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Brief Article

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Hypoxia-Selective O^6-Alkylguanine-DNA Alkyltransferase Inhibitors: Design, Synthesis, and Evaluation of 6-(Benzyloxy)-2-(aryldiazenyl)-9*H*-purines as Prodrugs of O^6-Benzylguanine

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ABSTRACT: O^6-Alkylguanine-DNA alkyltransferase (AGT) is a DNA repair protein which removes alkyl groups from the O-6 position of guanine, thereby providing strong resistance to anticancer agents which alkylate this position. The clinical usefulness of these anticancer agents would be substantially augmented if AGT could be selectively inhibited in tumor tissue, without a corresponding depletion in normal tissue. We report the synthesis of a new AGT inhibitor (**5c**) which selectively depletes AGT in hypoxic tumor cells.

INTRODUCTION

O^6-Alkylguanine-DNA alkyltransferase (AGT, MGMT), a ubiquitous DNA repair protein, effects the stoichiometric removal of alkyl groups from the O-6 position of guanine in DNA.[1] Expression of this protein in tumors is the primary mechanism of resistance to guanine O-6 chloroethylating agents such as the *N*-(2-chloroethyl)-*N*-nitrosoureas (e.g., BCNU and CCNU) and the sulfonylhydrazine prodrugs synthesized in our laboratory[2−5] (e.g., laromustine and KS119) and methylating agents (e.g., DTIC, Temozolomide, and procarbazine). Inhibition of AGT activity in tumors is currently of great interest to cancer researchers because it can substantially increase the efficacy of antitumor guanine O-6 alkylating agents. The current therapeutic strategy of using an AGT inhibitor such as O^6-benzylguanine[6] (O^6-BG) to sensitize AGT-expressing tumors to the cytotoxic effects of guanine O-6 alkylating agents has one inherent flaw. A global AGT inhibitor not only ablates AGT in tumor tissue where the repair protein is a hindrance to treatment, but it also significantly lowers AGT levels in normal tissues where the protein serves a protective function. For example, although nontoxic doses of O^6-BG have been shown in patients to deplete the AGT content of tumors, this action also sensitizes host tissue, necessitating a considerable decrease in the dosage of BCNU, the guanine O-6 alkylating agent used in this trial, because of myelosuppression, leading to an ineffective blood level of BCNU.[7,8] Therefore, for an AGT inhibitor to have a meaningful impact on cancer therapy involving guanine O-6 alkylating agents, it is necessary for it to be delivered selectively or preferentially to the tumor target. This will ensure that the normal tissues are spared without severely compromising therapeutic efficacy.

Repeated studies have demonstrated that oxygen-deficient tumor cells resulting from the inherently abnormal tumor vasculature create an environment conducive to reductive processes. Several anticancer agents, particularly alkylating agents, latentiated as bioreductive prodrugs, have been tested and found to be preferentially toxic to oxygen deficient cells compared to their aerobic counterparts. The substrates examined to date have been predominantly quinones, nitro-aromatics, and *N*-oxides (for a review see ref 9). Although azoreduction has been used as a therapeutic strategy in designing prodrugs of nitrogen mustard[10,11] and 5-amino-salicylic acid,[12,13] very little work, if any, has been done on the possible use of hypoxia in solid tumors to activate azo prodrugs for therapeutic purposes. This paper describes the synthesis and evaluation of 6-(benzyloxy)-2-(aryldiazenyl)-9*H*-purines as prodrugs of O^6-BG activated under hypoxic conditions. The 2-amino group in these compounds, which is essential for the AGT inhibitory activity of O^6-BG, is latentiated as an azo linkage. Unmasking of the 2-amino group occurs in the hypoxic fraction of a solid tumor via reduction of the azo linkage.[14] Once O^6-BG is formed, it is expected to sensitize not only the hypoxic tumor cells in which it is released but also the surrounding aerobic tumor cells due to diffusion across membranes.

