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Design of a hypoxia-activated prodrug inhibitor of O^6-alkylguanine-DNA alkyltransferase

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ABSTRACT

The efficacy of agents that alkylate the *O*-6 position of guanine is inhibited by O^6-alkylguanine-DNA alkyltransferase (AGT) which removes these lesions from the tumor DNA. To increase differential toxicity, inhibitors must selectively deplete AGT in tumors, while sparing normal tissues where this protein serves a protective function. A newly synthesized prodrug of the AGT inhibitor O^6-benzylguanine (O^6-BG) with an α,α-dimethyl-4-nitrobenzyloxycarbonyl moiety masking the essential 2-amino group has demonstrated the feasibility of targeting hypoxic regions that are unique to solid tumors, for drug delivery. However, these modifications resulted in greatly decreased solubility. Recently, new potent global AGT inhibitors with improved formulatability such as O^6-[(3-aminomethyl)benzylguanine (**1**) have been developed. However, acetylamino (*N*-(3-(((2-amino-9*H*-purin-6-yl)oxy)methyl)benzyl)acetamide) (**2**) exhibits a pronounced decrease in activity. Thus, **1** would be inactivated by *N*-acetylation and probably *N*-glucuronidation. To combat potential conjugational inactivation while retaining favorable solubility, we synthesized 6-((3-((dimethylamino)methyl)benzyl)oxy)-9*H*-purin-2-amine (**3**) in which the 3-aminomethyl moiety is protected by methylation; and to impart tumor selectivity we synthesized 2-(4-nitrophenyl)propan-2-yl(6-((3-((dimethylamino)methyl)benzyl)oxy)-9*H*-purin-2-yl)carbamate (**7**), a hypoxia targeted prodrug of **3** utilizing an α,α-dimethyl-4-nitrobenzyloxycarbonyl moiety. Consistent with this design, **7** demonstrates both hypoxia selective conversion by EMT6 cells of **7** to **3** and hypoxic sensitization of AGT containing DU145 cells to the cytotoxic actions of laromustine, while exhibiting improved solubility.

A number of antitumor agents alkylate the *O*-6 position of guanine residues in DNA.[1] These include the nitrosoureas carmustine (BCNU) and lomustine (CCNU) and the methylating agents temozolomide (TMZ), procarbazine, dacarbazine (DTIC) and streptozocin,[1] as well as the 1,2-bis(sulfonyl)hydrazine prodrug class of antitumor chloroethylating agents exemplified by laromustine and KS119 developed in this laboratory.[2,3] The repair protein O^6-alkylguanine-DNA alkyltransferase (AGT), stoichiometrically transfers alkyl and chloroethyl groups from the *O*-6 position of guanine in DNA to cysteine 145 in its active site.[4,5] The *O*-6 position of guanine in DNA is restored to its native state and the alkylated form of AGT is then degraded. Thus, one AGT molecule repairs only a single guanine *O*-6 position lesion. This action represents the primary mechanism of tumor and host cell resistance to agents that alkylate the *O*-6 position of guanine in DNA.[1]

The AGT inhibitor O^6-benzylguanine (O^6-BG) reacts with the active site of AGT to form *S*-benzylcysteine, inactivating the protein and increasing the sensitivity of cells to agents that alkylate the *O*-6 position of guanine.[6,7] However, global inactivation of AGT, in most cases, does not result in a therapeutic advantage as this sensitizes both normal and tumor tissues.[8,9] In instances where the therapeutic effect of the alkylator is largely due to a lower tumor AGT activity, global AGT ablation would be expected to be therapeutically deleterious, as it would collapse the very differential responsible for efficacy. Thus, non-toxic doses of O^6-BG have been shown in patients to deplete the AGT content of tumors, enhancing their sensitivity to BCNU, but also sensitizing host tissues, necessitating a reduction in BCNU dosage to ineffective levels.[8,9] Selectively targeting the release of AGT inhibitors to tumor tissue would result in differential sensitization and circumvent this weakness.[10] We have attempted to exploit hypoxic

Abbreviations: AGT, O^6-alkylguanine-DNA alkyltransferase; O^6-BG, O^6-benzylguanine; BCNU, carmustine; CCNU, lomustine; DTIC, dacarbazine; TMZ, temozolomide.

