Inactivation of O^6-Alkylguanine-DNA Alkyltransferase by Folate Esters of O^6-Benzyl-2′-deoxyguanosine and of O^6-[4-(Hydroxymethyl)benzyl]guanine

Sahar Javanmard,[†] Natalia A. Loktionova,[‡] Qingming Fang,[‡] Gary T. Pauly,[†] Anthony E. Pegg,*[,‡] and Robert C. Moschel[†,§]

Laboratory of Comparative Carcinogenesis, National Cancer Institute at Frederick, Post Office Box B, Building 538, Frederick, Maryland 21702, and Departments of Cellular and Molecular Physiology and Pharmacology, The Pennsylvania State University, College of Medicine, Post Office Box 850, Hershey, Pennsylvania 17033

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O^6-Alkylguanine-DNA alkyltransferase (alkyltransferase) provides an important source of resistance to some cancer chemotherapeutic alkylating agents. Folate ester derivatives of O^6-benzyl-2′-deoxyguanosine and of O^6-[4-(hydroxymethyl)benzyl]guanine were synthesized and tested for their ability to inactivate human alkyltransferase. Inactivation of alkyltransferase by the γ-folate ester of O^6-[4-(hydroxymethyl)benzyl]guanine was similar to that of the parent base. The γ-folate esters of O^6-benzyl-2′-deoxyguanosine were more potent alkyltransferase inactivators than the parent nucleoside. The 3′-ester was considerably more potent than the 5′-ester and was more than an order of magnitude more active than O^6-benzylguanine, which is currently in clinical trials to enhance therapy with alkylating agents. They were also able to sensitize human tumor cells to killing by 1,3-bis(2-chloroethyl)-1-nitrosourea, with O^6-benzyl-3′-*O*-(γ-folyl)-2′-deoxyguanosine being most active. These compounds provide a new class of highly water-soluble alkyltransferase inactivators and form the basis to construct more tumor-specific and potent compounds targeting this DNA repair protein.

Introduction

O^6-Alkylguane-DNA alkyltransferase (alkyltransferase[a]) is a widespread DNA repair protein that acts by transferring adducts such as methyl- or 2-chloroethyl- from the O^6-position of guanine in DNA to a cysteine acceptor site.[1−3] The alkyltransferase-mediated repair of DNA adducts in cells exposed to therapeutic alkylating agents such as temozolomide or 1,3-bis-(2-chloroethyl)-1-nitrosourea (BCNU) provides a major mechanism of resistance to these drugs. Inactivation of alkyltransferase therefore improves the response to these agents. A prototype inactivator O^6-benzylguanine is undergoing clinical trials in the U.S.[3−5] and a similar compound, O^6-(4-bromothenyl)guanine, is being tested in the U.K.[6,7] Although there have been some responses in the trials with O^6-benzylguanine, it is apparent that the lack of selectivity of the drug toward the tumor alkyltransferase may be a significant factor in limiting its effectiveness. Several approaches are in progress to render alkyltransferase inactivation more tumor-specific. These include attempts to make prodrugs that would be preferentially activated in tumors to generate alkyltransferase inhibitors[8] and to make compounds that would be selectively taken up by tumors. A promising candidate for the later approach is O^4-benzylfolic acid (Figure 1), which is a powerful alkyltransferase inhibitor that is considerably more active than O^6-benzylguanine and is likely to be taken up by the folate receptor system.[9] In the present work, we describe the synthesis and the properties of a number of other folate derivatives, which are also shown in Figure 1. These were γ-folate esters of O^6-benzyl-2′-deoxyguanosine attached through the 3′- or 5′-hydroxyl (**1** and **2**) and the folic acid γ-ester of O^6-[4-(hydroxymethyl)benzyl]-guanine (**3**).

Previous studies with glucuronic acid derivatives of O^6-benzylguanine and O^6-benzyl-2′-deoxyguanosine linked via the N^2- position showed that these were inactive prodrugs but were converted by β-glucuronidase to the active compounds.[8] It was possible that the folate esters would act as similar prodrugs that could be accumulated via the folate transport mechanism. However, the studies described here show that all of the folate esters tested were active as alkyltransferase inactivators without need for metabolic activation and that **1** was a very potent compound. Both **1** and **2** were more active than the parent O^6-benzyl-2′-deoxyguanosine in vitro. In contrast, the addition of a folic acid moiety via a γ-ester to O^6-[4-(hydroxymethyl)-benzyl]guanine forming **3** only slightly reduced the ability to inactivate alkyltransferase. The esters were all converted to the parent compounds by esterases found in tumor cells. The ability of compounds **1**−**3** to sensitize cells to killing by BCNU was also investigated using tumor cells that differ in their folate receptor status.

Results and Discussion

Synthesis of Folate Ester Derivatives. For the synthesis of the 3′- and 5′-γ-folate esters of O^6-benzyldeoxyguanosine (compounds **1** and **2**), the starting material was O^6-benzyl-5′-*O*-(4,4′-dimethoxytrityl)-N^2-phenoxyacetyl-2′-deoxyguanosine (**4**). This compound had been previously prepared in this laboratory for use in the synthesis of oligonucleotides containing O^6-benzyldeoxyguanosines.[10] Protecting groups on the nucleoside were manipulated to give O^6-benzyldeoxyguanosine having either a free 3′-hydroxyl (Scheme 1) or free 5′-hydroxyl (Scheme 2), which could be selectively coupled to the γ-carboxylate of bis-silyl-protected folic acid[11] using a carbodiimide.

Compound **3** was prepared similarly by carbodiimide coupling of the hydroxyl group on O^6-[4-(hydroxymethyl)benzyl]guanine (**12**), which had been prepared as described[12] to the same silyl-

* To whom correspondence should be addressed. Phone: 717-531-8152. Fax: 717-531-5157. E-mail: aep1@psu.edu.
[†] National Cancer Institute at Frederick.
[‡] The Pennsylvania State University.
[§] Deceased April 20, 2007.

[a] Abbreviations: alkyltransferase, O^6-alkylguane-DNA alkyltransferase; PAc, phenoxyacetyl; DMAP, 4-dimethylaminopyridine; TBAF, tetrabutylammonium fluoride; EDC, 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride.

