2-Amino-O^4-benzylpteridine Derivatives: Potent Inactivators of O^6-Alkylguanine-DNA Alkyltransferase

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2-Amino-O^4-benzylpteridine (**1**), 2-amino-O^4-benzyl-6,7-dimethylpteridine (**2**), 2-amino-O^4-benzyl-6-hydroxymethylpteridine (**4**), 2-amino-O^4-benzylpteridine-6-carboxylic acid (**5**), 2-amino-O^4-benzyl-6-formylpteridine (**6**), and O^4-benzylfolic acid (**7**) are shown to be as potent or more potent inactivators of the human DNA repair protein O^6-alkylguanine-DNA alkyltransferase (alkyltransferase) in vitro than O^6-benzylguanine, the prototype alkyltransferase inactivator currently in clinical trials. Additionally, the negatively charged (at physiological pH) inactivators 2-amino-O^4-benzylpteridine-6-carboxylic acid (**5**) and O^4-benzylfolate (**7**) are far more water soluble than O^6-benzylguanine. The activity of O^4-benzylfolic acid (**7**) is particularly noteworthy because it is roughly 30 times more active than O^6-benzylguanine against the wild-type alkyltransferase and is even capable of inactivating the P140K mutant alkyltransferase that is resistant to inactivation by O^6-benzylguanine. All the pteridine derivatives except 2-amino-O^4-benzylpteridine-6-carboxylic acid are effective in enhancing cell killing by 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU). However, the effectiveness of O^4-benzylfolate as an adjuvant for cell killing by BCNU appears to be a function of a cell's α-folate receptor expression. Thus, O^4-benzylfolate is least effective as an adjuvant in A549 cells (which express little if any receptor), is moderately effective in HT29 cells (which express low levels of the receptor), but is very effective in KB cells (which are known to express high levels of the α-folate receptor). Therefore, O^4-benzylfolic acid shows promise as an agent for possible tumor-selective alkyltransferase inactivation, which suggests it may prove to be superior to O^6-benzylguanine as a chemotherapy adjuvant.

Introduction

O^6-Benzylguanine derivatives,[1,2] some O^6-benzylpyrimidines,[3] and related compounds[4,5] are known to be inactivators of the human DNA repair protein O^6-alkylguanine-DNA alkyltransferase (alkyltransferase).[6] This repair protein is the primary source of resistance many tumor cells exhibit to chemotherapeutic agents that modify the O^6-position of DNA guanine residues.[6] Therefore, inactivation of this protein can bring about a significant improvement in the therapeutic effectiveness of these chemotherapy drugs. The prototype inactivator O^6-benzylguanine is currently in clinical trials in the U.S. as an adjuvant in combination with the chloroethylating agent 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) and the methylating agent temozolomide.[7,8] A similar alkyltransferase inactivator, O^6-(4-bromothenyl)guanine is in clinical trials in the UK.[9]

Although O^6-benzylguanine is a promising chemotherapy adjuvant, it is not an ideal drug because its inactivating potency is modest, it exhibits only limited water solubility. and it is not a specific inactivator for alkyltransferase in tumor cells vs normal cells of a host. Therefore, we have sought to prepare additional alkyltransferase inactivators that are more potent, better formulated, more water-soluble, or more tumor-specific than O^6-benzylguanine.[10] Our ongoing studies have led to the discovery of a new class of potent alkyltransferase inactivators, i.e., 2-amino-O^4-benzylpteridine derivatives (**1**, **2**, and **4**−**7**) that readily sensitize cells to killing by BCNU. Furthermore, some members of this class offer significant advantages over O^6-benzylguanine with respect to water solubility and tumor cell selectivity. In particular, O^4-benzylfolic acid (**7**), a very water-soluble inactivator, shows promise for selectively inactivating alkyltransferase in tumor cells that overexpress folic acid receptors. Therefore, 2-amino-O^4-benzylpteridine derivatives represent a promising new class of alkyltransferase inactivator with representatives that may be superior to O^6-benzylguanine as a chemotherapy adjuvant.

