β-Glucuronidase-Cleavable Prodrugs of O^6-Benzylguanine and O^6-Benzyl-2′-deoxyguanosine

Guangping Wei,[†] Natalia A. Loktionova,[‡] Anthony E. Pegg,[‡] and Robert C. Moschel*,[†]

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

Received July 30, 2004

Glucuronic acid linked prodrugs of O^6-benzylguanine and O^6-benzyl-2′-deoxyguanosine were synthesized. The prodrugs were found to be quite stable at physiological pH and were more than 200-fold less active as inactivators of O^6-alkylguanine-DNA alkyltransferase (alkyltransferase) than either O^6-benzylguanine or O^6-benzyl-2′-deoxyguanosine. β-Glucuronidase from both *Escherichia coli* and bovine liver cleaved the prodrugs efficiently to release O^6-benzylguanine and O^6-benzyl-2′-deoxyguanosine, respectively. In combination with 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), the prodrugs were not effective adjuvants for HT29 cell killing. However, as expected, incubation of these prodrugs with β-glucuronidase in the culture medium led to much more efficient cell killing by BCNU as a result of the liberation of the more potent inactivators, O^6-benzylguanine and O^6-benzyl-2′-deoxyguanosine. These prodrugs may be useful for prodrug monotherapy of necrotic tumors that liberate β-glucuronidase or for antibody-directed enzyme prodrug therapy with antibodies that can deliver β-glucuronidase to target tumor cells.

Introduction

Enzymatic conversion of chemotherapeutic prodrugs to active drugs in or in the vicinity of tumors provides a means for delivering active drugs more selectively to tumor cells versus normal cells of a host.[1−5] The antibody directed enzyme prodrug therapy (ADEPT)[4] and prodrug monotherapy (PMT)[5,6] approaches are examples of such delivery systems. The former system involves tethering an activating enzyme to a tumor-specific antibody so that prodrugs can be activated in the vicinity of tumor cells while the latter approach relies on activation by endogenous enzymes that are expressed by tumors. β-Glucuronidase is an example of a lysosomal hydrolase that is overexpressed by tumor cells and released from necrotic tumor cells found within poorly vascularized regions of tumor masses.[5,7,8] To exploit the presence of this enzyme for prodrug activation, several prodrugs composed of a glucuronic acid residue linked through a self-immolating linker to active drugs (e.g. anthracyclines and nitrogen mustards) have been synthesized.[9−16] When these prodrugs are cleaved by tumor cell β-glucuronidase, the linker undergoes spontaneous decomposition to release the active drug that enters nearby tumor cells preferentially, thereby minimizing the more widespread toxicity associated with systemic delivery of the unmodified drugs.

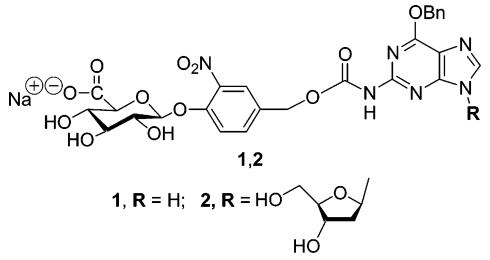
We have previously shown that O^6-benzylguanine, O^6-benzyl-2′-deoxyguanosine, and related derivatives can inactivate the DNA repair protein, O^6-alkylguanine-DNA alkyltransferase (alkyltransferase),[17,18] and we have shown that this inactivation can markedly improve the effectiveness of chemotherapeutic drugs that modify the O^6-position of DNA guanine residues, such as 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) and temozolomide.[17,19−23] However, this adjuvant therapy approach is not tumor specific which can lead to undesirable side effects in clinical trials.[24] To bring about more selective toxicity toward tumor cells by this adjuvant approach, either the DNA repair inactivator or the antitumor agent or both should be more selectively delivered to tumor cells in preference to normal cells of a host. Toward this end, we have prepared β-glucuronidase-cleavable prodrugs (**1** and **2**) of two potent DNA repair inactivators, O^6-benzylguanine and O^6-benzyl-2′-deoxyguanonsine. Both **1** and **2** have the glucuronic acid residue attached through a linker to the exocyclic amino group of the base or nucleoside, respectively. The choice for this site of attachment of a cleavable substituent was motivated by our previous work which showed that substitution on the N^2-position of O^6-benzylguanine greatly reduced its activity as an alkyltransferase inactivator.[18,25−27] We therefore anticipated that prodrugs with bulky substituents at N^2 would be poor inactivators in their own right but would release potent inactivators if the substituent were removed by enzymatic conversion to an unsubstituted derivative. The linker in both **1** and **2** is a nitrobenzylphenoxy carbamate linker[28] which has been shown for doxorubicin[13] and 5-fluorouracil[14] prodrugs to self-immolate efficiently once the respective glucuronides are cleaved.