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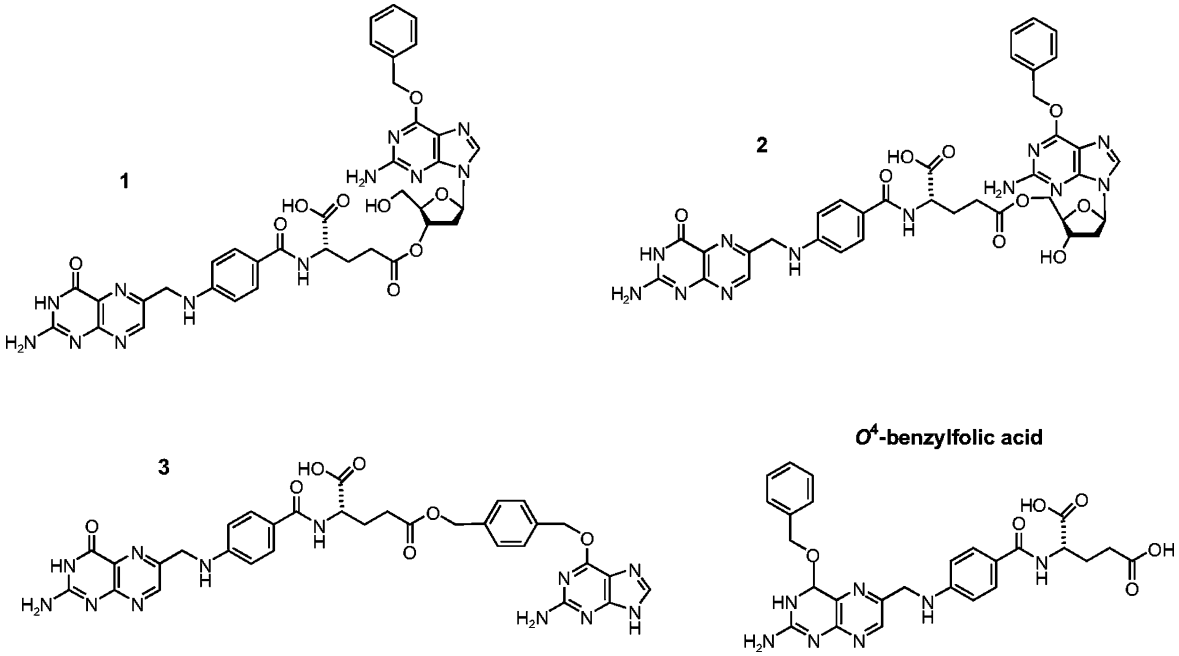
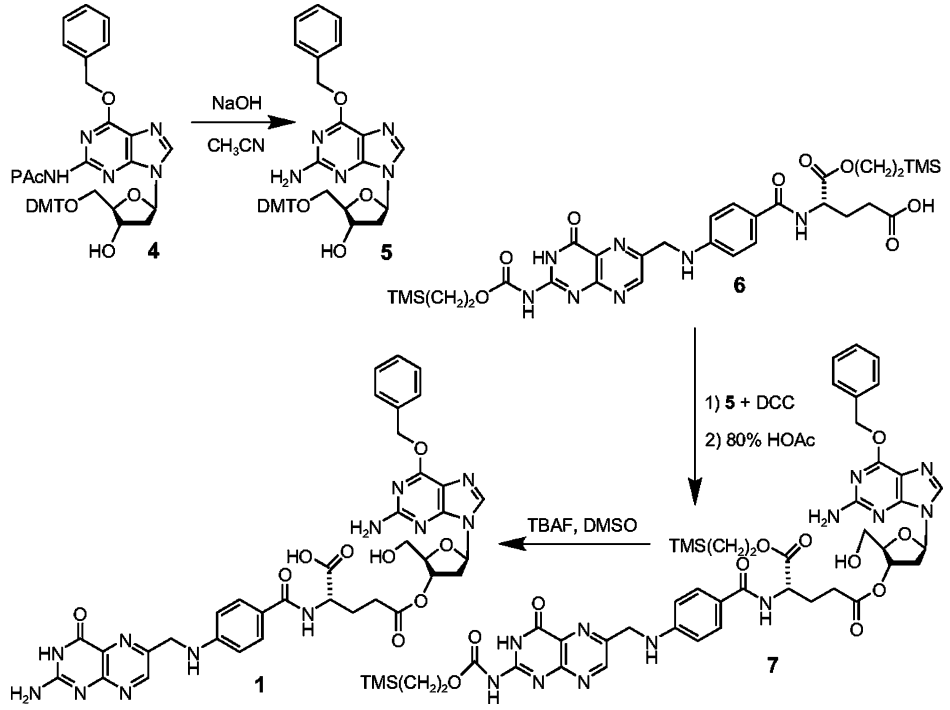


Figure 1. Structures of folate conjugates.

Scheme 1. Synthesis of 3′-γ-Folate Ester of O^6-Benzyldeoxyguanosine (**1**)[a]



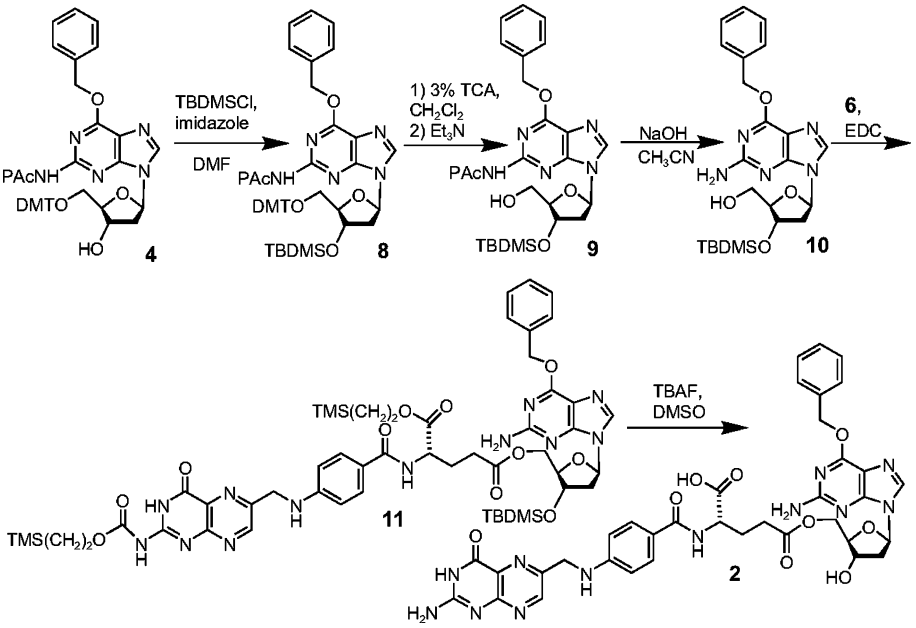
[a] PAc indicates phenoxyacetyl.

protected folate (Scheme 3). The resulting esters were purified, deprotected, and subsequently isolated by mild acid precipitation.

Inactivation of Purified Human Alkyltransferase In Vitro. Previous studies have shown that O^6-benzyl-2′-deoxyguanosine is about an order of magnitude less active than the free base O^6-benzylguanine in the ability to inactivate purified alkyltransferase in vitro.[13] When assays were conducted in the presence of DNA, this difference was considerably larger (>100-fold) because the inactivation by O^6-benzylguanine is facilitated by DNA, whereas it is reduced in the case of O^6-benzyl-2′-deoxyguanosine.[14] Both **1** and **2** were more active than O^6-benzyl-2′-deoxyguanosine itself in the inactivation of alkyltransferase (Table 1). Remarkably, the 3′-ester (**1**) was an extremely potent alkyltransferase inactivator with an ED_{50} value in the absence of DNA of 16 nM, which is 125 times lower than the parent O^6-benzyl-2′-deoxyguanosine and 19 times lower than O^6-benzylguanine. When DNA was present, the ED_{50} value of **1** was increased to 0.68 μM, but this is still much less than that of O^6-benzyl-2′-deoxyguanosine itself (40 μM). Compound **1** was also able to inactivate the P140K mutant of alkyltransferase, which is totally resistant to O^6-benzylguanine or O^6-benzyl-2′-deoxyguanosine,[15,16] but quite high concentrations were required (ED_{50} of about 100 μM; results not shown).

The 5′-ester (**2**) was also a potent inactivator of alkyltransferase that was more effective in the absence of DNA but it

Scheme 2. Synthesis of 5′-γ-Folate Ester of O^6-Benzyldeoxyguanosine (**2**)



Scheme 3. Synthesis of Folic Acid γ-Ester of O^6-[4-(Hydroxymethyl)benzyl]guanine (**3**)

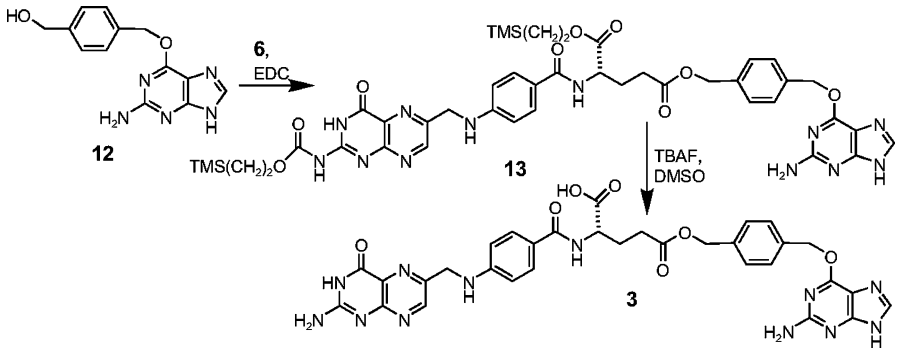


Table 1. Inactivation of Purified Human Alkyltransferase In Vitro

cmpd	ED_{50} for inactivation of alkyltransferase[a] (μM) −DNA	+DNA
O^6-benzylguanine[b]	0.3	0.1
O^6-benzyl-2′-deoxyguanosine[c]	2.0	40.0
1	0.016 ± 0.002	0.68 ± 0.02
2	0.64 ± 0.05	3.06 ± 0.18
3	0.70	0.24
12[d]	0.4	0.1