Results and Discussion

The 2-amino-O^4-benzylpteridine derivatives studied are 2-amino-O^4-benzylpteridine (**1**), 2-amino-O^4-benzyl-6,7-dimethylpteridine (**2**), 2-amino-O^4-benzyl-6-hydroxymethylpteridine (**4**), 2-amino-O^4-benzylpteridine-6-carboxylic acid (**5**), 2-amino-O^4-benzyl-6-formylpteridine (**6**), and O^4-benzylfolic acid (**7**). Pteridines **1** and **2** were prepared by the method of Pfleiderer and Lohrmann from 2,4,5-triamino-O^6-benzylpyrimidine (**3**) (structure shown in Scheme 1) and glyoxal or diacetyl, respectively.[11] Pteridines **4**−**7** were prepared as illustrated in Scheme 1. Thus, treatment of the triaminopyrimidine

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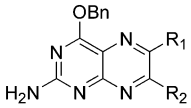
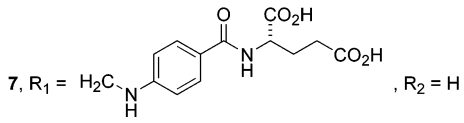


1, $R_1 = R_2 = H$
2, $R_1 = R_2 = CH_3$
4, $R_1 = CH_2OH$, $R_2 = H$
5, $R_1 = CO_2H$, $R_2 = H$
6, $R_1 = CHO$, $R_2 = H$



7, $R_1 =$, $R_2 = H$

(**3**) with dihydroxyacetone in dimethylacetamide (DMA)/H_2O in the presence of sodium ascorbate and air afforded the hydroxymethylpteridine (**4**), which was oxidized to the 6-carboxyl derivative (**5**) with permanganate in acetone water solution. Alternatively, **4** was oxidized to the 6-formylpteridine derivative **6** by treatment with iodoxybenzoic acid (IBX) in dimethyl sulfoxide. Treatment of **6** with *p*-aminobenzoylglutamate (pAB-glu) followed by reduction with sodium cyanoborohydride in dimethylformamide led to formation of O^4-benzylfolic acid (**7**).

The ability of these various compounds to inactivate the human alkyltransferase protein in the presence and absence of calf thymus DNA is summarized Table 1. Data for O^6-benzylguanine are included for comparison. The data are expressed as concentration of inactivator required to reduce the activity of the alkyltransferase protein by 50% (i.e., ED_{50}). As indicated, in the absence of calf thymus DNA, pteridine derivatives **2**, **4**, and **6** exhibit activity similar to that of O^6-benzylguanine whereas derivatives **1**, **5**, and **7** are superior to O^6-benzylguanine as alkyltransferase inactivators. In particular, O^4-benzylfolic acid (**7**) is roughly 30 times more effective than O^6-benzylguanine against the wild-type human alkyltransferase and displays an ED_{50} in the nanomolar range. Interestingly, this same compound is also effective against the P140K mutant alkyltransferase (although at significantly higher concentrations). This protein is essentially resistant to inactivation by O^6-benzylguanine and related derivatives.[10] Previously, only oligodeoxyribonucleotides containing O^6-benzylguanine residues were known to inactivate the P140K and other mutant alkyltransferase proteins.[10]

In the presence of calf thymus DNA, the ED_{50} values for alkyltransferase inactivation increase significantly, suggesting that DNA binding of the protein hinders access of these 2-amino-O^4-benzylpteridines to the protein's active site. This contrasts with the situation for O^6-benzylguanine (Table 1), which exhibits an enhanced ED_{50} in the presence of calf thymus DNA.[12] However, previous studies with 9-substituted O^6-benzylguanine derivatives, which are also sterically hindered from entering the alkyltransferase active site by DNA, show that these are effective inactivators of alkyltransferase in vivo, suggesting that the amount of cellular alkyltransferase bound to DNA must be small or readily exchangeable with the free form.[12]

