7.74 (d, J = 8.7 Hz, 2H), 7.61−7.49 (m, 2H), 7.46−7.26 (m, 3H), 5.59 (s, 2H), 5.34 (s, 2H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 151.8, 151.7, 147.0, 144.7, 136.3, 128.9, 128.5, 128.3, 123.6, 67.5, 64.5. HRMS, calculated for $C_{20}H_{16}N_6O_5$, m/z, 421.1255 [(M + H)$^+$]; found, 421.1257.

1-(4-Nitrophenyl)ethyl (6-(Benzyloxy)-9*H*-purin-2-yl)carbamate (2). *Method 1.* To an ice-cooled solution of **7** (1.2 g, 2.2 mmol) in ethanol (60 mL) was added an ice-cooled solution of 1 M sodium hydroxide (10 mL), and the mixture was stirred at 0 °C for 30 min. The reaction mixture was neutralized with 10% acetic acid and was evaporated with 5.8 g of silica gel to dryness in vacuo. The residue was chromatographed on a silica gel column (60 Å, 70−230 mesh) and eluted with CH_2Cl_2/EtOH, 20:1 (v/v), to give 0.57 g (60%) of the title compound as a white solid.

Method 2. Compound **2** was also synthesized using a procedure analogous to the one described for **1** except that 1 M ammonia in methanol was used as a base in lieu of 0.1 M ammonia and was obtained as a white solid: yield, 79%; mp 134−135 °C (dec); ^{1}H NMR (400 MHz, DMSO-d_6) δ 13.22 (s, 1H), 10.44 (s, 1H), 8.42−8.12 (m, 3H), 7.76 (d, J = 8.7 Hz, 2H), 7.56 (dd, J = 7.9, 1.5 Hz, 2H), 7.46−7.26 (m, 3H), 5.96 (q, J = 6.6 Hz, 1H), 5.59 (s, 2H), 1.56 (d, J = 6.6 Hz, 3H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 151.7, 151.3, 146.9, 136.3, 128.9, 128.5, 128.3, 126.9, 123.7, 71.2, 67.5, 22.5. HRMS, calculated for $C_{21}H_{18}N_6O_5$, m/z, 435.1411 [(M + H)$^+$]; found, 435.1414.

2-(4-Nitrophenyl)propan-2-yl (6-(Benzyloxy)-9*H*-purin-2-yl)carbamate (3). Compound **3** was synthesized using procedures analogous to the ones described for **2** and was obtained as a white solid: yield, 63% (method 1), 82% (method 2); mp 246 °C (dec); ^{1}H NMR (400 MHz, DMSO-d_6) δ 13.21 (s, 1H), 10.36 (s, 1H), 8.34−8.19 (m, 3H), 7.79 (dd, J = 9.2, 2.1 Hz, 2H), 7.57 (dd, J = 7.8, 1.4 Hz, 2H), 7.46−7.27 (m, 3H), 5.61 (s, 2H), 1.84 (s, 6H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 154.1, 151.8, 150.5, 146.3, 136.3, 128.9, 128.4, 128.3, 125.8, 123.5, 80.2, 67.5, 28.4. HRMS, calculated for $C_{22}H_{20}N_6O_5$, m/z, 449.1568 [(M + H)$^+$]; found, 449.1571.