[a] ED_{50} values were calculated from graphs of the percentage of remaining alkyltransferase activity against inhibitor concentration. Values are the mean ± SEM for four estimations or the mean of two experiments. [b] Previously published.[14,17] [c] Previously published.[13,14] [d] Previously published.[12,18]

was considerably less active than **1**, with an ED_{50} value of 640 nM compared to 16 nM for **1** (Table 1). When DNA was present, the ED_{50} of **2** increased 5-fold to about 3 μM. These results suggest that the addition of a folate moiety increases the ability of O^6-benzyl-2′-deoxyguanosine to bind to human alkyltransferase and that this binding occurs more favorably when the folate is attached to the 3′- rather than the 5′-position.

The ability of DNA to reduce the effectiveness of O^6-benzyl-2′-deoxyguanosine and other 9-substituted O^6-benzylguanine derivatives has been attributed to a competition between the DNA and the 9-substituent for binding at the active site.[14] Such competition would still be expected to occur with the folate esters, but the fact that they are still more active than O^6-benzyl-2′-deoxyguanosine itself is consistent with the concept that these folate derivatives also interact with the alkyltransferase at residues not involved in the binding of DNA. Although it was originally envisaged that **1** and **2** might act as prodrugs that would be converted to an active alkyltransferase inhibitor, it appears that this conversion to O^6-benzyl-2′-deoxyguanosine is not necessary and would actually reduce their effectiveness.

Compound **3**, the folic acid γ-ester of **12**, was slightly less effective than the free base parent compound **12** in the inactivation of alkyltransferase (Table 1). Both compounds resemble O^6-benzylguanine in that inactivation is slightly greater when DNA is present. This result is in agreement with a number of studies that have shown that adducts can be added to the *para*-position of O^6-benzylguanine without greatly affecting the ability to interact with alkyltransferase[12,19] including large fluorescent derivatives.[20] This finding is consistent with models of the binding of O^6-benzylguanine to alkyltransferase, which shows that there is a space available around the *para*-position of the benzyl group that is contiguous with the opening to the active site pocket.[21,22] Large adducts can therefore fit into this space without interactions with the protein. The small increase in reactivity when DNA is present, which is seen with O^6-benzylguanine, **12** and **3** (Table 1) is likely to be due to an activation of the cysteine acceptor part of the protein that occurs when DNA is bound facilitating the alkyl transfer.[23]

Stability and Enzymatic Hydrolysis of Compounds 1−3. Compounds **1**−**3** were relatively stable when incubated in neutral solution. The rate of release of folate was approximately

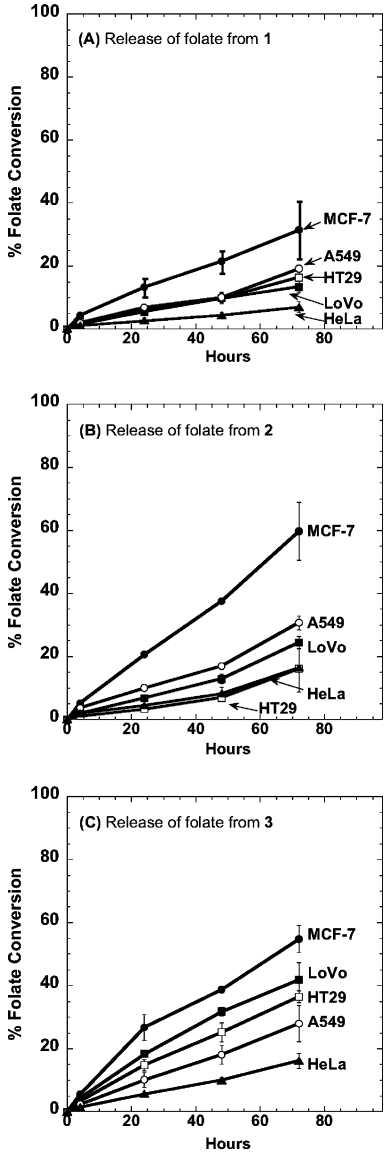


Figure 2. Release of folate from **1**, **2**, and **3** by cell extracts.

2.2%/day from **1**; 3.2%/day from **2**; and 0.9%/day from **3**. Extracts from a variety of human tumor cells and porcine liver esterase were able to release folate from **1**, **2**, and **3** (Figure 2). Compound **3** was a much better substrate for porcine liver esterase than **2** or **1** with 592 pmol/h/unit converted to folate. This rate is 10−20 times that of the conjugates with O^6-benzyl-2′-deoxyguanosine, where 20 pmol/h/unit of **1** was converted to folate and 46 pmol/h/unit of **2** was hydrolyzed. The extent of conversion to folate by human tumor cell extracts varied slightly according to the cell type with MCF-7 cells showing the highest rate of conversion. All tumor cells tested had esterase activity toward the compounds, but this activity was quite weak, with more than 50% of the compound remaining after 48 h. The 5′-ester (**2**) was a slightly better substrate than the 3′-ester (**1**). There was very little cleavage (less than 2%) of either compound in culture medium containing 10% serum for 24 h.

Sensitization of Tumor Cells to Killing by BCNU. The ability of the ester inhibitors to sensitize human tumor cells to killing by BCNU was investigated using HT29, A549, and KB cells. As shown in Figure 3, both **1** and **2** were able to sensitize HT29 cells to killing by BCNU. However, their activity appears to be limited by a low uptake. Both esters were less effective than their O^6-benzyl-2′-deoxyguanosine parent despite being

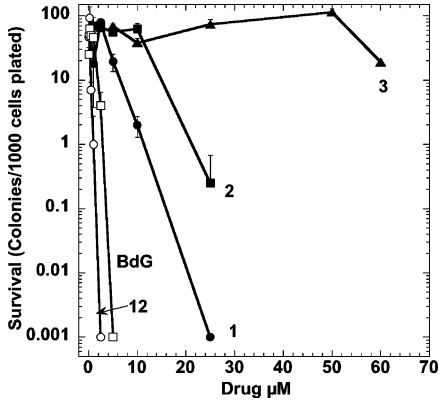


Figure 3. Killing of HT29 cells by BCNU plus alkyltransferase inhibitors. Results are shown for HT29 cells grown in RPMI medium and exposed to the drugs indicated for 2 h prior to addition of 40 μM BCNU for 2 h. Results are shown for treatment with O^6-benzyl-2′-deoxyguanosine (BdG) and with compounds **1**, **2**, **3**, and **12**.

better alkyltransferase inactivators in vitro. The observation that **1** is more active than **2** in all of the cells tested (Figure 4) is consistent with the more potent inactivation of alkyltransferase in vitro by the 3′-ester. This also supports the concept that their effects are due to the esters themselves rather than their conversion to O^6-benzyl-2′-deoxyguanosine because **2** was a better esterase substrate than **1**.

Compound **3** was much less effective than **1** and **2** in increasing the killing of HT29 cells by BCNU (Figure 3). In contrast, its parent **12** was very active. This result is consistent with limited uptake of **3** and poor conversion to **12** in either the cells or the culture medium.