Here we report the synthesis of **1** and **2** together with a description of the kinetics of their enzymatic cleavage by β-glucuronidase from both *Escherichia coli* and bovine liver. We also show that the prodrugs are not effective adjuvants for HT29 cell killing by BCNU unless they are incubated with β-glucuronidase to liberate O^6-benzylguanine and O^6-benzyl-2′-deoxyguanosine, re-

* To whom correspondence should be addressed. Phone: 301-846-5852. Fax: 301-846-6146. E-mail: moschel@mail.ncifcrf.gov.
[†] National Cancer Institute at Frederick.
[‡] The Pennsylvania State University College of Medicine.

10.1021/jm0493865 CCC: $30.25
Published on Web 12/13/2004

spectively. These prodrugs may be superior to O^6-benzylguanine or O^6-benzyl-2′-deoxyguanosine as chemotherapy adjuvants since their use in PMT or ADEPT therapies should reduce side effects associated with systemic alkyltransferase inactivation.



Results and Discussion

For the synthesis of **1**, the starting material was O^6-benzyl-9-[(pivaloyloxy)methyl]guanine (**3**)[29] (Scheme 1) while the synthesis of **2** began with 3′,5′-di-*O*-acetyl-O^6-benzyl-2′-deoxyguanosine (**4**).[30] When **3** or **4** was reacted with an equivalent of phosgene, unstable intermediates, presumably the isocyanates **5** and **6,** respectively, were produced. These were reacted individually without isolation with 4-*O*-(2′,3′,4′-tri-*O*-acetyl-6′-methyl-β-D-glucopyranuronosyl)-3-nitrobenzyl alcohol (**7**)[13] in one pot to produce the respective coupled products **8** and **9.** Deprotection of these derivatives with methanolic sodium hydroxide and neutralization with 10% acetic acid led to the formation of viscous colloidal suspensions which were slowly suction-filtered to produce crude samples or either **1** or **2**. Analytically pure samples of **1** and **2** were obtained after purification by Sephadex LH-20 column chromatography and lyophilization.

Stability and Enzymatic Hydrolysis of the Prodrugs. The stability of the prodrugs **1** and **2** was examined by incubating them at concentrations of 100 mM in phosphate-buffered saline (pH 7.2), a 50 mM Tris-HCl buffer (pH 7.5), a 50 mM mopholinopropane sulfonic acid (MOPS) buffer (pH 7.0), and a modified Dulbecco's medium at 37 °C for 7 days. Rate constants for the first-order disappearance of prodrugs were estimated from semilog plots of the concentration of prodrug as a function of time. Observed first-order rate constants for hydrolysis of these prodrugs are presented in Table 1. As indicated, the decomposition of **1** was faster than that of **2** in all buffers although decomposition rates for both compounds are fairly low. For example, under these aqueous conditions, the average half-time for decomposition of **1** is of the order of 100 days.