AGT Inactivation Assay. AGT substrate DNA was prepared by treating L1210 DNA (175 μg/mL) in 10 mM Tris-HCl buffer (pH ~7.4) with 0.2 mM 1,2-bis(methylsulfonyl)-1-(2-chloroethyl)hydrazine for 3 min at 37 °C. This substrate DNA was then stored at 0 °C until used. A 10 μL aliquot of substrate DNA containing O^6-(2-chloroethyl)guanine and N^1,O^6-ethanoguanine lesions (~50 fmol) was incubated for 30 min at 37 °C with 40 fmol of purified recombinant human AGT which was pretreated (or not) with various inhibitors for 30 min at 37 °C. This assay gives a moderately linear decrease in these DNA lesions in proportion to the level of AGT activity until the lesions have been largely titrated. The level of remaining cross-link precursors in this mixture was then determined and the level of inhibition of AGT calculated from this value. The procedure is as follows. About 40 fmol of AGT in AGT stabilization buffer of the following composition was used: 20 mM Tris-HCl, 1 mM EDTA, 1 mg/mL of bovine serum albumin, 0.1 mM dithiothreitol, and 2 μg/mL of L1210 DNA, pH 7.4 (the L1210 DNA greatly contributes to the AGT stability but does not add significantly to the total DNA in the final assay). This was mixed with an equal volume of 2× concentration of inhibitor in an equivalent amount of buffer to give a mixture of the desired inhibitor and AGT concentration. This mixture was then incubated for 30 min at 37 °C. Then an amount of 10 μL was added to 10 μL of substrate DNA, and the mixture was incubated for a further 30 min at 37 °C to allow the residual AGT activity to repair the DNA. This mixture was then diluted 10-fold with 5 mM Tris-HCl, 1 mM EDTA, and 1 mM NaN_3, pH 8.0 buffer, then incubated at 50 °C for 2−3 h to allow the unrepaired cross-link precursors to progress to cross-links. The level of cross-linking was then measured using a previously described fluorescence methodology.[6] This involved dilution of the sample to a volume of 1.5 mL with 5 mM Tris-HCl, 1.0 mM EDTA, and 1.0 mM NaN_3 buffer, pH 8.0, containing 0.1 μg/mL of Hoechst H33258 fluorescent dye, and measuring the fluorescence using a Hoefer Scientific Instruments TKO 100 fluorometer. The mixture was then heated in a 100 °C hot-block for 5 min and then plunged into a water bath at room temperature for 5 min, and the fluorescence measured again. The percentage of DNA molecules that were cross-linked (i.e., containing at least one cross-link per molecule) was then calculated from the change in fluorescence.

Polarographic Determination of Half-Wave Reduction Potentials. Differential pulse polarography (DPP) voltagrams of the synthesized agents were obtained using a pH 7.0 buffer composed of 100 mM potassium chloride and 50 mM potassium phosphate as the supporting electrolyte containing 10% DMSO by volume. Compounds were added as 0.5% of a 10 mM solution in DMSO to give final concentrations of 50 μM. The samples were purged with nitrogen to remove dissolved oxygen, and DPP voltammograms were obtained using a Princeton Applied Research electrochemical trace analyzer model 394, linked to a model 303A static mercury drop electrode (Princeton Applied Research, Oak Ridge, TN, U.S.). Scans were performed from 0 to −900 mV (2 mV/s) using a platinum counter electrode versus an Ag/AgCl reference electrode (saturated KCl/AgCl electolyte). A pulse amplitude of 50 mV was used, and the polarographic half-wave reduction potential $E_{1/2}$ was calculated from the peak current potential (E_P) according to the following equation: $E_{1/2} = E_P$ − pulse amplitude/2.[27]

Determination by HPLC. HPLC measurements of the concentrations of nitrobenzene, *N*-phenylhydroxylamine, aniline, **1**, **2**, and **3** were performed using a Beckman 127P solvent module and a Beckman 168 UV/vis detector (Beckman, Fullerton, CA, U.S.). A standard curve for estimation of total agent was established, and a linear relationship between concentration and the area under the curve was found in all cases. Samples in 50% v/v acetonitrile were separated on a 5 μm, 220 mm × 4.6 mm Applied Biosystems RP-18 C-18 reverse phase column (Applied Biosystems, Carlsbad, CA, U.S.) by elution with 34% acetonitrile in buffer (0.03 M KH_2PO_4, 1.0 mM NaN_3, pH 5.4) for 5 min followed by a 34−70% acetonitrile linear gradient in buffer, at a flow rate of 0.6 mL/min from 5 to 35 min. After this point the concentration of acetonitrile was maintained at this level for 5 min and then returned to the starting concentration over an additional 5 min. Absorbance was monitored at 280 nm using a Beckman 168 UV/vis detector.

Reduction of Nitrobenzene with Zn/EDTA. A solution of nitrobenzene at 1 mM in 50 mM Tris-HCl, 10 mM EDTA, pH 7.0, was quickly shaken with approximately 4 mg/mL Zn dust and allowed to settle at room temperature, then centrifuged at 10000*g* for 4 min. The supernatant was then diluted 10-fold with 30 mM potassium phosphate buffer, pH 5.4, and analyzed by HPLC.