RESULTS AND DISCUSSION

Chemistry. **5a−e** were synthesized as shown in Figure 1. 2-Nitroso-6-benzyloxy-9-Boc-purine (**3**) was synthesized from 6-benzyloxypurine (**1**) in four steps using published proce-

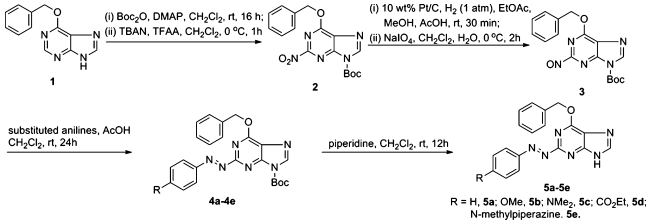

Figure 1. **5a−e** were synthesized as shown. 2-Nitroso-6-benzyloxy-9-Boc-purine (**3**) was synthesized from 6-benzyloxypurine (**1**) in four steps using published procedures.[15,16] **3** was then condensed with the appropriate aniline in dichloromethane in the presence of acetic acid at room temperature to give the corresponding **4a−e**.[17] Deprotection of the 9-*t*-Boc group was carried out using an equivalent amount of piperidine in dichloromethane to give the target azo prodrug **5a−e**.[16]

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dures.[15,16] **3** was then condensed with the appropriate aniline in dichloromethane in the presence of acetic acid at room temperature to give the corresponding **4a−e**.[17] Deprotection of the 9-*t*-BOC group was carried out using an equivalent amount of piperidine in dichloromethane to give the target azo prodrug **5a−e**.[16]

Determination of Half-Wave Reduction Potentials. Initially, five compounds were synthesized (**5a−e**, Figure 1) and their half-wave reduction potentials determined by differential pulse polarography (Table 1). The $E_{1/2}$ values

Table 1. Determination of Half-Wave Reduction Potentials[a]

agent	concentration (μM)	half-wave reduction potential mV ± SE (mV)
5d	10	−92 ± 7
5a	10	−156 ± 1
2-NBP	50	−213 ± 2
5e	10	−245 ± 1
nitrofurazone	50	−279 ± 4
5c	10	−286 ± 2
mitomycin C	10	−307 ± 1
misonidazole	50	−453 ± 3
BG-M2	50	−475 ± 2
metronidazole	50	−611 ± 7

[a]Polarographic half-wave reduction potentials of agents in relation to four well-known reference compounds; nitrofurazone, a topical bactericide; mitomycin C, a cancer chemotherapeutic agent preferentially targeting hypoxic regions; misonidazole, a radiosensitizer, ^{18}F derivatives of which are used for oxygen deficient region imaging; and metronidazole, an antibiotic targeting anaerobic bacteria and protozoa. 2-NBP and BG-M2 are prototype O^6-BG prodrugs designed to target hypoxic tumors. Half-wave reduction potentials were measured versus an Ag/AgCl reference electrode. All values are the result of at least three determinations ± SE.

versus an Ag/AgCl reference electrode under the tested conditions for **5a−e** were −156, −221, −286, −92, and −245 mV, respectively. Two candidates (**5c** and **5e**, Figure 1) with the lowest half-wave potentials (−286 and −245 mV, respectively) were chosen for further study. This was done not only to ensure that the candidates were relatively refractory to reductive activation under aerobic conditions but also to ensure facile activation under hypoxic conditions.

5c Sensitizes Cancer Cells to Laromustine. **5c** and **5e** were tested in clonogenic assays against DU145 human prostate carcinoma cells, which express relatively high levels of the resistance protein AGT[18] (42000 molecules per cell), to determine their ability to sensitize these cells to laromustine by selective release of O^6-BG. **5e**, which had a reduction potential slightly higher than **5c**, only weakly sensitized DU145 cells to the guanine O-6 alkylator laromustine (Figure 2) under hypoxic conditions and did not sensitize these cells under oxic conditions (Figure 2). **5c** was also tested in DU145 cells and demonstrated significant sensitization of these cells to laromustine under conditions of hypoxia with little or no effect seen under oxygenated conditions (Figure 3). No further increase in sensitization was observed at concentrations >10 μM (data not shown); this is likely the result of the limited aqueous solubility of this agent.