To test whether compounds **1**−**3** entered cells via the folate receptor mechansim, studies were carried out to examine the killing by BCNU of A549, HT29, and KB cells grown for 48 h in the absence of folate prior to the addition of the inhibitors (Figure 4). The inhibitors were added for 8 h prior to treatment with BCNU to allow increased time for alkyltransferase inactivation to occur, but other experiments (not shown) with 2 h or 4 h drug exposure times gave similar results. Both **1** and **2** were able to sensitize all three tumor cells to killing by BCNU, and **1** was more potent than **2**. Approximately the same degree of sensitization was seen with the three cell lines even though A549 cells have very low levels of folate receptors, and KB cells have a very high folate receptor carrier activity. This suggests that the compounds are not taken up via a folate receptor mediated mechanism. This is supported by comparison of the effects of an 8 h exposure to drugs on HT29 cells grown in the folate-free medium shown in Figure 4B with the effects of a 2 h exposure on HT29 cells grown in the folate containing medium in Figure 3. There was little difference in the effect under the two conditions. Experiments in which the addition of 100 μM folate 30 min prior to the inhibitor did not affect the results with **1** and **2** on KB or HT29 cells (results not shown) provide additional evidence that their effect is not dependent on a folate carrier. In contrast, previous studies with O^4-benzyfolate[9] showed that growing cells in folate-free medium increased the killing effects of BCNU when O^4-benzylfolate was added to KB or HT29 cells but not A549 cells and that the addition of 100 μM folate 30 min prior to the inhibitor blocked this effect.

Compound **3** was tested only in HT29 and KB cells grown in the folate-free medium (Figure 4B,C), and this compound was very poorly active in both cells. It was slightly more effective in KB cells. This suggests that it may be taken up in

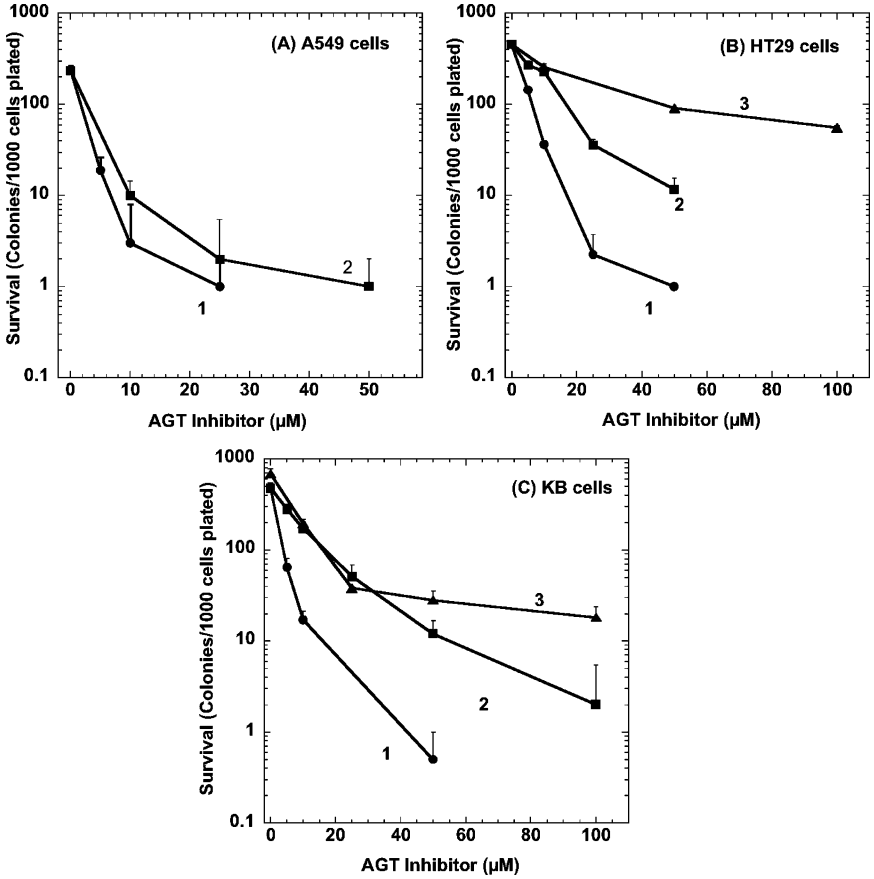


Figure 4. Killing of A549, HT29, and KB cells grown in folate-free medium by BCNU plus alkyltransferase inhibitors. Cells were grown in RPMI medium minus folate for 48 h and exposed to the drugs for 8 h prior to addition of BCNU for 2 h. The amount of BCNU used was the maximum dose that gave no decrease in survival in the absence of alkyltransferase inhibition. Panel A shows results for A549 cells treated with **1** or **2** and 20 μM BCNU. Panel B shows results for HT29 cells treated with **1**, **2**, or **3** and 20 μM BCNU. Panel C shows results for KB cells treated with **1**, **2**, or **3** and 40 μM BCNU.

a folate carrier related manner but that such uptake is very limited. There was little enhancement of cell killing by BCNU even at 50 μM, whereas **12** and O^6-benzyl-2′-deoxyguanosine (Figure 3) and O^6-benzylguanine itself were effective at 5 μM concentrations.[15,17,24] This contrasts strikingly with the in vitro inactivation of alkyltransferase in which **3** is more active than O^6-benzyl-2′-deoxyguanosine and only slightly less potent than O^6-benzylguanine and **12.** Although **3** was the best substrate for porcine liver esterase and for cellular esterases among the compounds tested, it is clear that very little of the drug added to the cell culture medium is converted to **12** by serum esterases, because this metabolite was >100× more effective than **3** itself in increasing cell killing by BCNU.

Conclusions

The in vitro studies of the inactivation of purified human alkyltransferase with compounds **1** and **2** indicate that the addition of folate adducts to the deoxyribose of O^6-benzyl-2′-deoxyguanosine increases the ability to reduce alkyltransferase activity. This effect is much greater when the folate is attached to the 3′-position than when attached to the 5′-position. In fact, addition of folate to the 3′-position of O^6-benzyl-2′-deoxyguanosine to form **1** produces one of the most potent alkyltransferase inhibitors so far described with an ED_{50} of 16 nM. This value is similar to that found for O^4-benzylfolate.[9] At present, it is not known how these folate groups strengthen the interaction with alkyltransferase. Further study of the interaction of O^4-benzylfolate and of **1** and **2** with alkyltransferase by binding studies, modeling, and crystallographic investigation should provide a mechanistic explanation for this effect. This could then be tested experimentally with studies of appropriate mutants of key residues. Such an understanding would allow for the synthesis of other very potent alkyltransferase inhibitors. The addition of folate groups to O^6-benzylguanine derivatives also has the important advantage of greatly increasing the water solubility of these compounds. This increased solubility is also seen with **3**, the γ-folate ester of **12**, but addition to this position had little effect on the ability to inactivate alkyltransferase.

Surprisingly, based on the amount needed to sensitize tumor cells to killing by BCNU, the uptake of compounds **1** and **2** into tumor cells occurred to only a limited extent and was not greatly affected by the folate receptor status. Compound **3** also had much less effect on increasing sensitivity to BCNU than expected from its potency as an alkyltransferase inhibitor, suggesting that uptake is poor even in the KB cells with a high folate receptor content. The results for the enhancement of cell killing by the folate esters **1**−**3** described here differ from those for O^4-benzylfolate. This compound, which is also a highly potent alkyltransferase inhibitor, was much more potent in KB cells than in A549 cells,[9] and its effects were increased by growing KB or HT29 cells in a folate-free media and antagonized by the addition of folate (unpublished observations). These observations with O^4-benzylfolate are consistent with its efficient uptake by a folate receptor carrier mechanism. Despite the excellent alkyltransferase inactivation activity of the folate esters described in this paper, their ability to sensitize cells to alkylating agents was limited. This is most likely due to a low level of uptake rather than rapid degradation because their conversion

to folate by cellular esterases was quite slow (Figure 2). However, they provide a very useful lead for the design of other highly water soluble, specific, and potent alkyltransferase inhibitors based on the ability to interact with additional residues in the alkyltransferase active pocket.