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regions, which are unique to solid tumors, to target the release of O^6-BG by masking its essential 2-amino group.[10] This should sensitize not only the hypoxic regions themselves but also adjacent normoxic regions of the tumor by a bystander effect resulting from the diffusion of the released inhibitor into the surrounding tumor tissue. This targeting is based on the observation that nitro aromatic groups are reduced in mammalian tissues in a one electron manner to generate a nitro radical anion as the initial product.[11] Nitro radical anions are rapidly reoxidized by molecular oxygen to regenerate the parental compound, so reduction beyond this stage, to hydroxylamino or amino functionalities, only occurs in very low oxygen environments.[11] When the nitro group is reduced to a hydroxylamino or amino functionality the masking group fragments and liberates O^6-BG.[10] Using a similar strategy, nitroheterocycles have been successfully used diagnostically to selectively label hypoxic regions in human tumors using agents such as [F-18]fluoromisonidazole.[12] We initially synthesized three nitrobenzyloxycarbonyl prodrug derivatives of O^6-BG (**4**–**6**)[10] (Fig. 1) and evaluated their ability to undergo reductive fragmentation to generate O^6-BG under normoxic and oxygen deficient conditions using both enzyme systems and intact cells.[10] Of these, the *gem*-dimethyl analogue (**6**) emerged as the most promising agent, giving the highest yield of O^6-BG under oxygen deficient conditions, with little or no activation under normoxia. Compound **6** produced a significant enhancement of the cytotoxicity of laromustine selectively under hypoxic conditions to DU145 human prostate carcinoma cells, which express very high levels (42,000 molecules/cell) of AGT.[10] However, the utility of **6** was strongly compromised by the fact that the addition of the lipophilic α,α-dimethyl-4-nitrobenzyloxycarbonyl moiety to O^6-BG reduced its aqueous solubility by >70-fold (Table 1), hampering formulation and therapeutic utility. Therefore, it is essential for an efficacious prodrug to contain a hydrophilic (solubilizing) moiety to counter the hydrophobicity of this hypoxic region targeting moiety.

Recently Pauly et al.[13] reported that O^6-[(3-aminomethyl) benzyl]guanine (**1**) (Fig. 1) was superior to O^6-BG as an inactivator of human AGT. This finding is particularly important since this modification not only results in an increase in potency but it also permits salt formation, thereby aiding in formulation. Thus, it would be anticipated that prodrugs of compound **1**, containing the α,α-dimethyl-4-nitrobenzyloxycarbonyl moiety, could be formulated as a consequence of improved aqueous solubility. However, the primary 3-aminomethyl group on the benzyl moiety may be susceptible to conjugation reactions (i.e., *N*-acetylation and *N*-glucuronidation;[14]) in patients, potentially resulting in a decrease in activity (*N*-acetylation to form compound **2**) (Fig. 1) and/or rapid excretion (*N*-glucuronidation), decreasing the advantages gained by the aminomethyl substitution. Such conjugation reactions can be prevented by replacing the protons on the amino group in compound **1** with protective methyl groups to give the *N*,*N*-dimethylaminomethyl analogue **3** (Fig. 1) while retaining the ability to be converted to a relatively water-soluble salt to permit formulation for clinical usage. We have, therefore, synthesized **2** and **3**, to test the effects of acetylation and methylation of the 3-aminomethyl moiety of **1** on its AGT inhibitory activity; and have synthesized **7**, as a α,α-dimethyl-4-nitrobenzyloxycarbonyl based hypoxia selective prodrug of **3**, as shown in Figure 2. Compounds **1**, **4**–**6** were synthesized as described earlier.[10,13] Compound **7** is a hypoxia selective AGT inhibitor prodrug that preferentially liberates **3** under hypoxic conditions, while exhibiting improved solubility, and resistance to potential conjugational inactivation. We also prepared **7W** the isethionic acid salt of **7** to further increase water-solubility.