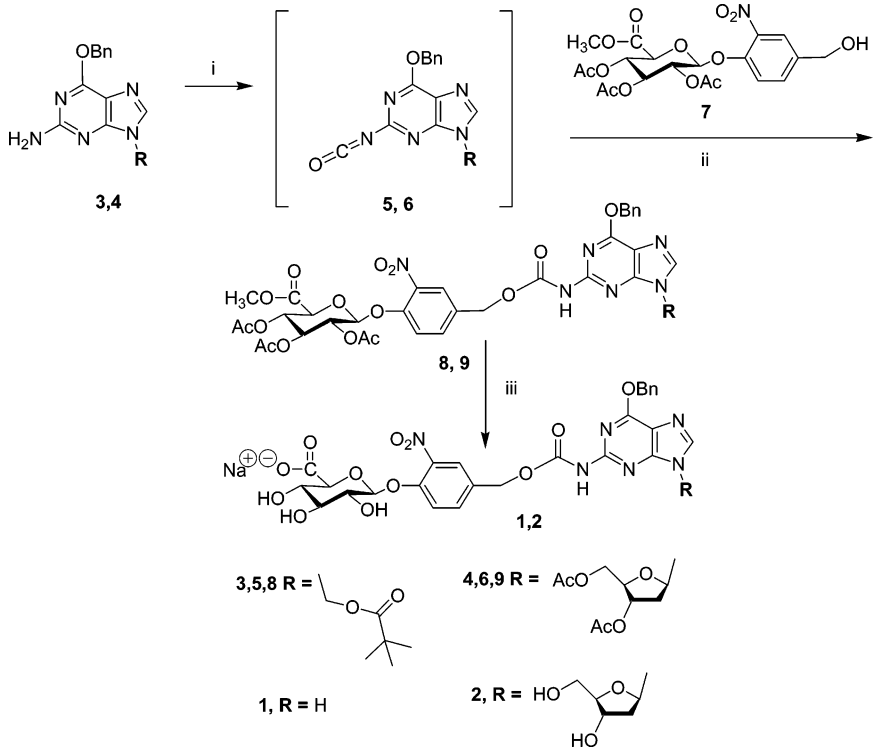
Table 1. Observed First Order Rate Constants ($\times 10^6$, min^{-1}) for Hydrolysis of Prodrugs in Aqueous Buffers

prodrug	k_{PBS}[a]	k_{Tris}[b]	k_{MOPS}[c]	$k_{Dulbecco}$[d]
1	3.86	4.06	3.77	4.29
2	0.646	0.155	0.188	0.468

[a] PBS = phosphate-buffered saline, pH 7.2. [b] Tris = 50 mM Tris-HCl (pH 7.5), 5 mM dithiothreitol, and 0.1 mM EDTA, pH 7.5. [c] MOPS = 50 mM morpholinopropane sulfonic acid, pH 7.0, 0.1% bovine serum albumin, 0.01% NaCl. [d] Dulbecco = modified Dulbecco's medium.

The enzymatic hydrolysis of the prodrugs was investigated with both *E. coli* and bovine liver β-glucuronidase in MOPS buffer at pH 7.0. Representative data for hydrolysis of **1** and **2** with the bovine liver enzyme are presented in Figure 1A. Hydrolysis by the *E. coli* enzyme is shown in Figure 1B. As indicated, hydrolysis by both the bovine liver enzyme (10 units/mL) or the *E. coli* enzyme (0.2 unit/mL) led to rapid disappearance of both **1** and **2** accompanied by the formation of O^6-benzylguanine and O^6-benzyl-2′-deoxyguanosine, re-

Scheme 1[a]



[a] Reagents and conditions: (i) Phosgene, CH_2Cl_2/pyridine, 0 °C to room temperature, 20 h; (ii) rt, 2 h; (iii) MeOH/2 N NaOH (1:1), 0 °C, 30 min, followed by neutralization with 10% acetic acid.