It is not totally clear yet why compounds **1**, **5**, and **7** are so significantly better than O^6-benzylguanine against the alkyltransferase in the absence of calf thymus DNA. For these compounds it may be that the respective pteridine derivative "leaving groups" are easier to displace by the active site cysteine residue of the alkyltransferase than is guanine from O^6-benzylguanine. Compound **1** is known to be sensitive to acid hydrolysis,[11] indicating that reaction of the benzyl group with water to displace the protonated pteridine leaving group is also facile. Interestingly, **1** is more acid-labile than **2**,[11] which may reflect why **2** exhibits a higher ED_{50} than **1** (Table 1). Clearly, there is not enough data available at present to conclude that ease of leaving group displacement as reflected by relative rates of acid hydrolysis is responsible for the very low ED_{50} values exhibited by **1**, **5**, and **7**. To draw such conclusions, extensive comparisons of the rates of acid hydrolysis of **1**, **2**, and **4**−**7** with hydrolysis rates for O^6-benzylguanine under identical conditions will be required. With respect to O^4-benzylfolic acid (**7**), a combination of ease of displacement and favorable steric interactions with the protein probably contributes to its remarkable effectiveness as an alkyltransferase inactivator. It is possible that the glutamyl residue of **7** (particularly the carboxyl groups) provides an additional binding site on the alkyltransferase protein surface. This would be consistent with the inactivation of mutant P140K because the resistance of this mutant to O^6-benzylguanine results from its prevention of the binding of the benzyl group to the protein via stacking with Pro140. At present we do not know the alkyltransferase site(s) that interact with the folate but have ruled out Arg128 because mutant R128L is inactivated with an ED_{50} of 0.02, very similar to the behavior of the wild type.

We have confirmed that benzylation of the active site cysteine is indeed involved in the mechanism of inactivation of the alkyltransferase by **7** as it is with O^6-benzylguanine. To demonstrate this, peptide fragments

Scheme 1

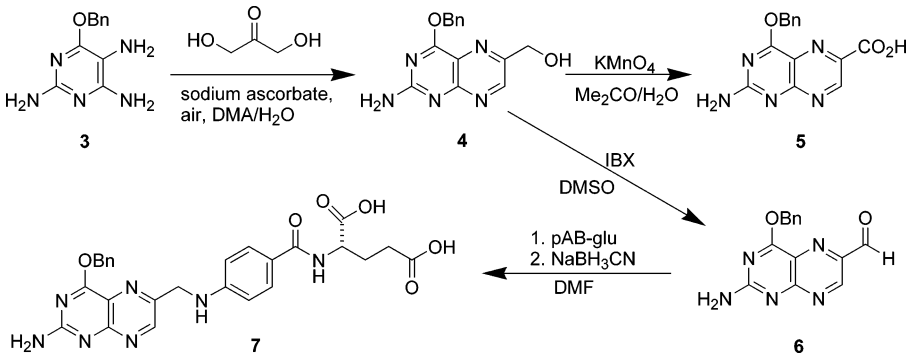


Table 1. Inactivation of Human O^6-Alkylguanine-DNA Alkyltransferase in Vitro in the Absence and Presence of Calf Thymus (ct) DNA

inactivator	-ctDNA[a]		+ctDNA[a]	
	ED_{50} (μM)	*n*	ED_{50} (μM)	*n*
O^6-benzylguanine	0.32 ± 0.08	4	0.12 ± 0.02	3
2-amino-O^4-benzylpteridine (**1**)	0.045 ± 0.01	4	0.45 ± 0.05	6
2-amino-O^4-benzyl-6,7-dimethylpteridine (**2**)	0.4	1	0.5	1
2-amino-O^4-benzyl-6-hydroxymethylpteridine (**4**)	0.2	1	0.4	1
2-amino-O^4-benzyl-6-carboxypteridine (**5**)	0.09	2	1.83 ± 0.62	3
2-amino-O^4-benzyl-6-formylpteridine (**6**)	0.19	2	1.05	2
O^4-benzylfolic acid (**7**)[b]	0.01 ± 0.001	3	0.47 ± 0.05	3

[a] The ED_{50} value was calculated from a graph of residual alkyltransferase activity over a range of 8−10 concentrations, and the experiment was repeated 1−4 times as indicated by *n*. Results are shown as the mean for two separate estimations and the mean ± SD for three more separate estimations. [b] ED_{50} against the P140K mutant alkyltransferase in the absence of ctDNA is 12 μM. In the presence of ctDNA, the compound is inactive at concentrations of ≤1 mM.