5c Selectively Produces O^6-Benzylguanine under Hypoxic Conditions. **5c**, which sensitizes DU145 cells to laromustine, was further studied to determine the levels of O^6-BG released under oxic and hypoxic conditions. **5c** was

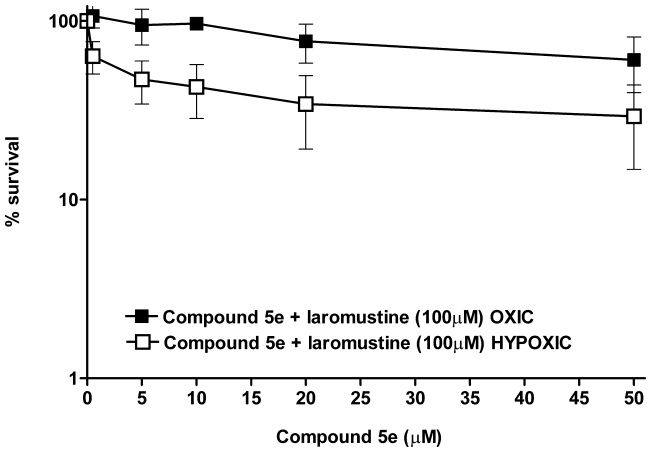

Figure 2. Survival (clonogenic) assays in DU145 human prostate cancer cells pretreated for 4 h with graded concentrations of **5e** then dosed with 100 μM laromustine for a total of 24 h. Cells were treated under either oxic (■) or hypoxic (□) conditions before staining and quantification. The horizontal axis indicates the concentration of **5e** in μM. The vertical axis indicates the percent survival. All points represent three independent determinations ± SEM.

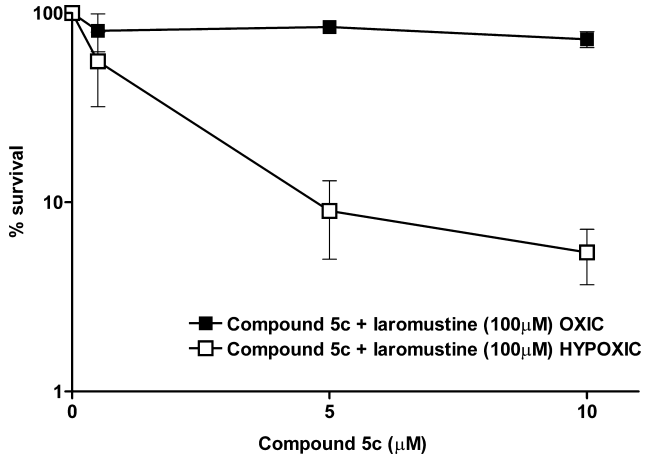

Figure 3. Survival (clonogenic) assays using DU145 cells pretreated for 4 h with graded concentrations of **5c** then exposed to 100 μM laromustine for a total of 24 h. Cells were treated under either oxic (■) or hypoxic (□) conditions before staining and quantification. The *x* axis indicates the concentration of **5c** in μM. The *y* axis indicates the percent survival. All points represent three independent determinations ± SEM.

incubated under aerobic conditions and under hypoxic conditions in the presence of EMT6 mouse mammary tumor cells (1×10^7/ml) at 37 °C, and samples were taken at hourly intervals and analyzed by HPLC as described in Figure 4. Over the course of 3 h, approximately 50% of the initial starting amount of 50 μM of the prodrug **5c** was converted to O^6-BG under hypoxic conditions with essentially no production of O^6-BG detected under oxic conditions. Under hypoxic conditions in EMT6 cells, the loss of prodrug exceeded the production of O^6-BG, likely due to loss from nonreductive metabolism or precipitation. Studies of other O^6-BG prodrugs designed by this laboratory have yielded similar incomplete conversions.[19] **5c** was also tested for the ability to generate O^6-BG when incubated with DU145 cells. As can be seen in Figure 5, DU145 cells converted over 84% of the initial input of **5c** to O^6-BG under hypoxic conditions with only minimal production of O^6-