Initially, compounds **1**–**3** were assessed for AGT inhibitory activity using two methods; O^6-benzylguanine (O^6-BG) was also included to serve as a positive control in these studies. In the first protocol, the sensitivity of purified cloned human recombinant AGT to direct inactivation by the inhibitors in a simple model system was determined (Fig. 3). In the second protocol, the ability of the inhibitors to inactivate AGT in intact cells (HL-60 promyelocytic leukemia which naturally expresses ~17,000 AGT molecules/cell), where it resides in the nucleus largely bound to DNA, was assayed (Fig. 4). Both of these protocols gave very similar results, suggesting that access to AGT was approximately equivalent for compounds **1**–**3**. While all four compounds were found to possess AGT inhibitory activity, compound **2** was considerably less active than the other three agents, demonstrating that *N*-acetylation of the aminomethyl group markedly reduces efficacy. In addition, compound **3** was comparable in activity to the aminomethyl analogue **1**. In agreement with Pauly et al.,[13] compound **1**, the most potent of the four analogues, was found to be more effective than O^6-BG. In the intact HL-60 cell experiments, 2 h exposure to O^6-BG and compounds **1**, **2** and **3** gave IC_{50} values of 0.039, 0.009, 0.086, and 0.025 μM, respectively. Thus, compound **3** retains reasonable activity as an inhibitor of AGT, and the solubility advantage of compound **1** (Table 1), i.e., its ability to form a water-soluble salt, while avoiding the possibility of being largely inactivated by metabolic N-acetylation. The methylation of the amino function also permits a significant decrease in the cost of goods by eliminating a blocking and unblocking step in the synthesis of compound **3**. O^6-BG, **1**, **2** and **3** were also evaluated for their ability to enhance the cytotoxic effects of laromustine against an EMT6 cell line (murine mammary carcinoma) that had been transfected with the human AGT gene (Fig. 5). This cell line (EMT6hAGT18) expresses ~18,000 AGT

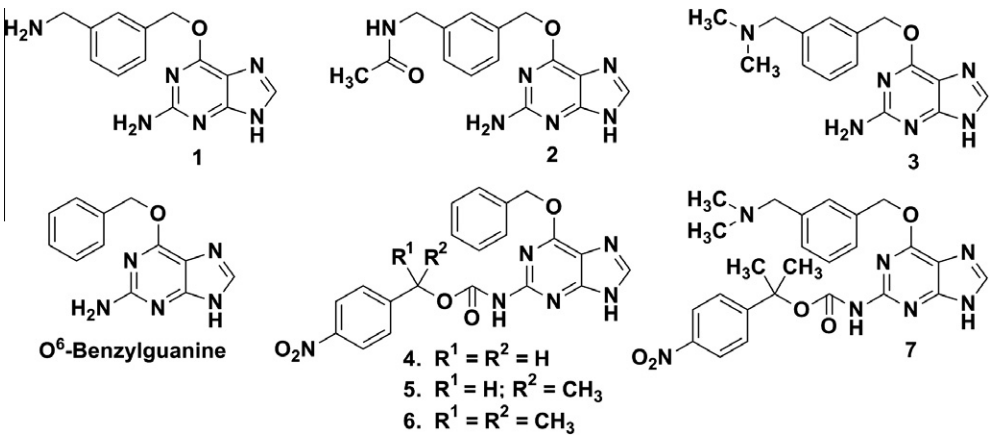

Figure 1. Chemical structures and names of key compounds.