Table 2. Concentrations of 2-Amino-O^4-benzylpteridine Derivatives Required To Kill 90% of HT29 Cells (ED_{90}) with BCNU (40 μM)[a]

inactivator	ED_{90} (μM)
O^6-benzylguanine	0.4,[b] 0.7[c]
2-amino-O^4-benzylpteridine (**1**)	0.2[b]
2-amino-O^4-benzyl-6,7-dimethylpteridine (**2**)	0.6[b]
2-amino-O^4-benzyl-6-hydroxymethylpteridine (**4**)	0.7[b]
2-amino-O^4-benzyl-6-carboxypteridine (**5**)	inactive at 30 μM[b]
2-amino-O^4-benzyl-6-formylpteridine (**6**)	0.7[c]
O^4-benzylfolic acid (**7**)	15,[d] 24[c]

[a] Cells were completely resistant to 40 μM BCNU treatment in the absence of alkyltransferase inactivator. [b] Studies carried out in Dulbecco's medium (which contains 9.1 μM folic acid). The alkyltransferase inactivator was present before, during, and after treatment with BCNU for 16−18 h before replating (see Experimental Section). [c] Studies carried out in RPMI medium (which contains 2.3 μM folic acid). The alkyltransferase inactivator was present before and during BCNU treatment only. [d] Studies carried out in folate-free RPMI medium. The alkyltransferase inactivator was present before and during BCNU treatment only.

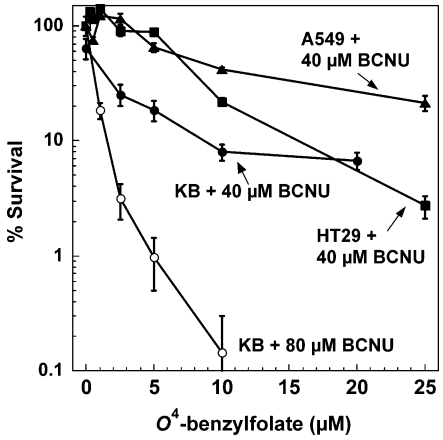


Figure 1. Cell killing by O^4-benzylfolic acid and BCNU. The plating efficiency was 606 ± 33 colonies per 1000 cells for HT29, 700 ± 98 colonies per 1000 cells for KB, and 689 ± 8 colonies per 1000 cells for A549 cells and was not affected by the doses of BCNU used.

obtained by digestion of the alkyltransferase protein with trypsin were separated and analyzed by MALDI-TOF mass spectroscopy on an Applied Biosystems 4700 Proteomics analyzer, using α-cyanohydroxycinnamic acid as the sample matrix. A peak of *m*/*z* 1315.75, corresponding to the theoretical mass of the unmodified tryptic fragment GNPVPILIPCHR, was observed in the spectrum from the unmodified protein. This peak was not observed in the tryptic digest of either the O^6-benzylguanine or O^4-benzylfolic acid treated protein, but a new peak at *m*/*z* 1405.78 or 1405.75, respectively, appeared instead, corresponding to the theoretical mass of GNPVPILIP(benzyl-C)HR. This 1405.75 peak was then subjected to collision-induced fragmentation in a tandem MS/MS analysis on the same instrument, and the dominant peaks observed in the resulting spectra corresponded to the theoretical immonium ions, y-ions, and internal fragment ions that would be expected from fragmentation of the modified peptide containing *S*-benzylcysteine.