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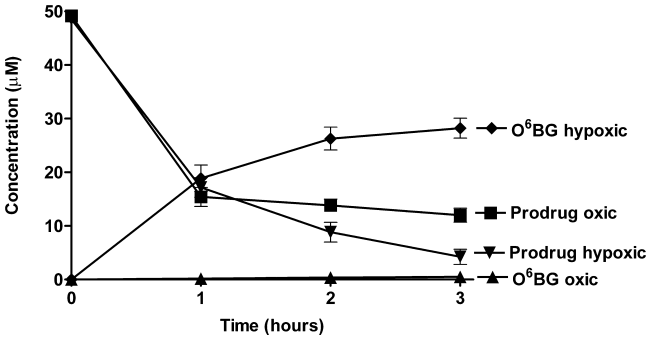

Figure 4. The generation of O^6-BG, and parental prodrug loss (**5c**) over a 3 h time interval. Prodrug was incubated with 10^7/mL of EMT6 mouse mammary tumor cells, and samples were analyzed at 1 h intervals by HPLC. The *x* axis indicates time in h. The *y* axis indicates the concentration in μM. All points are the results of at least three determinations ± SEM.

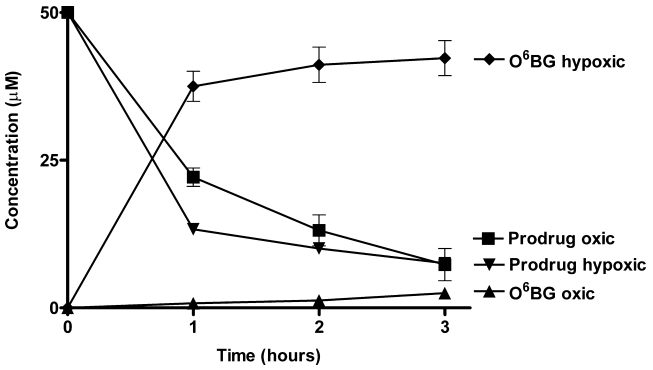

Figure 5. The generation of O^6-BG and parental prodrug (**5c**) loss over a 3 h time interval. Prodrug was incubated with 10^7/mL of DU145 human prostate carcinoma cells, and samples were analyzed at 1 h intervals by HPLC. The *x* axis indicates time in h. The *y* axis indicates the concentration in μM. All points are the results of at least three determinations ± SEM.

BG under oxic conditions. These studies using high cell densities indicate that significant quantities of the AGT inhibitor O^6-BG are released selectively under hypoxic conditions by the novel azo prodrug **5c**. The yields of O^6-BG obtained under hypoxic conditions following reductive fragmentation of **5c** are significantly higher than those obtained from 2-(4-nitrophenyl)propan-2-yl (6-(benzyloxy)-9*H*-purin-2-yl)carbamate (BG-M2) under similar conditions.[19] A significant decrease in the concentration of **5c** was seen with both cell lines under aerobic conditions, but this was not associated with the production of O^6-BG and was likely the result of parental material lost to precipitation or nonreductive routes of metabolism.