Table 1
Comparison of the aqueous solubilities of key compounds

Agent	Compound description	Aqueous solubility 22 °C	Solubility ratio w.r.t. O^6-BG
O^6-BG	O^6-Benzylguanine standard AGT inhibitor	718 ± 25 μM	1.00
1	(O^6-[(3-Aminomethyl)benzylguanine)	1.77 ± 0.11 mM	2.47
3	(O^6-[(3-*N*,*N*-dimethylaminomethyl)benzylguanine)	6.49 ± 0.07 mM	9.04
6	O^6-BG with α,α-dimethyl-4-nitrobenzyloxycarbonyl moiety attached to the 2-amino group of guanine	9.8 ± 0.7 μM	0.014
7	**3** With α,α-dimethyl-4-nitrobenzyloxycarbonyl moiety attached to the 2-amino group of guanine	326 ± 10 μM	0.454
7W	Hydroxyethylsulfonate salt of 7	>10 mM [a]	>13.9[a]

The aqueous solubility at room temperature was determined by adding 1% by volume of a saturated solution of agent to 0.99 mL of distilled water at 22 °C with very rapid mixing. To this mixture ~200 mg of white quartz (50 + 70 mesh) was added and the mixture shaken periodically for 10 min to stimulate precipitation from super saturated solutions. The mixture was centrifuged for 2 min at 10,000 g then an aliquot of the supernatant was diluted 10-fold with 30 mM potassium phosphate pH 5.4 buffer containing 30% CH_3CN and the sample analyzed by HPLC.[10] The concentrations were then calculated by comparisons with standards of known concentration.
Values represent the mean of 3 determinations ± S.E.M.
[a] Aqueous solubility limit not reached.