Not surprisingly, the 2-amino-O^4-benzylpteridine derivatives **1**, **2**, **4**, **6**, and **7** are all capable of enhancing HT29 cell killing by BCNU (Table 2), which is a common property of alkyltransferase inactivators.[1−6,13] 2-Amino-O^4-benzylpteridine-6-carboxylic acid (**5**) is not effective probably because the negative charge on the molecule at physiological pH prevents its easy entry into cells. Even though O^4-benzylfolic acid is also anionic at physiological pH, it does enhance cell killing by BCNU but it was much less effective than compounds **1**, **2**, **4**, and **6**, suggesting that its uptake is brought about by some active process but that uptake is limited. This would be consistent with uptake occurring via the α-form of the folic acid receptor, and **7** was more effective when tested in a folate-free medium (Table 2). Further support for this concept is shown in Figure 1, which shows A549 lung tumor, HT29 colon tumor, and KB nasopharyngeal tumor cell killing by 40 μM BCNU following a 2 h exposure to **7** in folate-free growth medium. KB cell killing by 80 μM BCNU in combination with **7** is also illustrated. The A549 and HT29 cells are completely resistant to killing by 40 μM BCNU in the absence of modulator because of their alkyltransferase activity. The KB cells, which have a higher alkyltransferase level, are completely resistant to even 80 μM BCNU. As shown, the effectiveness of **7** as an adjuvant was lowest in A549 cells, was somewhat greater in HT29 cells, and was greatest in KB cells. A549 cells express little, if any, α-folate receptor, HT29 cells express low levels of the receptor, and KB cells express high levels of the receptor.[14−17] Thus, O^4-benzylfolic acid may be a useful agent for selectively inactivating alkyltransferase in tumors that overexpress the α-folate receptor. These tumors are numerous and include adenocarcinomas, ovarian, endometrial, and bronchioloalveolar carcinomas, some non-small-cell lung carcinomas, small-cell lung carcinomas, squamous cell carcinomas, colorectal carcinomas, gastric carcinomas, and kidney tumors.[17] Such tumor selectivity would be very advantageous because the side effects associated with systemic alkyl-

transferase inactivation[7,8] could be significantly reduced.

Experimental Section

Materials and Methods. Unless otherwise stated, chemicals were obtained from Aldrich (Milwaukee, WI) or Sigma (St. Louis, MO) and were used without further purification. UV spectra were determined on a Beckman Coulter DU 7400 spectrophotometer. ^{1}H and ^{13}C NMR spectra were recorded in DMSO-d_6 with a Varian INOVA 400 MHz spectrometer. Chemical shifts are reported as δ values in parts per million relative to TMS as internal standard. The splitting pattern abbreviations are as follows: s = singlet, d= doublet, dd = double doublet, t = triplet, m = multiplet. Elemental analyses, performed by Atlantic Microlab, Inc. (Norcross, GA) were within 0.4% of the theoretical values calculated for C, H, and N. Thin-layer chromatographic analyses were performed using precoated, aluminum-backed silica gel plates, and the spots were visualized with UV light. All silica gel column chromatography was carried out using Davisil grade 633, 200−425 mesh, 60 Å. Compounds **1**−**3** were prepared by the method of Pfleiderer and Lohrmann.[11]