CONCLUSION

Compound **5c**, conceived as a hypoxia-activated prodrug of O^6-BG, has the following desirable properties: (a) **5c** has little or no activity under normoxic conditions in tissue culture as measured by its ability to sensitize AGT expressing DU145 cells to the cytotoxic effects of laromustine, a DNA guanine O-6 alkylating agent. However, significant sensitization of DU145 cells to laromustine occurs under hypoxic conditions. (b) Reduction of **5c** under hypoxic conditions by DU145 and EMT6 cells results in excellent yields of O^6-BG. In fact, the yields of O^6-BG obtained were significantly higher, under similar conditions, than those obtained with our previously designed O^6-BG prodrugs in many cases. The yields of O^6-BG from **5c** using EMT6 cells were approximately 10-fold greater than that from 2-(4-nitrophenyl)propan-2-yl (6-(benzyloxy)-9*H*-purin-2-yl)carbamate (BG-M2) and comparable to those from the more readily reduced 2-nitro-6-benzyloxypurine (2-NBP). In the case of DU145 cells, the yields of O^6-BG from **5c** exceeded those from either BG-M2 or 2-NBP by >40-fold. The high hypoxic azo-reduction dependent activation of **5c** by DU145 cells is particularly noteworthy as this cell line tends to feebly activate nitro-reduction dependent prodrugs. The poor water solubility of **5c** is a potential drawback, and we are in the process of synthesizing analogues of this agent which are considerably more water soluble yet retain the desirable characteristics of **5c**, with the intent of conducting in vivo studies on suitable compounds.

EXPERIMENTAL SECTION

Chemical Syntheses. Melting points were determined on a Thomas–Hoover Unimelt melting point apparatus and are uncorrected. ^{1}H NMR spectra were recorded on a Bruker Avance DPX-400 spectrometer (400 MHz) with tetramethylsilane as an internal standard. High resolution mass spectra were recorded on a Bruker Daltonics 9.4 T APEXQe FT-ICR and Ultimate mass spectrometer. 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. All the reported test compounds possess a purity of at least 95% as determined by HPLC.

*tert-Butyl-6-(benzyloxy)-2-(phenyldiazenyl)-9H-purine-9-carboxylate (**4a**).* To a solution of aniline (0.28 g, 3 mmol) in CH_2Cl_2 (10 mL) containing acetic acid (0.5 mL) was added 2-nitroso-6-benzyloxy-9-Boc-purine (**3**) (0.71 g, 2 mmol) at room temperature. The reaction mixture was stirred for 24 h. The solvent was removed by vacuum, and the residue was purified by flash chromatography using CH_2Cl_2:EtOAc (20:1) as eluent, which gave **4a** as a yellow solid (0.43g, 61%); mp 230–232 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.45 (s, 1H), 8.20–8.01 (m, 2H), 7.68–7.50 (m, 5H), 7.45–7.30 (m, 3H), 5.80 (s, 2H), 1.71 (s, 9H).

4b–4e were synthesized using procedures analogous to the one described above.

*tert-Butyl-6-(benzyloxy)-2-((4-methoxyphenyl)diazenyl)-9H-purine-9-carboxylate (**4b**).* **4b** was obtained in a 60% yield; mp 143–144 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.42 (s, 1H), 8.20–8.09 (m, 2H), 7.67–7.56 (m, 2H), 7.45–7.32 (m, 3H), 7.11–7.02 (m, 2H), 5.80 (s, 2H), 3.93 (s, 3H), 1.71 (s, 9H).

*tert-Butyl-6-(benzyloxy)-2-((4-(dimethylamino)phenyl)diazenyl)-9H-purine-9-carboxylate (**4c**).* **4c** was obtained in a 55% yield; mp 226–227 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.38 (s, 1H), 8.10 (t, J = 9.2 Hz, 2H), 7.60 (d, J = 6.9 Hz, 2H), 7.44–7.30 (m, 3H), 6.77 (d, J = 9.2 Hz, 2H), 5.81 (s, 2H), 3.15 (s, 6H), 1.71 (s, 9H).

*tert-Butyl-6-(benzyloxy)-2-((4-(ethoxycarbonyl)phenyl)diazenyl)-9H-purine-9-carboxylate (**4d**).* **4d** was obtained in a 52% yield; mp 138–140 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.46 (s, 1H), 8.28–8.09 (m, 3H), 7.59 (d, J = 7.7 Hz, 2H), 7.43–7.29 (m, 3H), 5.79 (s, 2H), 4.43 (q, J = 7.1 Hz, 2H), 1.71 (s, 9H), 1.43 (t, J = 7.1 Hz, 3H).