Reduction of Nitrobenzene with Xanthine/Xanthine Oxidase. A solution of nitrobenzene at 100 μM in 100 mM potassium phosphate buffer, pH 7.4, containing 2.5 mM xanthine added as 1% of a 250 mM solution in 1 M NaOH was purged with nitrogen and 0.16 units/mL xanthine oxidase (Sigma, bovine milk, X4500-5UN) added and the mixture incubated at 37 °C. Samples were taken at 2 h and analyzed by HPLC for aniline, nitrobenzene, and *N*-phenylhydroxylamine. Nitrobenzene, *N*-phenylhydroxylamine, and aniline eluted at approximately 20, 10, and 8 min, respectively.

Reduction of Nitrobenzene with NADPH:Cytochrome P450 Reductase. A solution of nitrobenzene at 100 μM in 100 mM

potassium phosphate buffer, pH 7.85, the pH optimum for NADPH: cytochrome P450 reductase (Sigma, human recombinant C8113), containing 10 mM NADPH, 10 mM glucose, 2 units/mL glucose oxidase, 120 units/mL catalase, and 4 units/mL of NADPH:cytochrome P450 reductase, added after 5 min to allow the oxygen to be depleted, was incubated for 2 h in a sealed tube at 37 °C. Then the mixture was analyzed by HPLC for aniline, nitrobenzene, and *N*-phenylhydroxylamine.

Reduction of 1, 2, and 3 with Zn/EDTA. A solution of each agent at 50 μM in 50 mM Tris-HCl, 10 mM EDTA, pH 7.0, was quickly shaken with approximately 4 mg/mL Zn dust and allowed to settle at room temperature, then centrifuged at 10000*g* for 4 min. The supernatant was then analyzed by HPLC.

Reduction of 1, 2, and 3 by NADPH:Cytochrome P450 Reductase and Xanthine Oxidase. In these experiments, oxygen deficiency was enzymatically generated as previously described.[28] Briefly, glucose/glucose oxidase was used to rapidly consume the available free oxygen and catalase was employed to remove the generated hydrogen peroxide. In the absence of other components, this system itself does not measurably reduce **1**, **2**, and **3** in the time frames employed in these experiments. The reaction mixtures in a total volume of 0.5 mL were as follows: 100 mM potassium phosphate buffer, pH 7.4, containing 10 mM glucose, 5 μL of a solution of 200 units/mL glucose oxidase and 12 000 units/mL catalase, 20 μM test agent added as a 100× solution in DMSO plus either 5 μL of 104 units/mL NADPH: cytochrome P450 reductase plus 1 mM NADPH or 0.16 units/mL xanthine oxidase plus 1 mM xanthine. The aerobic reaction was identical except that the glucose needed for the oxygen deficiency system to operate was omitted. Furthermore, the aerobic reaction volumes were scaled up 10-fold and the mixture was incubated as a shallow layer with shaking in sealed 25 cm^2 plastic culture flasks to ensure that full air saturation was maintained and evaporation prevented. The reaction mixtures were incubated at 37 °C, and samples were removed at 0 and 1 h after initiation, mixed with an equal volume of CH_3CN, and centrifuged at 10000*g* for 10 min to sediment any precipitated protein. The supernatant was then analyzed as described above for the remaining agent and reduction products using HPLC.

Cellular Activation of 1, 2, and 3 under Normoxic and Oxygen Deficient Conditions. Experiments, following the time course of prodrug loss and O^6-BG generation when ~20 μM **1**, **2**, or **3** was incubated with either EMT6 or DU145 cells, were conducted as follows. Cells at 10^7/mL in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum (EMT6) or in α-minimum essential medium with 10% fetal bovine serum (DU145) containing 10 mM glucose were shaken as cell suspensions as shallow layers (5 mL) in 25 cm^2 plastic culture flasks for the normoxic incubations. For the oxygen deficient condition incubations, the cells were incubated in sealed microfuge tubes with an additional 10 mM glucose and 2 units/mL glucose oxidase plus 120 units/mL catalase as additional components. Samples were removed at 0, 1, 2, and 3 h after the start of the incubations and mixed with an equal volume of acetonitrile to lyze the cells, precipitate the macromolecules, and extract the remaining prodrug and generated O^6-BG. The mixture was allowed to stand for 15 min at room temperature, centrifuged at 10000*g* for 10 min and the supernatant analyzed by HPLC for parental prodrug and O^6-BG.