Experimental Section

Chemistry. 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 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 an internal standard. Splitting pattern abbreviations are as follows: s = singlet, d = doublet, dd = double doublet, ddd = a doublet of doublet of doublets, t = triplet, td = triplet of doublets, m = multiplet. Coupling constants are in hertz. Mass spectra were collected on a Thermo Finnigan TSQ Quantum spectrometer in positive ion electrospray mode scanning m/z = 100 to 1500 in 1 second. The electrospray voltage was 3.5 kV and the transfer tube was at 350 °C. Elemental analyses, performed by Atlantic Microlab, Inc. (Norcross, GA), were within 0.4% of theoretical values calculated for C, H, and N. All silica gel chromatography was carried out using Davisil, grade 633, 200−425 mesh 60 Å. Synthesis and purification of folate-containing compounds was performed under reduced (yellow) light, and these materials should be considered light-sensitive.[25]

***O*^{6}-Benzyl-5′-*O*-(4,4′-dimethoxytrityl)-2′-deoxyguanosine (5).** Nucleoside **4** was synthesized according to methods reported earlier.[10] NaOH (1.0 M, 50 mL) was added to a solution of nucleoside **4** (3.93 g, 4.96 mmol) in CH_3CN (100 mL) and stirred at room temperature for 18 h. The reaction was neutralized with HCl (1.0 M) and extracted with CH_2Cl_2 (3 × 50 mL). The resulting organic layers were combined, dried with $MgSO_4$, and filtered, and the solvent was removed under reduced pressure to yield **5** as a white solid (3.18 g, 97.2%). ^{1}H NMR (DMSO-d_6): δ 2.29 (1H, ddd, J = 11.2, J = 6.4, J = 4.8, H-2′), 2.70 (1H, ddd, J = 13.2, J = 6.4, J = 6.4, H-2′), 3.13 (2H, d, J = 5.5, H-5′), 3.70 (3H, s, DMT-O-CH_3), 3.71 (3H, s, DMT-O-CH_3), 3.93 (1H, dd, J = 8.8, J = 4.4, H-4′), 4.39 (1H, ddd, J = 9.6, J = 4.4, J = 4.4, H-3′), 5.32 (1H, br d, J = 4.4, 3′-OH, exchanges with D_2O), 5.49 (2H, s, CH_2−Ph), 6.24 (1H, t, J = 6.4, H-1′), 6.46 (2H, br s, N^2H_2, exchanges with D_2O), 6.78−6.84 (4H, m, DMT Ph and DMT Ar), 7.15−7.26 (7H, m, DMT Ph and DMT Ar), 7.32−7.42 (5H, m, DMT Ar and Bn Ar), 7.51−7.94 (2H, m, Bn Ar), 7.94 (1H, s, H-8).

***O*^{6}-Benzyl-3′-*O*-[γ-[α-[2-(trimethylsilyl)ethoxy]]-2-*N*-[2-(trimethylsilyl)-ethoxycarbonyl]folyl]-2′-deoxyguanosine (7).** Folate **6** was synthesized according to methods reported by Nomura et al.[11] 4-Dimethylaminopyridine (DMAP; 0.0324 g, 0.265 mmol) and 1,3-dicyclohexylcarbodiimide (1.09 g, 5.30 mmol) were added to a solution of **6** (1.82 g, 2.65 mmol) in CH_2Cl_2 (75 mL) and stirred at room temperature for 1.5 h. Nucleoside **5** (1.75 g, 2.65 mmol) was then added to the solution and stirred for an additional 18 h. The solvent was removed under reduced pressure to yield a yellow foam. The solid foam was dissolved in EtOAc and filtered to remove insoluble dicyclohexylurea and dried under reduced pressure to afford a yellow solid. This solid was dissolved in 80% acetic acid (25 mL) and stirred for 30 min. Ethanol (250 mL) was added, and the solvent was removed under reduced pressure to afford an orange foam that was purified by column chromatography (silica gel, 7:3: 0.25 CH_2Cl_2/EtOAc/MeOH) to give **7** (1.1 g, 40.4%). ^{1}H NMR (DMSO-d_6): δ 0.003 (9H, s, Si(CH_3)$_3$), 0.058 (9H, s, Si(CH_3)$_3$), 0.92−0.96 (2H, m, TMS-C**H$_2$**CH_2), 1.03−1.07 (2H, m, TMS-C**H$_2$**-CH_2), 1.94−2.03 (1H, m, gluβ-CH_{2b}), 2.11 (1H, ddd, J = 20.8, J = 8.0, J = 6.0, gluβ-CH_{2a}), 2.40 (1H, br dd, J = 13.2, J = 5.6, H-2′), 2.48 (2H, br t, J = 7.6, gluγ-CH_2), 2.84 (1H, ddd, J = 14.8, J = 9.2, J = 6.0, H-2′), 3.52−3.62 (2H, m, H-5′), 4.00 (1H, td, J = 4.4, J = 1.6, H-4′), 4.11−4.15 (2H, m, TMS-CH_2C**H$_2$**), 4.27−4.32 (2H, m, TMS-CH_2C**H$_2$**), 4.41 (1H, ddd, J = 12.8, J = 7.6, J = 5.2, gluα-CH), 4.59 (2H, br d, J = 6.0, folate 6-C**H$_2$**NH, s in D_2O), 5.16 (1H, br t, J = 5.6, 5′-OH, exchanges with D_2O), 5.32 (1H, br d, J = 6.0, H-3′), 5.50 (2H, s, CH_2−Ph), 6.19 (1H, dd, J = 9.2, J = 5.6, H-1′), 6.50 (2H, br s, guanine N^2H_2, exchanges with D_2O), 6.66 (2H, d, J = 8.8, pAB Ar), 7.03 (1H, t, J = 6.0, folate 6-CH_2N**H**, exchanges with D_2O), 7.32−7.42 (3H, m, Bn Ar), 7.50 (2H, br dd, J = 8.4, J = 1.6, Bn Ar), 7.66 (2H, br d, J = 8.8, pAB Ar), 8.10 (1H, s, guanine H-8), 8.26 (1H, br d, J = 7.6, glu NH, exchanges with D_2O), 8.84 (1H, s, folate H-7), 11.71 (2H, br s, folate N^3H and folate N^2H, exchanges with D_2O). MS m/z 1025.6 [M + H]$^+$; Anal. ($C_{47}H_{60}N_{12}O_{11}Si_2$) C, H, N.