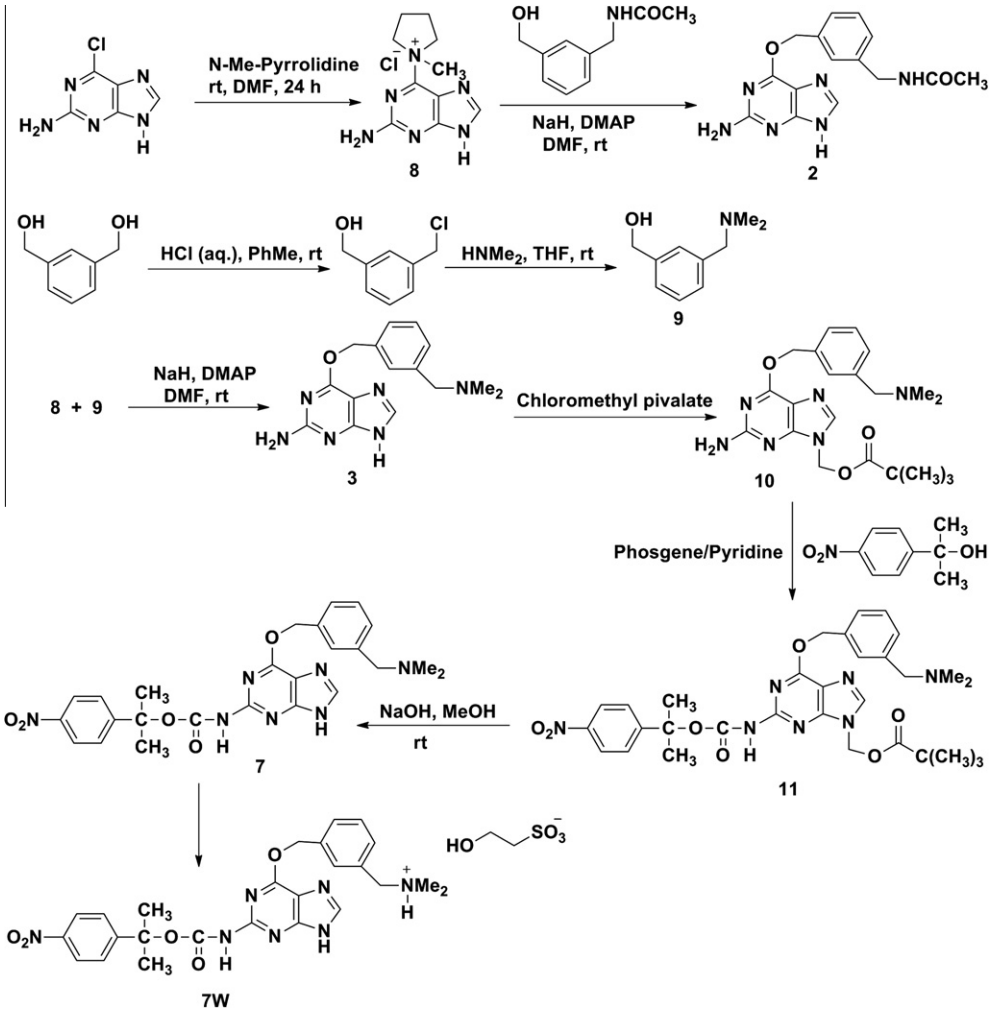

Figure 2. The synthesis of key compounds. Compound **2** was prepared by reacting the sodium salt of 3-(acetylamino)methylbenzyl alcohol with 1-(2-amino-9*H*-purin-6-yl)-1-methylpyrrolidin-1-ium chloride (**8**) in *N*,*N*-dimethylformamide in the presence of 4-(*N*,*N*-dimethylamino)pyridine. Compound **3** was synthesized using an analogous procedure in which 3-(*N*,*N*-dimethylamino)methylbenzyl alcohol (**9**) was used *in lieu* of 3-(acetylamino)methylbenzyl alcohol.[16] Compound **7** was prepared from compound 3 using a procedure analogous to the one employed for the synthesis of the 3-nitrophenoxycarbonyl derivative of O^6-BG.[10] The free amine was then converted to the water-soluble hydroxyethylsulfonate salt (**7W**) by reaction with isethionic acid.

molecules/cell. As expected, all four AGT inhibitors produced insignificant cytotoxicity on their own, but pretreatment of cells with these agents resulted in a significant potentiation of the cytotoxic effects of laromustine (Fig. 5). In this intact cell system, compound **3** was significantly more potent than both O^6-BG and **1**, which gave essentially equivalent enhancements of cytotoxicity. The greater potency of the dimethylamino analogue in this assay may reflect differences in the permeability, export, metabolism or other factors not relating directly to their relative potencies as inactivators of human AGT in this cell line. Compound **2** was by far the least effective, supporting the contention that conjugation of the primary amino group on the benzyl moiety of compound **1** in vivo would reduce its potency. These findings suggest that compound **3** offers the greatest potential of these O^6-BG analogues.