Cytotoxicity Studies. Cell survival (clonogenic) assays were performed using a previously described method.[3] The 25 cm^2 plastic tissue culture flasks were seeded with 2.5×10^5 cells each and 3 days later cells were pretreated for 6 h in the presence of **3** prior to the addition of laromustine dissolved in 10 mL of medium for 24 h at 37 °C. All agents were initially dissolved in DMSO and then diluted to the required concentration. For oxygen-deficient conditions, cells were incubated with laromustine in the presence of 2 units/mL glucose oxidase (Sigma G6641), 120 units/mL catalase (Sigma, C1345) in high glucose DMEM (Invitrogen).[28,29] Flasks were flushed with nitrogen for 10 s and the caps screwed on tightly. This action facilitated oxygen depletion of the medium by glucose oxidase through removal of residual oxygen containing air and denial of the entry of additional air. After treatment, monolayers were rinsed with phosphate buffered saline, and cells were detached by trypsinization, suspended in culture medium, and counted and sequential cell dilutions were plated in duplicate into six-well plates at a density of 1×10^2, 1×10^3, and 1×10^4 cells per well. At 10−14 days later, colonies were fixed, stained with 0.25% crystal violet in 80% methanol, and quantified. DMSO concentrations were ≤0.05% and nontoxic. Cells under aerobic conditions were treated under similar conditions and cytotoxic agent concentrations but in unsealed flasks without glucose oxidase and catalase. Cells were then washed, harvested by trypsinization, and assayed for survival using a clonogenic assay described previously.[3,30,31] In the absence of cells, no measurable direct metabolism of **3** or of laromustine was detected in the presence of the glucose oxidase and catalase enzyme components of the oxygen deficiency generating system.[28]

AUTHOR INFORMATION

Corresponding Author

*Telephone: (203) 785-4524. Fax: (203) 737-2045. E-mail: philip.penketh@gmail.com.

ACKNOWLEDGMENT

This work was supported in part by U.S. Public Health Service Grants CA090671, CA122112, and CA129186 from the National Cancer Institute and a grant from the National Foundation for Cancer Research.

ABBREVIATIONS USED

O^6-BG, O^6-benzylguanine; AGT, O^6-alkylguanine-DNA alkyltransferase

REFERENCES

(1) Shyam, K.; Penketh, P. G.; Loomis, R. H.; Rose, W. C.; Sartorelli, A. C. Antitumor 2-(aminocarbonyl)-1,2-bis(methylsulfonyl)-1-(2-chloroethyl)hydrazines. *J. Med. Chem.* **1996**, *39*, 796–801.

(2) Finch, R. A.; Shyam, K.; Penketh, P. G.; Sartorelli, A. C. 1, 2-Bis(methylsulfonyl)-1-(2-chloroethyl)-2-(methylamino)carbonylhydrazine (101M): a novel sulfonylhydrazine prodrug with broad-spectrum antineoplastic activity. *Cancer Res.* **2001**, *61*, 3033–3038.

(3) Baumann, R. P.; Seow, H. A.; Shyam, K.; Penketh, P. G.; Sartorelli, A. C. The antineoplastic efficacy of the prodrug cloretazine is produced by the synergistic interaction of carbamoylating and alkylating products of its activation. *Oncol. Res.* **2005**, *15*, 313–325.

(4) Ishiguro, K.; Shyam, K.; Penketh, P. G.; Sartorelli, A. C. Role of O^6-alkylguanine-DNA alkyltransferase in the cytotoxic activity of cloretazine. *Mol. Cancer Ther.* **2005**, *4*, 1755–1763.

(5) Ishiguro, K.; Seow, H. A.; Penketh, P. G.; Sartorelli, A. C. Mode of action of the chloroethylating and carbamoylating moiety of the prodrug cloretazine. *Mol. Cancer Ther.* **2006**, *5*, 969–976. PMCID: PMC2680221.

(6) Penketh, P. G.; Baumann, R. P.; Ishiguro, K.; Shyam, K.; Seow, H. A.; Sartorelli, A. C. Lethality to leukemia cell lines of DNA interstrand cross-links generated by cloretazine derived alkylating species. *Leuk. Res.* **2008**, *32*, 1546–1553. PMCID: PMC2888535.