2-Amino-*O*^{4}-benzyl-6-hydroxymethylpteridine (4). 2,4,5-Triamino-*O*^{6}-benzylpyrimidine (**3**) (3.26 g, 14.1 mmol) was dissolved in DMA/H_2O (1:1, 28 mL) and stirred at room temperature. Sodium ascorbate (2.85 g, 14.4 mmol) was added followed by dihydroxyacetone dimer (2.57 g, 14.3 mmol). The reaction mixture was heated to 40 °C, and air was bubbled into the reaction flask through a Pasteur pipet. The reaction was monitored by TLC (CH_2Cl_2/MeOH, 10:1). After 4 h all the starting material was consumed, and the reaction mixture was poured into 250 mL of H_2O to produce a yellow-orange solid. This solid was collected by filtration, was dissolved in CH_2Cl_2/MeOH (3:1, 500 mL), and was dried over $MgSO_4$. The solution was filtered and evaporated onto silica gel (100 mL). Product was eluted from a silica gel column with CH_2Cl_2/MeOH (20:1) and fractions containing product were pooled and evaporated to produce 2-amino-*O*^{4}-benzyl-6-hydroxymethylpteridine (**4**) (1.12 g, 28.1%). UV (MeOH/0.05 M phosphate, pH 6.8, 5:95) λ_{max} 234 nm (ϵ = 18 800), 264 nm (ϵ = 9200), 366 (ϵ = 6900); ^{1}H NMR δ 8.88 (1H, s, H-7), 7.56 (2H, m, ArH), 7.40 (3H, m, ArH), 7.28 (2H, s, N^2H_2, exchange with D_2O), 5.58 (1H, t, J = 5.9 Hz, 6-CH_2O**H**, exchanges with D_2O), 5.55 (2H, s, $ArCH_2$), 4.62 (2H, d, J = 5.9 Hz, 6-C**H$_2$**OH changes to a singlet in D_2O); ^{13}C NMR (100 MHz) δ 166.4, 161.2, 156.5, 151.1, 150.0, 135.8, 128.9, 128.5, 128.3, 121.2, 68.4, 62.7. Anal. ($C_{14}H_{13}N_5O_2$·0.5H_2O) C, H, N.

2-Amino-*O*^{4}-benzylpteridine-6-carboxylic Acid (5). 2-Amino-*O*^{4}-benzyl-6-hydroxymethylpteridine (**4**) (0.24 g, 0.84 mmol) was suspended in acetone/0.5 M phosphate buffer, pH 7 (1:1, 14 mL), and stirred at room temperature. Potassium permanganate (0.34 g, 2.18 mmol) was added in four portions at 30 min intervals. The resulting suspension was then stirred at room temperature for an additional 3 h. The reaction mixture was diluted with H_2O (50 mL). Sodium sulfite was added until all of the permanganate was consumed, producing a brown-black precipitate, which was removed by filtration, leaving a clear, yellow solution. The pH was adjusted to 2.5 by the addition of 2 M HCl, producing a yellow solid, which was collected by filtration. The solid was dissolved in H_2O (50 mL) by adjusting the pH to 7.0 through the addition of 0.1 M NaOH until the pH remained constant for 30 min. Any suspended solid material was filtered and the solution was evaporated to give the sodium salt. This product was purified on a 3 cm × 80 cm Sephadex LH-20 column and was eluted with H_2O (1 mL/min). UV absorption was monitored continuously at 280 nm. Fractions (10 mL) 34−44 containing the product were combined, and the pH was adjusted to 2.5 with HCl to precipitate the product, which was collected by filtration and dried under vacuum to afford **5** (0.17 g, 67%). ^{1}H NMR δ 13.52 (1H, s, CO_2H, exchanges with D_2O), 9.25 (1H, s, H-7), 7.83 (1H, N^2H_a, exchanges with D_2O), 7.70 (1H, N^2H_b, exchanges with D_2O), 7.58 (2H, m, ArH), 7.41 (3H, m, ArH), 5.60 (2H, s, $ArCH_2$); ^{13}C NMR (100 MHz) δ 166.9, 164.8, 162.6, 158.1, 151.3, 137.4, 135.5, 129.0, 128.5, 128.45, 122.5, 68.75. Anal. ($C_{14}H_{11}N_5O_3$·H_2O) C, H, N.