***O*^{6}-Benzyl-3′-*O*-(γ-folyl)-2′-deoxyguanosine (1).** Tetrabutylammonium fluoride (TBAF; 1.0 M in THF, 2.15 mL) was added to a solution of **7** (0.220 g, 0.215 mmol) dissolved in DMSO (2.15 mL) and stirred at room temperature for 2 h. Water (25 mL) was added to the reaction, and the pH of the solution was adjusted to 3 with HCl. The yellow precipitate was filtered off and suspended in 2:1 H_2O/MeOH (50 mL). $NaHCO_3$ (0.036 g, 0.430 mmol) was added to the suspension and stirred until the solid was completely dissolved (∼2 h). The solution was acidified to pH 3 with HCl, and the resulting solid was filtered and dried under vacuum (0.161 g, 95.8%). UV (0.05 M phosphate, pH 7.4) λ_{max} = 253 nm (ϵ = 1.23 × 10^4), λ_{max} = 284 nm (ϵ = 2.00 × 10^4), λ_{max} = 362 nm (ϵ = 5.20 × 10^3). ^{1}H NMR (DMSO-d_6): δ 1.91−2.02 (1H, m, gluβ-CH_{2b}), 2.09−2.18 (1H, m, gluβ-CH_{2a}), 2.41 (1H, br dd, J = 12.8, J = 5.6, H-2′), 2.47 (2H, br t, J = 7.2, gluγ-CH_2), 2.84 (1H, ddd, J = 14.8, J = 9.2, J = 6.0, H-2′), 3.57 (2H, br s, H-5′), 4.01 (1H, td, J = 4.0, J = 1.2, H-4′), 4.35−4.40 (1H, m, gluα-CH), 4.48 (2H, br d, J = 5.2, folate 6-C**H$_2$**NH, s in D_2O), 5.16 (1H, br t, J = 5.6, 5′-OH, exchanges with D_2O), 5.32 (1H, br d, J = 6.0, H-3′), 5.50 (2H, s, CH_2−Ph), 6.19 (1H, dd, J = 9.2, J = 5.6, H-1′), 6.50 (2H, br s, guanine N^2H_2, exchanges with D_2O), 6.66 (2H, d, J = 8.8, pAB Ar), 6.92 (3H, t, J = 6.0, folate 6-CH_2N**H** and folate N^2H_2, exchanges with D_2O), 7.33−7.42 (3H, m, Bn Ar), 7.50 (2H, br dd, J = 8.4, J = 1.6, Bn Ar), 7.65 (2H, br d, J = 8.8, pAB Ar), 8.10 (1H, s, guanine H-8), 8.13−8.14 (1H, m, glu-NH, exchanges with D_2O), 8.65 (1H, s, folate H-7), 11.48 (1H, br s, folate N^3H exchanges in D_2O), 12.54 (1H, br s, CO_2H, exchanges in D_2O). MS m/z 781.3 [M + H]$^+$; Anal. ($C_{36}H_{36}N_{12}O_9$·1.6H_2O) C, H, N.

***O*^{6}-Benzyl-3′-*O*-(*t*-butyldimethylsilyl)-5′-*O*-(4,4′-dimethoxytrityl)-*N*^{2}-phenoxyacetyl-2′-deoxyguanosine (8).** Imidazole (0.607 g, 8.92 mmol) was added to a solution of **4** (1.77 g, 2.23 mmol) in DMF (6 mL) and stirred until completely dissolved. *tert*-Butyldimethylsilyl chloride (1.01 g, 6.70 mmol) was added, and the reaction was stirred at room temperature for 18 h. The solvent was removed under reduced pressure, water (20 mL) was added to the residue, and the residue was extracted with CH_2Cl_2 (3 × 30 mL). The organic extracts were combined, dried over $MgSO_4$, and filtered, and the solvent was removed under reduced pressure. The resulting oil was purified by column chromatography (silica, 70:30 EtOAc/Hex) to yield **8** as a white solid (1.84 g, 90.9%). ^{1}H NMR (DMSO-d_6): δ −0.031 (3H, s, Si−CH_3), 0.035 (3H, s, Si−CH_3), 0.816 (9H, s, Si−C(CH_3)$_3$), 2.38 (1H, ddd, J = 12.8, J = 6.8, J = 5.2, H-2′), 2.96 (1H, ddd, J = 13.2, J = 6.4, J = 6.4, H-2′), 3.20−3.28 (2H, m, H-5′), 3.73 (6H, s, DMT-(O-CH_3)$_2$), 3.88 (1H, br dd, J = 9.6, J = 5.2, H-4′), 4.69 (1H, br dd, J = 10.8, J = 5.2, H-3′), 5.04 (1H, d, J = 18.0, diastereotopic CH_2−Ph), 5.05 (1H, d, J = 18.0, diastereotopic CH_2−Ph), 5.66 (2H, s, PAc-CH_2), 6.40 (1H, br dd, J = 6.0, J = 6.8, H-1′), 6.77−6.83 (4H, m DMT Ar), 6.94−7.10 (3H, m, PAc Ar), 7.17−7.25 (7H, m, DMT Ph and DMT Ar), 7.29−7.35 (4H, m, Bn Ar and DMT Ar), 7.38−7.46 (3H, m, Bn Ar), 7.57−7.60 (2H, m, PAc Ar), 8.44 (1H, s, H-8), 10.69 (1H, br s, N^2H, exchanges with D_2O). Anal. ($C_{52}H_{57}N_5O_8Si$) C, H, N.

***O*^{6}-Benzyl-3′-*O*-(*t*-butyldimethylsilyl)-*N*^{2}-phenoxyacetyl-2′-deoxyguanosine (9).** A solution of 3% TCA (67.8 mL, 19.9 mmol) in CH_2Cl_2 was added to a solution of **8** (4.51 g, 4.97 mmol) dissolved in CH_2Cl_2 (100 mL) and stirred at room temperature for 4 min. Et_3N (2.77 mL, 19.9 mmol) was added to the solution and the solvent was removed under reduced pressure. The resulting solid was purified by column chromatography (silica, 90:10 CH_2Cl_2/EtOAc) to afford **9** as an off-white foam (2.56, 85.0%). ^{1}H NMR (DMSO-d_6): δ 0.088 (3H, s, Si−CH_3), 0.094 (3H, s, Si−CH_3),

0.875 (9H, s, Si−C(CH_3)$_3$), 2.28 (1H, ddd, J = 13.2, J = 6.0, J = 3.2, H-2′), 2.82 (1H, ddd, J = 13.2, J = 6.0, J = 2.0, H-2′), 3.51 (1H, ddd, J = 11.2, J = 5.2, J = 4.8, H-5′), 3.57 (1H, ddd, J = 11.6, J = 5.6, J = 5.6, H-5′), 3.84 (1H, ddd, J = 4.8, J = 4.8, J = 2.8, H-4′), 4.60 (1H, ddd, J = 5.6, J = 2.8, J = 2.8, H-3′), 4.94 (1H, t, J = 5.4, 5′-OH, exchanges with D_2O), 5.04 (2H, s, PAc-CH_2), 5.63 (2H, s, CH_2−Ph), 6.33 (1H, t, J = 6.8, H-1′), 6.92−6.97 (3H, m, PAc Ar), 7.26−7.31 (2H, m, Bn Ar), 7.34−7.42 (3H, m, Bn Ar), 7.54 (2H, dd, J = 8.4, J = 1.6, PAc Ar), 8.47 (1H, s, H-8), 10.69 (1H, s, N^2H, exchanges with D_2O). Anal. ($C_{31}H_{39}N_5O_6$-Si) C, H, N.

***O*^{6}-Benzyl-3′-*O*-(*t*-butyldimethylsilyl)-2′-deoxyguanosine (10).** NaOH (2 M, 21 mL, 42.3 mmol) was added to a solution of **9** (2.56 g, 4.23 mmol) dissolved in CH_3CN (13 mL) and stirred at room temperature for 21 h. Water (20 mL) was added to the reaction and the pH was adjusted to 7 with HCl. The solution was extracted with CH_2Cl_2 (2 × 30 mL), and the organic extracts were combined, dried over $MgSO_4$, and filtered. The solvent was removed under reduced pressure to yield **10** as a white solid (1.86 g, 93.4%). ^{1}H NMR (DMSO-d_6): δ 0.11 (6H, s, Si-(CH_3)$_2$), 0.90 (9H, s, Si−C(CH_3)$_3$), 2.21 (1H, ddd, J = 12.8, J = 5.6, J = 2.4, H-2′), 2.70 (1H, ddd, J = 13.2, J = 8.0, J = 5.6, H-2′), 3.49 (1H, ddd, J = 11.6, J = 5.2, J = 4.8, H-5′), 3.55 (1H, ddd, J = 11.6, J = 5.2, J = 5.2, H-5′), 3.82 (1H, ddd, J = 4.4, J = 4.4, J = 2.4, H-4′), 4.53 (1H, ddd, J = 5.2, J = 2.4, J = 2.4, H-3′), 5.04 (1H, t, J = 5.6, 5′-OH, exchanges with D_2O), 5.50 (2H, s, CH_2−Ph), 6.21 (1H, dd, J = 8.0, J = 6.0, H-1′), 6.50 (2H, br s, N^2H_2, exchanges with D_2O), 7.33−7.42 (3H, m, Bn Ar), 7.48−7.51 (2H, m, Bn Ar), 8.11 (1H, s, H-8). Anal. ($C_{23}H_{33}N_5O_4Si$) C, H, N.