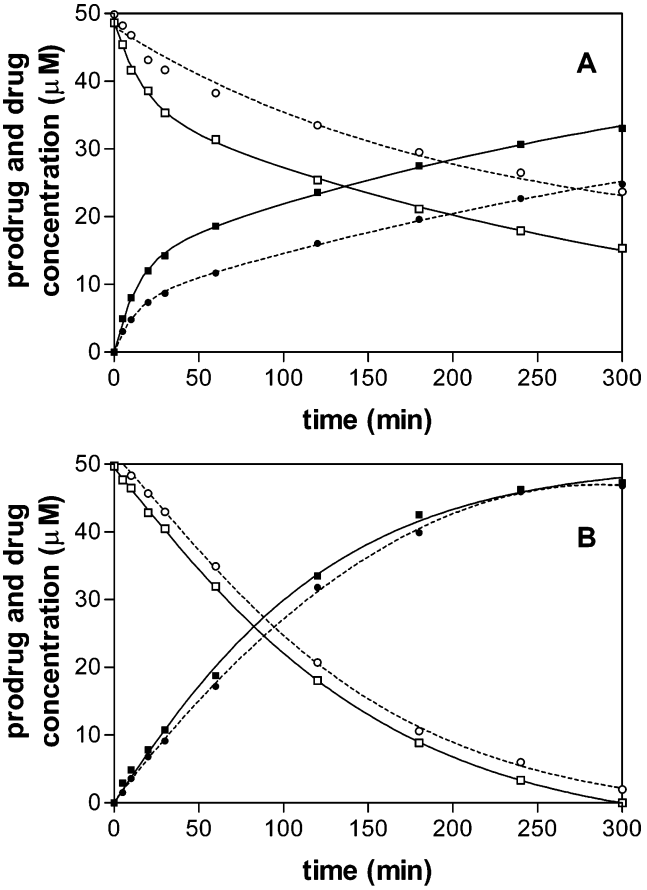


Figure 1. Prodrug cleavage by β-glucuronidase. (A) Cleavage by 10 units/mL bovine liver β-glucuronidase. Open squares, prodrug **1**; open circles, prodrug **2**; closed squares, O^6-benzylguanine; closed circles, O^6-benzyl-2′-deoxyguanosine. (B) Cleavage by 0.2 units/mL *E. coli* β-glucuronidase. Open squares, prodrug **1**; open circles, prodrug **2**; closed squares, O^6-benzylguanine; closed circles, O^6-benzyl-2′-deoxyguanosine.

Table 2. Enzyme Kinetic Parameters for Prodrugs[a]

	E. coli β-glucuronidase		bovine liver β-glucuronidase	
prodrug	K_M (μM)	V_{max} (μmol·mg^{-1}·min^{-1})	K_M (μM)	V_{max} (μmol·mg^{-1}·min^{-1})
1	17	7.8	2.4×10^2	0.27
2	41	11	3.6×10^2	0.39

[a] Data obtained at 37 °C in MOPS buffer, pH 7.0.

spectively. Although **1** and **2** were cleaved more rapidly by the *E. coli* protein than the bovine liver protein, and **1** was hydrolyzed more rapidly than **2** by both proteins, the hydrolyses followed Michaelis−Menten kinetics (Table 2) in all cases. These data (Table 2) are comparable to data for other glucuronic acid conjugates such as DOX-GA3[31] and epicurubicin-glucuronide[32] although the testing conditions are different.

Alkyltransferase Inactivation. For inactivation of the human O^6-alkylguanine-DNA alkyltransferase, prodrugs **1** and **2** were inactive up to a concentration of 50 μM (Figure 2) while the ED_{50} for O^6-benzylguanine is 0.2 μM.[17] This indicates that both **1** and **2** are intrinsically very poor alkyltransferase inactivators compared to O^6-benzylguanine. However, incubation of these drugs in the presence of bovine liver β-glucuronidase for 30 min led to efficient alkyltransferase inactivation