2-Amino-*O*^{4}-benzyl-6-formylpteridine (6). Iodoxybenzoic acid (IBX) (1.7 g, 6.1 mmol) was stirred in DMSO (16 mL) until it dissolved. 2-Amino-*O*^{4}-benzyl-6-hydroxymethylpteridine (**4**) (1.16 g, 4.1 mmol) was added with constant stirring at room temperature to produce a dark-orange solution. The reaction was complete in 2 h as monitored by TLC (CH_2Cl_2/MeOH, 10:1). The reaction mixture was poured into H_2O (150 mL) to produce a pale-yellow precipitate, which was collected by filtration. This solid was stirred at 40 °C in CH_2Cl_2/acetone (1:1, 250 mL) for approximately 30 min and was filtered to remove the iodosobenzoic acid byproduct. This process was repeated twice. The dissolved product was evaporated onto silica (50 mL) and was eluted from a silica gel column with CH_2Cl_2/CH_3CN (7:3). Solvent was evaporated to give 2-amino-*O*^{4}-benzyl-6-formylpteridine (**6**) (0.26 g, 0.92 mmol, 22.4%). UV (MeOH/0.05 M phosphate, pH 6.8, 5:95) λ_{max} 236 nm (ϵ = 13 600), 261 (sh) (ϵ = 9600) 309 nm (ϵ = 5200), 370 (ϵ = 9300); ^{1}H NMR δ 9.96 (1H, s, 6-CHO), 9.19 (1H, s, H-7), 8.03 (1H, s, N^2H_a, exchanges with D_2O), 7.89 (1H, s, N^2H_b, exchanges with D_2O), 7.60 (2H, m, ArH), 7.42 (3H, m, ArH), 5.62 (2H, s, $ArCH_2$); ^{13}C NMR (100 MHz) δ 191.2, 166.9, 163.1, 159.0, 149.2, 141.0, 135.4, 129.1, 128.51, 128.49, 122.8, 69.0. Anal. ($C_{14}H_{11}N_5O_2$) C, H, N.

***O*^{4}-Benzylfolic Acid (7).** 2-Amino-*O*^{4}-benzyl-6-formylpteridine (**6**) (0.26 g, 0.92 mmol) and *p*-aminobenzoylglutamate (pAB-glu) (0.29 g, 1.1 mmol) were stirred in DMF (4.4 mL) until they were completely dissolved. $NaBH_3CN$ (0.08 g, 1.3 mmol) was added with stirring. After approximately 5 min, the reaction color changed from yellow-orange to red. TLC (CH_2Cl_2/MeOH/AcOH, 90:5:5) showed complete loss of **6**. The reaction mixture was poured into vigorously stirred water (50 mL), producing a yellow precipitate that dissolved when the pH of the suspension was adjusted to 7.2 by the addition of 2 M NaOH. Activated charcoal (20 mg) was added and was then filtered out. The solution pH was then adjusted to 3.0 by the addition of 2 M HCl, producing a yellow precipitate that was collected by filtration. The solid was dissolved in CH_2Cl_2/MeOH (3:1) and evaporated onto silica (30 mL). The product was eluted from a silica gel column with CH_2Cl_2/MeOH/AcOH (90:5:5). The solvent was evaporated and the product was suspended in vigorously stirred H_2O to produce a fine solid (**7**), which was filtered and dried under vacuum (87 mg, 0.16 mmol, 17.7%). UV (0.05 M phosphate, pH 6.8) λ_{max} 277 nm (ϵ = 19 000), 289 nm (sh) (ϵ = 18 100), 368 nm (ϵ = 8200); ^{1}H NMR δ 12.31 (2H, br s, $2CO_2H$), 8.79 (1H, s, 7-H), 8.12 (1H, d, J = 7.7 Hz, glu-NH, exchanges with D_2O), 7.64 (2H, d, J = 8.7 Hz, pABArH), 7.57 (2H, m, BnArH), 7.41 (3H, m, BnArH), 7.29 (2H, br-s, N^2H_2, exchange with D_2O), 6.95 (1H, t, J = 6.1 Hz, 6-CH_2N**H**, exchanges with D_2O), 6.64 (2H, d, J = 8.8 Hz, pABArH), 5.58 (2H, s, $BnCH_2$), 4.50 (2H, d, J = 5.7 Hz, 6-C**H$_2$**-NH, singlet in D_2O), 4.33 (1H, m, gluα-CH, dd in D_2O), 2.32 (2H, t, J = 7.5 Hz gluγ-CH_2), 2.04 (1H, m, gluβ-CH_{2a}), 1.90 (1H, m, gluβ-CH_{2b}); ^{13}C NMR (100 MHz) δ 173.9, 173.7, 166.4, 166.3, 161.3, 156.5, 150.8, 150.3, 149.1, 135.9, 129.0, 128.8, 128.5, 128.3, 121.6, 121.3, 111.2, 68.4, 51.7, 46.1, 30.4, 24.0. Anal. ($C_{26}H_{25}N_7O_6$·0.5H_2O) C, H, N. Alternatively, the crude product before silica gel chromatography (see above) was dissolved in H_2O, the pH of which was adjusted to 7.0 by the addition of 2 M NaOH, and the solution was evaporated to give the sodium salt. This product was purified on a Sephadex LH-20 column (3 cm × 80 cm) and was eluted with aqueous 0.1 M sodium acetate solution at 1 mL/min. UV absorption was monitored continuously at 280 nm. Fractions (10 mL) 54−90 containing the product were combined, and the pH was adjusted to 2.5 with HCl to precipitate the product, which was collected by filtration and dried under vacuum.