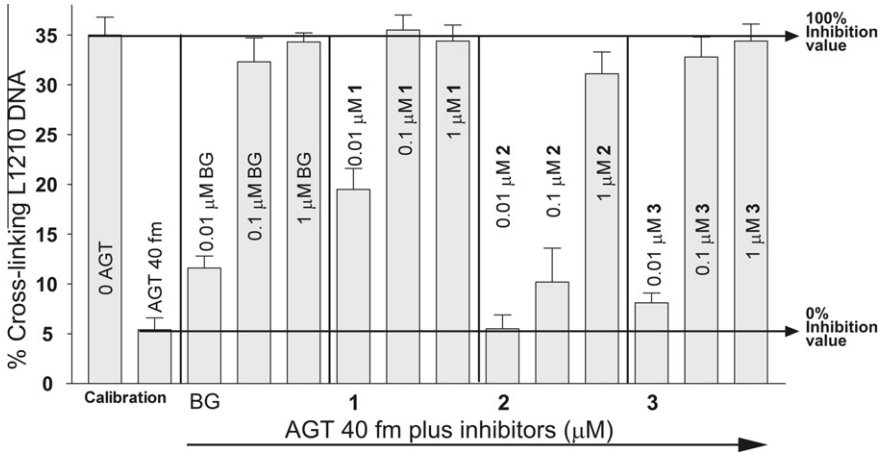

Figure 3. Comparative inhibition of purified recombinant human AGT activity by O^6-BG and the O^6-BG analogs **1**, **2**, **3**. The ability of O^6-BG, **1**, **2**, and **3** to inhibit AGT was assessed using an assay based upon the repair of DNA containing cross-link precursor lesions (O^6-(2-chloroethyl)guanine and N^1,O^6-ethanoguanine).[17] This DNA was prepared by reacting 1,2-bis(sulfonyl)-1-(2-chloroethyl)hydrazine, synthesized as previously described[15] with L1210 DNA.[10] Varying concentrations (0.01, 0.1, and 1.0 μM) of O^6-BG, **1**, **2**, and **3** were incubated with 40 fm of AGT (37 °C for 10 min) and the treated AGT assayed for its ability to repair the cross-link precursor lesions relative to AGT incubated in the absence of inhibitors.[10] Repair of the cross-link precursor lesions results in decreased DNA cross-linking. Therefore, inhibition of AGT repair results in an increase in the measured cross-linking. Values represent the means of **3** independent determinations (±S.E.M.)

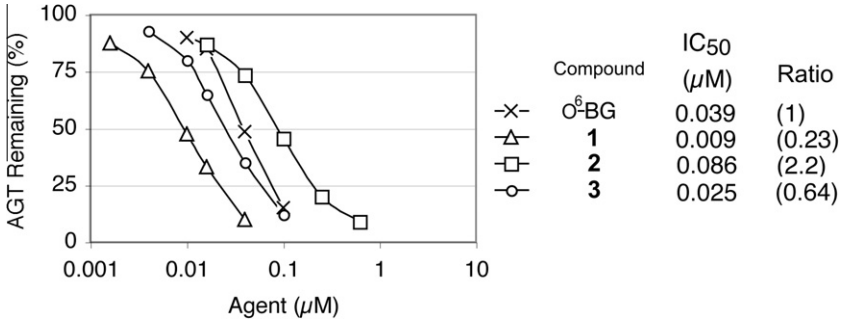

Figure 4. AGT inhibition in HL60 promyelocytic leukemia cells in vitro. Cells from the human HL60 leukemia cell line, were treated with graded concentrations of O^6-BG (×), **1** (Δ), **2** (□), or **3** (○) for 2 h and AGT levels were assessed using a [^{3}H]AGT based binding assay as described previously.[18] Dose response curves were generated and IC_{50} values were determined.

While compound **3** has an impressive ability to sensitize AGT expressing cells to laromustine in vitro, it retains the inherent flaw of global AGT inhibition, that untargeted inhibitors not only ablate AGT in the tumors, where the repair protein is a hindrance to treatment, but also in normal tissues where it serves a protective function. Therefore, for an AGT inhibitor to have a meaningful impact in cancer therapy as a modulator of guanine *O*-6 alkylating agents it is necessary for the inhibitor to be delivered preferentially to the tumor target. This ensures that normal tissues are largely spared sensitization and that the dosage of the guanine *O*-6 alkylating drug is maintained without substantially increased host toxicity. To accomplish this we synthesized compound **7** which utilizes the same α,α-dimethyl-4-nitrobenzyloxycarbonyl hypoxic region targeting moiety used in 6 to mask the essential 2-amino group of the more water soluble compound **3**. In support of this, compounds **7** and **7W** were found to have >30 and >1000 times the aqueous solubility of compound **6**, respectively (Table 1). Some of the advantage gained by the enhanced solubility of **7** and **7W** could be offset by the greater basicity of the tertiary amine as the extracellular pH is lower in hypoxic tumors than in normal tissue, while their intracellular pH is relatively unchanged and this can impair the uptake of basic chemotherapeutic agents.[19]