*tert-Butyl-6-(benzyloxy)-2-((4-(4-methylpiperazin-1-yl)phenyl)-diazenyl)-9H-purine-9-carboxylate (**4e**).* **4e** was obtained in a 50% yield; mp 192–194 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.40 (s, 1H), 8.09 (d, J = 9.2 Hz, 2H), 7.67–7.54 (m, 2H), 7.43–7.29 (m, 3H), 6.98 (d, J = 9.2 Hz, 2H), 5.80 (s, 2H), 3.53–3.43 (m, 4H), 2.63–2.53 (m, 4H), 2.37 (s, 3H), 1.71 (s, 9H).

*6-(Benzyloxy)-2-(phenyldiazenyl)-9H-purine (**5a**).* To a solution of *tert*-butyl 6-(benzyloxy)-2-(phenyldiazenyl)-9*H*-purine-9-carboxylate (**4a**) (0.43 g, 1 mmol) in CH_2Cl_2 (5 mL) was added dropwise piperidine (0.08 g, 1 mmol) at room temperature. The mixture was stirred overnight. The precipitate was collected and washed with CH_2Cl_2 and ether to give the target molecule as a yellow solid (0.30 g,

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90%); mp 242−243 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ13.74 (s, 1H), 8.55 (s, 1H), 8.05−7.98 (m, 2H), 7.74−7.56 (m, 5H), 7.41 (dt, J = 21.6, 7.1 Hz, 3H), 5.71 (s, 2H). HRMS: calcd for $C_{18}H_{14}N_6O$, m/z, 331.1302 [(M + H)$^+$]; found, 331.1303. HPLC: t_r = 35.26 min (97.1%).

5b−**5e** were synthesized using procedures analogous to the one described above.

*6-(Benzyloxy)-2-((4-methoxyphenyl)diazenyl)-9H-purine (**5b**).* **5b** was obtained in an 88% yield; mp 223−224 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 13.61 (s, 1H), 8.51 (s, 1H), 8.07−7.93 (m, 2H), 7.69−7.55 (m, 2H), 7.48−7.32 (m, 3H), 7.26−7.14 (m, 2H), 5.70 (s, 2H), 3.91 (s, 3H). HRMS: calcd for $C_{19}H_{16}N_6O_2$, m/z, 361.1408 [(M + H)$^+$]; found, 361.1407.

*4-((6-(Benzyloxy)-9H-purin-2-yl)diazenyl)-N,N -dimethylaniline (**5c**).* **5c** was obtained in an 80% yield; mp 245−246 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 13.52 (s, 1H), 8.44 (s, 1H), 7.88 (d, J = 9.1 Hz, 2H), 7.61 (d, J = 7.0 Hz, 2H), 7.40 (dt, J = 21.5, 7.1 Hz, 3H), 6.88 (d, J = 9.2 Hz, 2H), 5.68 (s, 2H), 3.11 (s, 6H). HRMS: calcd for $C_{20}H_{19}N_7O$, m/z, 374.1724 [(M + H)$^+$]; found, 374.1716.

*Ethyl 4-((6-(benzyloxy)-9H-purin-2-yl)diazenyl)benzoate (**5d**).* **5d** was obtained in a 90% yield. mp 228−230 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 13.80 (s, 1H), 8.58 (s, 1H), 8.28−8.18 (m, 2H), 8.15−8.06 (m, 2H), 7.66−7.56 (m, 2H), 7.50−7.35 (m, 3H), 5.71 (s, 2H), 4.38 (q, J = 7.1 Hz, 2H), 1.37 (t, J = 7.1 Hz, 3H). HRMS: calcd for $C_{21}H_{18}N_6O_3$, m/z, 403.1513 [(M + H)$^+$]; found, 403.1513.