***O*^{6}-Benzyl-3′-*O*-(*t*-butyldimethylsilyl)-5′-*O*-[γ-[α-[2-(trimethylsilyl)ethoxy]]-2-*N*-[2-(trimethylsilyl)-ethoxycarbonyl]folyl]-2′-deoxyguanosine (11).** DMAP (0.764 g, 6.25 mmol), 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC; 1.20 g, 6.25 mmol), and **10** (2.67 g, 5.68 mmol) were added to a solution of protected folate **6** (4.29 g, 6.25 mmol) in CH_2Cl_2 (180 mL) and stirred at room temperature for 3 h. Water (100 mL) was added to the solution, and the organic layer was extracted off and subsequently dried over $MgSO_4$ and filtered. The solution was dried under reduced pressure, and the resulting yellow foam was purified by column chromatography (silica gel, EtOAc) to afford **11** (2.62 g, 40.5%). ^{1}H NMR (DMSO-d_6): δ 0.012 (9H, s, Si-(CH_3)$_3$), 0.070 (9H, s, Si-(CH_3)$_3$), 0.080 (6H, s, Si-(CH_3)$_2$), 0.86 (9H, s, Si−C(CH_3)$_3$), 0.90−0.94 (2H, m, TMS−C**H**$_2$CH$_2$), 1.04−1.08 (2H, m, TMS−C**H**$_2$CH$_2$), 1.91−1.99 (1H, m, gluβ-CH_{2b}), 2.03−2.12 (1H, m, gluβ-CH_{2a}), 2.25 (1H, ddd, J = 13.6, J = 6.0, J = 3.6, H-2′), 2.44 (2H, br t, J = 7.4, gluγ-CH_2), 2.83 (1H, ddd, J = 7.6, J = 3.6, J = 2.8, H-2′), 3.96 (1H, ddd, J = 6.0, J = 6.0, J = 6.0, H-4′), 4.10−4.16 (3H, m, TMS−CH_2C**H**$_2$ and H-5′), 4.23 (1H, dd, J = 11.6, J = 6.0, H-5′), 4.29−4.33 (2H, m, TMS−CH_2C**H**$_2$), 4.37 (1H, ddd, J = 12.8, J = 5.2, J = 2.0, gluα-CH), 4.53 (1H, ddd, J = 6.0, J = 2.8, J = 2.8, H-3′), 4.59 (2H, d, J = 6.0, folate 6-C**H**$_2$-NH, s in D_2O), 5.50 (2H, s, CH_2−Ph), 6.22 (1H, br t, J = 7.0, H-1′), 6.52 (2H, br s, guanine N^2H_2, exchanges with D_2O), 6.66 (2H, d, J = 8.8, pAB Ar), 7.04 (1H, t, J = 6.2, folate 6-CH_2N**H**, exchanges with D_2O), 7.33−7.43 (3H, m, Bn Ar), 7.50 (2H, br dd, J = 8.4, J = 1.6, Bn Ar), 7.66 (2H, d, J = 8.8, pAB Ar), 8.09 (1H, s, guanine H-8), 8.26 (1H, d, J = 7.6, glu-NH, exchanges with D_2O), 8.84 (1H, s, folate H-7), 11.72 (2H, br s, folate N^3H and N^2H, exchanges with D_2O). Anal. ($C_{53}H_{74}N_{12}O_{11}Si_3$) C, H, N.

***O*^{6}-Benzyl-5′-*O*-(γ-folyl)-2′-deoxyguanosine (2).** TBAF (1.0 M in THF, 6.71 mL) was added to **11** (0.510 g, 0.448 mmol) dissolved in DMSO (5.0 mL). The reaction was stirred at room temperature for 3 h. Water (140 mL) was added to the reaction, and the pH of the solution was adjusted to 3 with HCl. The yellow precipitate was filtered off and suspended in H_2O (150 mL). $NaHCO_3$ (1 M, 0.896 mL) was added to the suspension and stirred until the solid was completely dissolved (∼2 h). The solution was acidified to pH 3 with HCl, and the resulting solid was filtered (0.297 g, 85.0%). The crude product, dissolved in 0.1 M $NaHCO_3$ at 10 mg/mL, was purified on a Sephadex LH-20 column eluted with 0.1 M NaCl at a flow rate of 1 mL/min. UV absorption was continuously monitored at 280 nm, and 10 mL fractions were collected. The combined fractions (110−140) were reduced to approximately 50 mL, and the pH of the solution was adjusted to 3 with HCl. The resulting yellow precipitate was filtered and dried to afford **2** as a yellow solid (0.110 g, 31.5% overall yield). UV (0.05 M phosphate, pH 7.4) λ_{max} = 253 nm (ϵ = 1.23 × 10^4), λ_{max} = 284 nm (ϵ = 2.00 × 10^4), λ_{max} = 362 nm (ϵ = 5.20 × 10^3). ^{1}H NMR (DMSO-d_6): δ 1.90−1.98 (1H, m, gluβ-CH_{2b}), 2.05−2.11 (1H, m, gluβ-CH_{2a}), 2.25 (1H, ddd, J = 13.2, J = 6.0, J = 3.6, H-2′), 2.41 (2H, br t, J = 7.6, gluγ-CH_2), 2.70 (1H, ddd, J = 7.6, J = 6.4, J = 6.4, H-2′), 3.93−3.97 (1H, m, H-4′), 4.13 (1H, dd, J = 11.6, J = 6.4, H-5′), 4.24 (1H, dd, J = 11.6, J = 4.8, H-5′), 4.29−4.35 (1H, m, gluα-CH), 4.37−4.40 (1H, m, H-3′), 4.48 (2H, d, J = 5.6, folate 6-C**H**$_2$-NH, s in D_2O), 5.44 (1H, br s, 3′-OH, exchanges with D_2O), 5.49 (2H, s, CH_2−Ph), 6.21 (1H, t, J = 6.8, H-1′), 6.51 (2H, br s, guanine N^2H_2, exchanges with D_2O), 6.64 (2H, d, J = 8.8, pAB Ar), 6.92 (3H, br t, J = 6.0, folate N^2H_2 and folate 6-CH_2N**H**, exchanges with D_2O), 7.32−7.41 (3H, m, Bn Ar), 7.49 (2H, dd, J = 8.4, J = 1.6, Bn Ar), 7.64 (2H, d, J = 8.8, pAB Ar), 8.04 (1H, s, guanine H-8), 8.12 (1H, br d, J = 6.4, glu-NH, exchanges with D_2O), 8.64 (1H, s, folate H-7), 11.49 (1H, br s, folate N^3H, exchanges with D_2O), 12.51 (1H, br s, CO_2H, exchanges with D_2O). MS *m*/*z* 781.3 [M + H]$^+$; Anal. ($C_{36}H_{36}N_{12}O_9$·1.5H_2O) C, H, N.