A comparison was made between the ability of compound **7** to potentiate the cytotoxic effects of laromustine under hypoxic and normoxic conditions in DU145 cells using a clonogenic cell survival assay. Cells received a 4 h pre-exposure to various concentrations of compound **7** under either normoxic or hypoxic conditions. The cells were then challenged with laromustine to ascertain the degree of sensitization. The high AGT activity (42,000 AGT molecules per cell) in the DU145 cell line results in substantial resistance to laromustine and other agents that rely on the alkylation of *O*-6 position of guanine residues in DNA for the bulk of their activity[10,20–22] so the loss of AGT activity should be readily apparent. The 4 h pretreatment resulted in only a moderate sensitization to the cytotoxic actions of laromustine under normoxic conditions, but a much more pronounced sensitization under hypoxic conditions. Thus, a 5 μM pretreatment with compound **7** resulted in normoxic and hypoxic survival fractions of 0.43 and 0.027, respectively (Fig. 6). This is consistent with the prodrug **7** liberating the AGT inhibitor **3** preferentially under hypoxic conditions. However, despite this ~16-fold hypoxic/oxic surviving fraction differential, corresponding to a >4-fold difference in LC_{90} concentration, compound **7** showed a significantly greater degree of normoxic sensitization than previously observed with **6**,[10] indicating the possible presence of more significant non-reductive routes of activation with compound **7** than **6**. The conversion of **7** to **3** by EMT6 cell suspensions under normoxic and hypoxic conditions was studied and a >60-fold differential in the formation of **3** was observed under hypoxic conditions (Table 2). The low IC_{50} value of **3** for AGT of 0.025 μM may account for these observations as very little

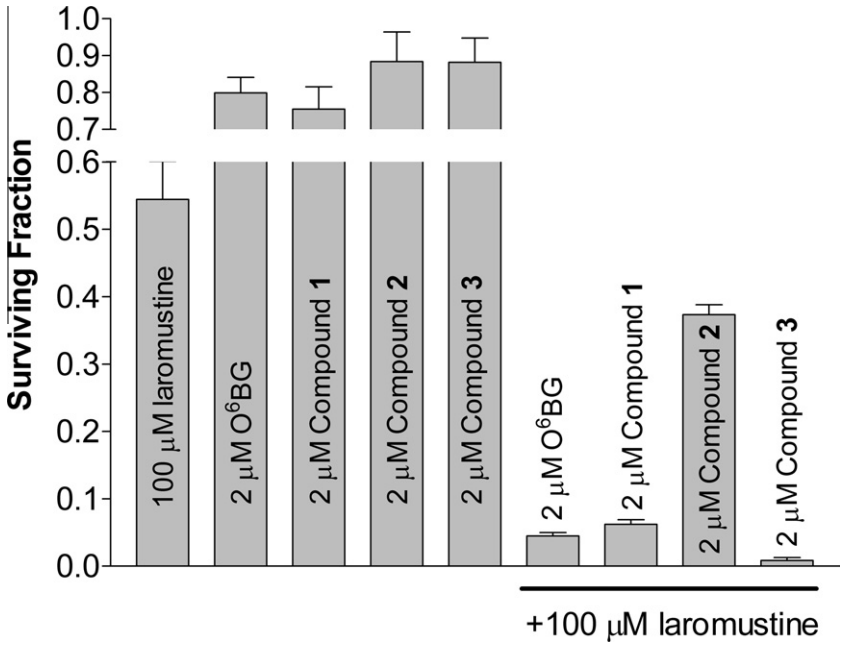

Figure 5. The effects of O^6-BG and compounds **1**, **2**, and **3** in the presence or absence of laromustine. EMT6hAGT18 cells, which express 18,000 molecules of AGT per cell, were treated with a 2 μM concentration of O^6-BG, **1**, **2**, or **3** in the presence or absence of laromustine (100 μM) for 18 h. In instances where cells were treated with two compounds, the AGT inhibitor was administered two hours before laromustine treatment (100 μM, 18 h). Cell survival was measured using a clonogenic assay described previously.[7] The maximum DMSO concentrations to which cells were exposed were ⩽0.05% and non-toxic. Points represent mean ± S.E.M of 3 to 5 individual experiments.