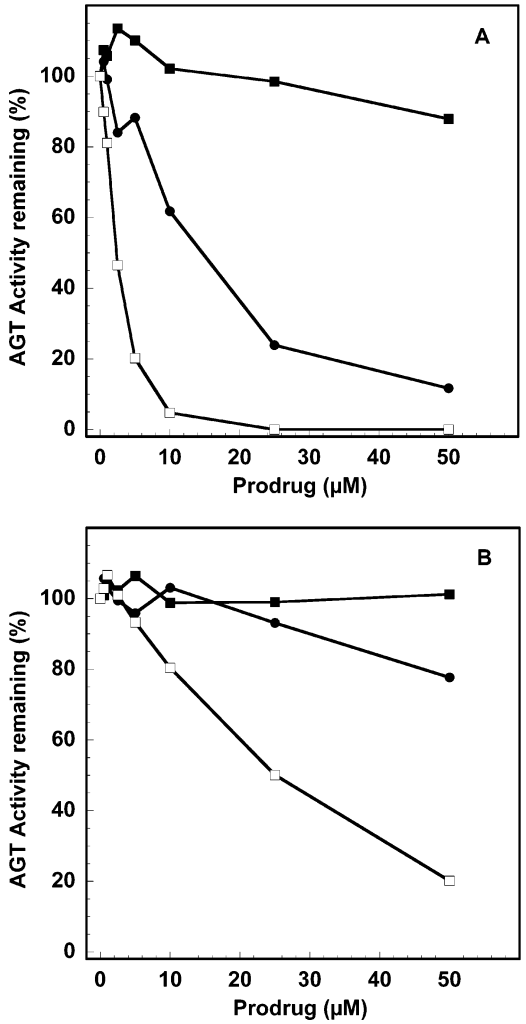


Figure 2. Alkyltransferase inactivation in the presence of bovine liver β-glucuronidase for 30 min. (A) Inactivation by **1** after treatment with no β-glucuronidase (closed squares), 20 units/mL of β-glucuronidase (closed circles), and 200 units/mL of β-glucuronidase (open squares). (B) Inactivation by **2** after treatment with no β-glucuronidase (closed squares), 20 units/mL of β-glucuronidase (closed circles), and 200 units/mL of β-glucuronidase (open squares).

due to liberation of O^6-benzylguanine from **1** (Figure 2A) or O^6-benzyl-2′-deoxyguanosine from **2** (Figure 2B).

HT29 cell killing by BCNU in combination with the prodrugs **1** and **2** is illustrated in Figure 3. As expected, cells treated for 5 h with increasing concentrations of prodrugs **1** and **2** were quite resistant to killing after a 2-h exposure to 40 μM BCNU. However, when cultures containing **1** were treated with bovine liver β-glucuronidase at 20 units/mL of medium for either 7 or 14 h, the cells were greatly sensitized to killing by BCNU. Similarly, incubation of **2** in cell cultures containing β-glucuronidase at 20 units/mL for 14 h also led to much greater cell killing by BCNU. These results were again a consequence of liberation of O^6-benzylguanine from **1** or O^6-benzyl-2′-deoxyguanosine from **2**, respectively.

Conclusions

These data suggest that if levels of β-glucuronidase secreted by necrotic human tumor cells are sufficiently high, the prodrugs **1** and **2** will be useful for selectively delivering O^6-benzylguanine and O^6-benzyl-2′-deoxygua-