*6-(Benzyloxy)-2-((4-(4-methylpiperazin-1-yl)phenyl)diazenyl)-9H-purine (**5e**).* **5e** was obtained in a 74% yield; mp 219−221 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 13.57 (s, 1H), 8.46 (s, 1H), 7.88 (d, J = 9.2 Hz, 2H), 7.61 (d, J = 6.9 Hz, 2H), 7.47−7.33 (m, 3H), 7.13 (d, J = 9.2 Hz, 2H), 5.68 (s, 2H), 3.47−3.39 (m, 4H), 2.49−2.41 (m, 4H), 2.24 (s, 3H). HRMS: calcd for $C_{23}H_{24}N_8O$, m/z, 429.2146 [(M + H)$^+$]; found, 429.2133. HPLC: t_r = 17.55 min (99.9%).

Cell Culture. DU145 human prostate carcinoma cells were cultured in α-MEM medium supplemented with 10% fetal bovine serum. EMT6 mouse mammary carcinoma cells were cultured in DMEM supplemented with 10% fetal bovine serum. Both cell lines were maintained at 37 °C in a 5% CO_2 atmosphere.

Cytotoxicity Assays. Cells survival (clonogenic) assays were performed as previously described.[5,20,21] DU145 cells were plated in 25 cm^2 plastic flasks at a density of 2 × 10^5 cells and used when near confluent.[5] Cells were pretreated for 4 h with graded concentrations of **5c** or **5e** under oxic or hypoxic conditions prior to the addition of 100 μM of laromustine for a total incubation time of 24 h at 37 °C. Hypoxia was generated by the direct depletion of oxygen in sealed flasks using the glucose oxidase (2 units/mL, Sigma G6641) and catalase (120 units/mL, Sigma C1345) dual enzyme system as previously described.[5,20,21]

Determination of Half-Wave Reduction Potentials ($E_{1/2}$). The $E_{1/2}$ values were determined by differential pulse polarography (DPP). The supporting electrolyte was 80% by volume 100 mM potassium chloride and 50 mM potassium phosphate (pH 7.0) and 20% by volume of CH_3CN in all cases. Agents were added as 1% by volume solutions in DMSO. The $E_{1/2}$ values of six reference compounds were also measured. Dissolved oxygen was removed by purging with nitrogen. DPP voltammograms were generated using a Princeton Applied Research electrochemical trace analyzer model 394, with a model 303A static mercury drop electrode (Princeton Applied Research, Oak Ridge, TN, USA) utilizing a platinum counter electrode and an Ag/AgCl reference electrode. Voltammograms were taken from 0 to −900 mV at a scan rate of 2 mV/s using a pulse amplitude of 50 mV. The $E_{1/2}$ value was determined from the peak current potential (E_P) using the following equation: $E_{1/2} = E_P$ − pulse amplitude/2.[22]

Cell Dependent O^6-BG Generation. Cell suspensions (10^7 cells/mL) were treated with **5c** (50 μM) under oxic or hypoxic conditions in DMEM (EMT6), or α-MEM (DU145) media containing 10% FBS. Plastic flasks (25 cm^2) with shallow 4 mL layers were employed for oxic studies and were shaken to maintain aeration. The glucose oxidase/catalase/glucose system was used to generate hypoxic conditions before the addition of **5c**; the mixtures were stirred gently in sealed tubes. Using this system, oxygen is depleted in ∼3 min and H_2O_2 is removed rapidly by a large excess of catalase.[5,20,21] It is expected that this low transient exposure to H_2O_2 will have no significant effect on the reduction of **5c** during these 1 h incubations. At various time intervals, samples were withdrawn and the cellular and medium components were precipitated by mixing with an equal volume of acetonitrile for 20 min at room temperature followed by centrifugation at 10000g for 15 min. The supernatant was then analyzed by HPLC for **5c** and O^6-BG.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

AGT, O^6-alkylguanine-DNA alkyltransferase; BCNU, bis(2-chloroethyl)-1-nitrosourea; O^6-BG, O^6-benzylguanine; BG-M2, 2-(4-nitrophenyl)propan-2-yl (6-(benzyloxy)-9H-purin-2-yl)-carbamate; DMEM, Dulbecco's Modified Eagle Medium; MEM, minimal essential media; 2-NBP, 2-nitro-6-benzyloxypurine

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