In Vitro Alkyltransferase Activity Assay. Purified recombinant human alkyltransferase was incubated with different concentrations of inactivator in 0.5 mL of reaction buffer (50 mM Tris-HCL, pH 7.6, 0.1 mM EDTA, 5.0 mM dithiothreitol) containing 50 μg of hemocyanin for 30 min at 37 °C. The remaining alkyltransferase activity was determined after

incubation with [^{3}H]methylated calf thymus DNA substrate for 30 min at 37 °C by measuring the [^{3}H]methylated protein formed, which was collected on nitrocellulose filters.[18] The results were expressed as the percentage of the alkyltransferase activity remaining. The concentration of inhibitor that led to a 50% loss of alkyltransferase activity (ED_{50}) was calculated from graphs of the percentage of remaining alkyltransferase activity against inactivator concentration. For assays in the presence of DNA, 10 μg of calf thymus DNA was added before incubation with the inactivators.

Cell Culture and Cytotoxicity Assays. Cells were grown either in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum plus 1.5 mM glutamine and 50 μg/mL gentamycin (HT29) or in RPMI 1640 medium in the presence of 10% fetal bovine serum (HT29, A549, and KB cells). The effect of alkyltransferase inactivators on the sensitivity of cells to BCNU was determined using a colony-forming assay.[13] Cells were plated at a density of 10^6 in 25 cm^2 flasks and 24 h later were incubated with different concentrations of alkyltransferase inactivator for 2 h before exposure to 40 μM (HT29 cells and A549 cells) or 80 μM (KB cells) of BCNU for 2 h. The BCNU was first dissolved in absolute ethanol at a concentration of 8 mM, was diluted with the same volume of ice-cold phosphate-buffered saline, and was immediately used to treat the cells. The medium was replaced with fresh medium containing alkyltransferase inactivator (unless otherwise indicated), and the cells were left to grow for an additional 16−18 h. The alkyltransferase inactivator was added to the medium after the treatment with BCNU to ensure that the inactivator was present during the entire period that DNA adducts are formed by BCNU. The cells were then replated at densities of 200−2000 cells per 25 cm^2 flasks and grown for 8 days until discrete colonies were formed. The colonies were washed with 0.9% saline solution, were stained with 0.5% crystal violet in ethanol, and counted. The plating efficiency of cells not treated with drugs was about 50% for HT29 and A549 cells and 80% for KB cells. In experiments to assess the effect of folate present in the medium on the sensitivity of cells to the alkyltransferase inactivators and BCNU, the cells were incubated with inactivators for 2 h and with BCNU for 2 h in RPMI 1640 folate-free medium. After this period, the medium was replaced with fresh RPMI 1640 medium, which contains 2.3 μM folic acid.

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