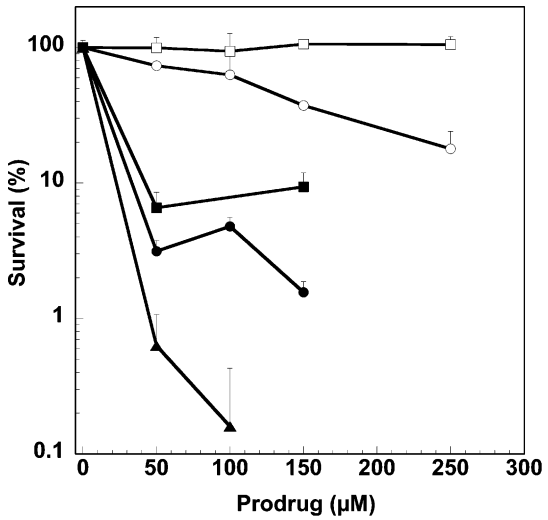


Figure 3. HT29 cell killing by a 2-h exposure BCNU (40 μM) after prodrug pretreatments. Prodrug **2** (open squares) and prodrug **1** (open circles) incubated for 5 h in cell medium containing no bovine liver β-glucuronidase; prodrug **2** incubated for 14 h in cell culture containing bovine liver β-glucuronidase at 20 units/mL of medium (closed squares); prodrug **1** incubated in cell cultures containing bovine liver β-glucuronidase at 20 units/mL of medium for 7 h (closed circles) or 14 h (closed triangles).

nosine to tumor cells compared to normal cells. This would greatly improve chemotherapy for human tumors with the combination of alkyltransferase inactivators and either chloroethylating or methylating drugs since possible side effects associated with widespread systemic alkyltransferase inactivation would be significantly reduced.

Experimental Section

Chemistry. 3′,5′-Di-*O*-acetyl-2′-deoxyguanosine was synthesized by the method of Schaller et al.[33] Previously unreported spectroscopic data for this compound are presented below. Unless otherwise stated, all other chemicals were obtained from Sigma, St. Louis, MO, or Aldrich Chemical Co., Milwaukee, WI, and were used without further purification. Melting points were determined using an Electrothermal apparatus and are uncorrected. ^{1}H NMR spectra were recorded in the indicated solvent with a Varian INOVA 400 MHz spectrometer. Chemical shifts are reported as δ values in ppm relative to TMS as internal standard. Mass spectra were obtained on a Thermo Finnigan TSQ Quantum LC mass spectrometer using electrospray ionization (ESI) and measuring either positive or negative ions. Elemental analyses were performed by Atlantic Microlab.

3′,5′-Di-*O*-acetyl-2′-deoxyguanosine.[33] 2′-Deoxyguanosine (Syngen, Inc., San Carlos, CA) (5.03 g, 17.6 mmol) was twice evaporated from 30 mL of anhydrous pyridine and was suspended in 200 mL of dry pyridine. Acetic anhydride (30 mL, 317 mmol) was added, and the mixture was stirred at room temperature for 3 days. The resulting suspended solid was collected by filtration and rinsed with ethyl ether to afford 5.73 g (93%) of 3′,5′-di-*O*-acetyl-2′-deoxyguanosine. ^{1}H NMR δ_H(DMSO-d_6) 10.67 (s, 1H, H-1, exchanges with D_2O), 7.91 (s, 1H, H-8), 6.50 (s, 2H, N^2H_2 exchange with D_2O), 6.13 (dd, J = 6.0, J = 8.7, 1H, H-1′), 5.30−5.29 (m, 1H, H-3′), 4.29−4.24 (m, 1H, H-4′), 4.21−4.16 (m, 2H, H-5′), 2.95−2.88 (m, 1H, H-1′α), 2.48−2.42 (m, 1H, H-2′β), 2.08 and 2.04 (two s, 6H, 2$COCH_3$).