(7) Shyam, K.; Penketh, P. G.; Shapiro, M.; Belcourt, M. F.; Loomis, R. H.; Rockwell, S.; Sartorelli, A. C. Hypoxia-selective nitrobenzyloxycarbonyl derivatives of 1,2-bis(methylsulfonyl)-1-(2-chloroethyl)hydrazine. *J. Med. Chem.* **1999**, *42*, 941–946.

(8) Seow, H. A.; Penketh, P. G.; Shyam, K.; Rockwell, S.; Sartorelli, A. C. 1,2-Bis(methylsulfonyl)-1-(2-chloroethyl)-2[[1-(4-nitrophenyl)-ethoxy]carbonyl]hydrazine (KS119): an anticancer agents targeting

hypoxic cells. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 9282–9287. PMCID: PMC1166587.

(9) Pegg, A. E. Mammalian O^6-alkylguanine-DNA alkyltransferase: regulation and importance in response to alkylating carcinogenic and therapeutic agents. *Cancer Res.* **1990**, *50*, 6119–6129.

(10) Brent, T. P.; Remack, J. S. Formation of covalent complexes between human O^6-alkylguanine-DNA alkyltransferase and BCNU-treated defined length oligodeoxynucleotides. *Nucleic Acids Res.* **1988**, *16*, 6779–6788.

(11) Baumann, R. P.; Shyam, K.; Penketh, P. G.; Remack, J.; Brent, T. P.; Sartorelli, A. C. 1,2-Bis(methylsulfonyl)-1-(2-chloroethyl)-2 mac_hyphen;[(methylamino)carbonyl]hydrazine (VNP40101M): II. Role of O^6-alkylguanine DNA alkyltransferase in cytotoxicity. *Cancer Chemother. Pharmacol.* **2004**, *53*, 288–295.

(12) Gerson, S. L.; Willson, J. K. O^6-Alkylguanine-DNA alkyltransferase. A target for the modulation of drug resistance. *Hematol. Oncol. Clin. North Am.* **1995**, *9*, 431–450.

(13) Schold, S., Jr.; Brent, T. P.; von Hofe, E.; Friedman, H. S.; Mitra, S.; Bigner, D. D.; Swenberg, J. A.; Kleihues, P. O^6-Alkylguanine-DNA alkyltransferase and sensitivity to procarbazine in human brain-tumor xenografts. *J. Neurosurg.* **1989**, *70*, 573–577.

(14) Dolan, M. E.; Moschel, R. C.; Pegg, A. E. Depletion of mammalian O^6-alkylguanine-DNA alkyltransferase activity by O^6-benzylguanine provides a means to evaluate the role of this protein in protection against carcinogenic and therapeutic alkylating agents. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 5368–5372.

(15) Dolan, M. E.; Mitchell, R. B.; Mummert, C.; Moschel, R. C.; Pegg, A. E. Effect of O^6-benzylguanine analogues on sensitivity of human tumor cells to the cytotoxic effects of alkylating agents. *Cancer Res.* **1991**, *51*, 3367–3372.

(16) Baer, J. C.; Freeman, A. A.; Newlands, E. S.; Watson, A. J.; Rafferty, J. A.; Margison, G. P. Depletion of O^6-alkylguanine-DNA alkyltransferase correlates with potentiation of temozolomide and CCNU toxicity in human tumor cells. *Br. J. Cancer* **1993**, *67*, 1299–1302.

(17) Magull-Seltenreich, A.; Zeller, W. J. Sensitization of human colon tumor cell lines to carmustine by depletion of O^6-alkylguanine-DNA alkyltransferase. *J. Cancer Res. Clin. Oncol.* **1995**, *121*, 225–229.

(18) Quinn, J. A.; Pluda, J.; Dolan, M. E.; Delaney, S.; Kaplan, R.; Rich, J. N.; Friedman, A. H.; Reardon, D. A.; Sampson, J. H.; Colvin, O. M.; Haglund, M. M.; Pegg, A. E.; Moschel, R. C.; McClendon, R. E.; Provenzale, J. M.; Guruangan, S.; Tourt-Uhlig, S.; Herndon, J. E., II; Bigner, D. D.; Freidman, H. S. Phase II trial of carmustine plus O^6-benzylguanine for patients with nitrosourea-resistant recurrent or progressive malignant glioma. *J. Clin. Oncol.* **2002**, *20*, 2277–2283.