***O*^{6}-[4-[γ-[[α-[2-(Trimethylsilyl)ethoxy]]-2-*N*-[2-(trimethylsilyl)-ethoxycarbonyl]folyl]-oxymethyl]benzyl]guanine (13).** O^6-[4-(Hydroxymethyl)benzyl]guanine (**12**) was synthesized as described.[12] Bis-silyl protected folic acid **6**, (600 mg, 0.88 mmol), **12** (270 mg, 1.0 mmol), and DMAP (109 mg, 0.90 mmol) were combined in 15 mL of DMF. EDC (173 mg, 0.9 mmol) was added and the reaction was stirred at room temperature for 2 h. The solvent was evaporated under reduced pressure. The residue was dissolved in 100 mL of CH_2Cl_2 and extracted with an equal volume of 0.05 M HCl in water followed by pure water. These extractions gave emulsions that required centrifugation to separate. The CH_2Cl_2 was then evaporated under reduced pressure. The resulting material was purified by column chromatography (silica gel, 19:1 chloroform: methanol). Upon evaporation of the solvent under reduced pressure, the desired bis-silyl protected product **13** was isolated as a yellow solid (300 mg, 36%). ^{1}H NMR (DMSO-d_6) with no TMS standard: δ 0.01 (9H, s, TMS-CH_3), 0.05 (9H, s, TMS-CH_3), 0.92 (2H, ddd, J = 8.4, J = 6.8, J = 4.4, TMS-C**H**$_2$CH$_2$), 1.04 (2H, ddd, J = 8.8, J = 6.8, J = 4.0, TMS-C**H**$_2$CH$_2$), 1.94−2.02 (1H, m, gluβ-CH_{2b}), 2.04−2.13 (1H, m, gluβ-CH_{2a}), 2.47 (2H, t, J = 7.6, gluγ-CH_2), 4.09−4.13 (2H, m, TMS-CH_2C**H**$_2$), 4.27−4.31 (2H, m, TMS-CH_2C**H**$_2$), 4.36 (1H, ddd, J = 12.4, J = 7.6, J = 5.2, gluα-CH), 4.58 (2H, d, J = 5.6, folate 6-C**H**$_2$-NH, s in D_2O), 5.08 (2H, s, benzyl-C**H**$_2$-folate), 5.47 (2H, s, benzyl-C**H**$_2$-guanine), 6.28 (2H, s, guanine N^2H_2, exchanges with D_2O), 6.65 (2H, d, J = 8.4, pAB Ar), 7.03 (1H, t, J = 6.0, folate 6-CH_2-N**H**, exchanges with D_2O), 7.35 (2H, d, J = 8.0, Bn Ar), 7.48 (2H, d, J = 8.0, Bn Ar), 7.64 (2H, d, J = 8.4, pAB Ar), 7.81 (1H, s, guanine H-8), 8.24 (1H, d, J = 7.6, glu-NH, exchanges with D_2O), 8.83 (1H, s, folate H-7), 11.7 (2H, br s, folate N^3H and folate N^2H, exchanges with D_2O), 12.4 (1H, br s, guanine N^9H, exchanges with D_2O). MS *m*/*z* 939.4 [M + H]$^+$.

***O*^{6}-[4-[(γ-Folyl)-oxymethyl]benzyl]guanine (3).** The above bis-protected O^6-[4-(hydroxymethyl)benzyl]guanine γ-folate ester **13** (290 mg, 0.31 mmol) was dissolved in 45 mL of dimethyl sulfoxide. TBAF (1 M in THF, 5 mL) was added, and the reaction was stirred for 2 h at room temperature. The reaction was terminated with the addition of 450 mL of water, and the suspension was acidified to pH 3 with HCl. Centrifugation of the resulting gelatinous mixture pelleted the product. The pellet was dissolved in 100 mL of 1 mM $NaHCO_3$ and precipitated by acidifying to pH 3 with HCl. The material was then repeatedly washed by suspending in water and pelleting by centrifugation prior to final drying under high vacuum to give **3** as a yellow powder (210 mg, 97%). UV in 0.1 M HCl, λ_{max} = 290 nm (ϵ = 2.95 × 10^4) and 364 nm (ϵ = 2.90 × 10^3), at pH 7 in 0.05 M phosphate buffer, λ_{max} = 283 nm (ϵ = 3.23 × 10^4) and 347 nm (ϵ = 6.60 × 10^3), in 0.1 M NaOH, λ_{max} = 256 nm (ϵ = 2.79 × 10^4), 285 nm (ϵ = 3.16 × 10^4), and 366 nm (ϵ = 8.30

$\times 10^3$), with decomposition in acid and base. ^{1}H NMR (DMSO-d_6): δ 1.91−2.01 (1H, m, gluβ-CH_{2b}). 2.06−2.15 (1H, m, gluβ-CH_{2a}), 2.47 (2H, t, J = 8.0, gluγ-CH_2), 4.35 (1H, ddd, J = 12.8, J = 8.0, J = 4.8, gluα-CH), 4.48 (2H, d, J = 6.0, folate 6-C**H**$_2$-NH, s in D_2O), 5.08 (2H, s, benzyl-C**H**$_2$-folate), 5.48 (2H, s, benzyl-C**H**$_2$-guanine), 6.30 (2H, s, guanine N^2H_2, exchanges with D_2O), 6.64 (2H, d, J = 8.8, pAB Ar), 6.90 (2H, br s, folate N^2H_2, exchanges with D_2O), 6.94 (1H, t, J = 6.0, folate 6-CH_2-N**H**, exchanges with D_2O), 7.36 (2H, d, J = 8.0, Bn Ar), 7.48 (2H, d, J = 8.0, Bn Ar), 7.65 (2H, d, J = 8.8, pAB Ar), 7.82 (1H, s, guanine H-8), 8.13 (1H, d, J = 7.6, glu-NH, exchanges with D_2O), 8.65 (1H, s, folate H-7), 11.5 (1H, br s, folate N^3H, exchanges with D_2O), 12.4 (2H, br s, guanine N^9H and folic acid CO_2H, exchanges with D_2O). MS m/z 695.2 $[M + H]^+$; Anal. ($C_{32}H_{30}N_{12}O_7 \cdot H_2O$) C, H, N.

Inactivation of Purified Recombinant Human Alkyltransferase. ED_{50} values for the inactivation of purified human alkyltransferase in vitro were obtained as previously described.[9,14] Purified recombinant human alkyltransferase was incubated with different concentrations of prodrugs 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 or 10 μg calf thymus DNA for 30 min at 37 °C.

Sensitization of Cells to Killing by BCNU. KB, HT29, and A549 cells were grown in RPMI 1640 medium or in RPMI medium lacking folate in the presence of 10% fetal bovine serum. The effect of alkyltransferase inactivators on the sensitivity of cells to BCNU was determined using a colony-forming assay.[8,9,12,17] Cells were plated at a density of 10^6 in 25 cm^2 flasks and 24 h later were incubated with different concentrations of potential inhibitors for 2−8 h as indicated before exposure to 20 or 40 μM BCNU for 2 h as previously described.[8,9] After 2 h, the medium was replaced with fresh medium containing the drug but no BCNU, and the cells were left to grow for an additional 16−18 h. The cells were then replated at densities of 250−1000 cells per 25 cm^2 flask and grown for 8 days until discrete colonies had formed. The colonies were washed with 0.9% saline solution, stained with 0.5% crystal violet in ethanol, and counted.

Conversion of Esters to Folate. Cells were trypsinized, washed with Hank's balanced salt solution, counted, and pelleted. On ice, the cell pellets were resuspended in 50 mM sodium phosphate, 5 mM DTT, pH 7.4 at a concentration of 10^8 cells/mL, and disrupted by sonication. The sonicated cells were centrifuged at 12 000 $\times$ g for 10 min and the supernatant was removed. Complete miniprotease inhibitor (Roche, Mannheim, Germany) was added as directed by the supplier. Lysate total protein concentration was determined, and the lysates were frozen until used at −20 °C. Reactions (600 μL) containing one of the ester substrates (200 μM) and 2 mg of lysate protein in 50 mM phosphate buffer (pH 7.4) were incubated at 37 °C. At various times, 50 μL were removed for analysis by HPLC. The amount of folic acid liberated was used to determine the extent of ester hydrolysis. The susceptibility of the compounds to hydrolysis by porcine liver esterase (Sigma, St. Louis, MO) was also measured in the same way.

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Supporting Information Available: Elemental analyses for compounds **1**−**3**. This material is available free of charge via the Internet at http://pubs.acs.org.

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