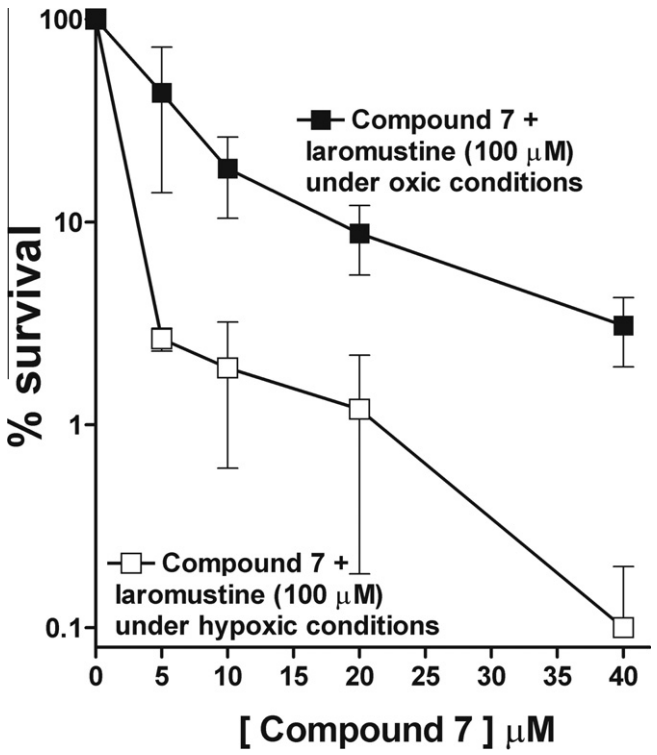

Figure 6. The effects of various concentrations of compound **7** on the cytotoxicity of laromustine under oxic and hypoxic conditions in DU145 cells. DU145 cells were pretreated with graded concentrations of **7** (5, 10, 20, 40 μM) for 4 h followed by 100 μM laromustine for a total exposure time of 24 h under normoxic (closed symbols) or hypoxic (open symbols) conditions and cell survival assessed using a clonogenic assay.[7] Oxygen deficiency was established using a nitrogen flush and seal combined with a dual enzyme oxygen scavenging system consisting of 2 U/ml of glucose oxidase, 120 U/ml of catalase, and 10 mM of added glucose to maintain hypoxia.[23] The maximum DMSO concentrations to which cells were exposed were ⩽0.05% and non-toxic. Points represent the mean ± S.E.M of 3 individual experiments.

Table 2
Liberation of **3** from **7** by incubation with EMT6 cells under oxic and hypoxic conditions

EMT6 activation	Oxic	Hypoxic
[**7**] μM T = 0 h	197.57 ± 3.8	199.78 ± 5.8
[**3**] μM T = 0 h	0.0[a]	0.0[a]
[**7**] μM T = 1 h	195.5 ± 2.9	149.6 ± 9.3
[**3**] μM T = 1 h	0.2 ± 0.04	12.3 ± 1.9

Production of **3** from **7** (~200 μM) by EMT6 cells (2×10^7/ml) under oxic and hypoxic conditions over a one hour incubation period at 37 °C. The concentrations were determined by HPLC as previously described[10] except the elution solvent's aqueous constituent buffer was 0.05 M Glycine, 1.0 mM NaN_3, pH 10.0. Compounds **3** and **7** elute at ~9.3 and ~29.7 min, respectively. Values represent the mean of 6 determinations ± S.E.M.

[a] Below the limit of detection (<0.1 μM).

activation would be required under oxic conditions to result in an enhancement of laromustine cytotoxicity. If the levels of non-reductive routes of activation are not too excessive in patients, the hypoxia-activated AGT inhibitor prodrug **7W**, in conjunction with laromustine, or other agents that rely on alkylation of the *O*-6 position of guanine residues in DNA for their activity, may be useful in a clinical setting.

Acknowledgments

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