(19) Schilsky, R. L.; Dolan, M. E.; Bertucci, D.; Ewesuedo, R. B.; Vogelzang, N. J.; Mani, S.; Wilson, L. R.; Ratain, M. J. Phase I clinical and pharmacological study of O^6-benzylguanine followed by carmustine in patients with advanced cancer. *Clin. Cancer Res.* **2000**, *6*, 3025–3031.

(20) Kennedy, K. A.; Teicher, B. A.; Rockwell, S.; Sartorelli, A. C. The hypoxic tumor cell: a target for selective cancer chemotherapy. *Biochem. Pharmacol.* **1980**, *29*, 1–8.

(21) Sartorelli, A. C. Therapeutic attack of hypoxic cells of solid tumors: presidential address. *Cancer Res.* **1988**, *48*, 775–778.

(22) Chae, M.-Y.; McDougall, M. G.; Dolan, M. E.; Swenn, K.; Pegg, A. E.; Moschel, R. C. Substituted O^6-benzylguanine derivatives and their inactivation of human O^6-alkylguanine DNA alkyltransferase. *J. Med. Chem.* **1994**, *37*, 342–347.

(23) Chae, M.-Y.; Swenn, K.; Kanugula, S.; Dolan, M. E.; Pegg, A. E.; Moschel, R. C. 8-Substituted O^6-benzylguanine, substituted 6(4)-(benzyloxy)pyrimidine derivatives, and related compounds as inhibitors of human O^6-alkylguanine DNA alkyltransferase. *J. Med. Chem.* **1995**, *38*, 359–365.

(24) Wei, G.; Loktionova, N. A.; Pegg, A. E.; Moschel, R. C. β-Glucuronidase-cleavable prodrugs of O^6-benzylguanine and O^6-benzyl-2′-deoxyguanine. *J. Med. Chem.* **2005**, *48*, 256–261.

(25) Penketh, P. G; Shyam, K; Baumann, R. P; Remack, J. S; Brent, T. P; Sartorelli, A. C. 1,2-Bis(methylsulfonyl)-1-(2-chloroethyl)-2-[(methylamino)carbonyl]hydrazine (VNP40101M): I. Direct inhibition of O^6-alkylguanine-DNA alkyltransferase (AGT) by electrophilic species generated by decomposition. *Cancer Chemother. Pharmacol.* **2004**, *53*, 279–287.

(26) Thomson, P.; Naylor, M. A.; Stratford, M. R. L.; Lewis, G.; Hill, S.; Patel, K. B.; Wardman, P.; Davis, P. D. Hypoxia-driven elimination of thiopurines from their nitrobenzyl prodrugs. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 4320–4322.

(27) Bianco, P.; Haladjian, J. Study of two forms of ferredoxin from *Desulfovibrio gigas* by differential pulse polarography. *Biochem. Biophys. Res. Commun.* **1977**, *78*, 323–327.

(28) Baumann, R.; Penketh, P.; Seow, H.; Shyam, K.; Sartorelli, A. Generation of oxygen deficiency in cell culture using a two-enzyme system to evaluate agents targeting hypoxic tumor cells. *Radiat. Res.* **2008**, *170*, 651–660. PMCID: PMC2667281.

(29) Baumann, R. P.; Penketh, P. G.; Ishiguro, K.; Shyam, K.; Zhu, Y. L.; Sartorelli, A. C. Reductive activation of the prodrug 1,2-bis-(methylsulfonyl)-1-(2-chloroethyl)-2-[[1-(4-nitrophenyl)ethoxy]carbonyl]hydrazine (KS119) selectively occurs in oxygen-deficient cells and overcomes O^6-alkylguanine-DNA alkyltransferase mediated KS119 tumor cell resistance. *Biochem. Pharmacol.* **2010**, *79*, 1553–1561. PMCID: PMC2885764.

(30) Rockwell, S. In vivo−in vitro tumor systems: new models for studying the response of tumors to therapy. *Lab. Animal Sci.* **1977**, *27*, 831–851.

(31) Belcourt, M. F.; Hodnick, W. F.; Rockwell, S.; Sartorelli, A. C. The intracellular location of NADH:cytochrome b5 reductase modulates the cytotoxicity of the mitomycins to Chinese hamster ovary cells. *J. Biol. Chem.* **1998**, *273*, 8875–8881. PMCID: PMC40257.