3′,5′-Di-*O*-acetyl-*O*^{6}-benzyl-2′-deoxyguanosine (4).[30] To a mixture of 3′,5′-di-*O*-acetyl-2′-deoxyguanosine (5.71 g, 16.3 mmol), triphenylphosphine (5.91 g, 22.5 mmol) and benzyl alcohol (2.5 mL, 24.1 mmol) in 100 mL of 1,4-dioxane under argon was slowly added diisopropylazodicarboxylate (4.5 mL, 22.8 mmol). The mixture was heated to 85 °C for two h and was then cooled and concentrated to a thick paste on a rotary evaporator. The product was isolated as a light brown solid (1.78 g, 24.8%) following silica gel column chromatography with 10% ethyl acetate/chloroform. ^{1}H NMR δ_H(DMSO-d_6) 8.09 (s, 1H, H-8), 7.51−7.35 (m, 5H, Ar), 6.54 (s, 2H, N^2H_2, exchange with D_2O), 6.24 (dd, J = 6.0, J = 8.5, 1H, H-1′), 5.50 (s, 2H, OCH_2Ar), 5.33−5.32 (m, 1H, H-3′), 4.32−4.27 (m, 1H, H-4′), 4.22−4.18 (m, 2H, H-5′), 3.06−2.98 (m, 1H, H-2′α), 2.50−2.44 (m, 1H, H-2′β), 2.09 (s, 3H, $COCH_3$), 2.02 (s, 3H, $COCH_3$); MS, m/z 442.1 $[M + H]^+$, 464.1 $[M + Na]^+$; Anal. ($C_{21}H_{23}N_5O_6$) C, H, N.

***O*^{6}-Benzyl-*N*^{2}-[[[[4′-[(2″,3″,4″-tri-*O*-acetyl-6″-methyl-β-*D*-glucopyranuronosyl)oxy]-3′-nitrophenyl]methyl]oxy]-carbonyl]-9-[(pivaloyloxy)methyl]guanine (8).** To an ice-cooled solution of *O*^{6}-benzyl-9-[(pivaloyloxy)methyl]guanine (**3**)[29] (1.97 g, 5.5 mmol) in 120 mL of anhydrous dichloromethane and 5 mL of pyridine was added a toluene solution of phosgene (2.8 mL, 5.3 mmol phosgene), and the mixture was stirred for 20 h while the ice bath was allowed to warm to room temperature. A solution of 4-*O*-(2′,3′,4′-tri-*O*-acetyl-6′-methyl-β-D-glucopyranuronosyl)-3-nitrobenzyl alcohol (**7**)[13] (2.31 g, 4.8 mmol) in 150 mL of dichloromethane was then added, and the solution was stirred at room temperature for 2 h. Purification by flash column chromatography (ethyl acetate:chloroform, 3:7) gave **8** (2.02 g, 42%). ^{1}H NMR δ_H-(DMSO-d_6) 10.63 (s,1H, N^2H, exchanges with D_2O), 8.29 (s, 1H, H-8), 8.03 (d, 1H, O_2NArH-2′), 7.79 (dd, J = 8.8, J = 2.1, 1H, O_2NArH-6′), 7.59 (dd, 2H, Ar-*o*), 7.46 (d, J 8.7, 1H, O_2-NArH-5′), 7.41−7.35 (m, 3H, Ar-*m*, *p*), 6.08 (s, 2H, 9-CH_2), 5.75 (d, J 7.8, 1H, H-1″), 5.61 (s, 2H, OCH_2Ar), 5.46 (t, J 9.6, 1H, H-2″), 5.23 (s, 2H, O_2NArCH_2), 5.15−5.08 (m, 2H, H-3″,4″), 4.75 (d, J 9.9, 1H, H-5″), 3.64 (s, 3H, CH_3), 2.02−2.00 (3 s, 9H, 3 $COCH_3$), 1.09 (s, 9H, $C(CH_3)_3$); MS m/z 867.5 $[M + H]^+$, 889.4 $[M + Na]^+$; Anal. ($C_{39}H_{42}N_6O_{17}$) C, H, N.

3′,5′-Di-*O*-acetyl-*O*^{6}-benzyl-*N*^{2}-[[[[4″-[(2‴,3‴,4‴-tri-*O*-acetyl-6‴-methyl-β-D-glucopyranuronosyl)oxy]-3″-nitrophenyl]methyl]oxy]carbonyl]-2′-deoxyguanosine (9). Using the above procedure for compound **8**, compound **9** was obtained in 54% yield. ^{1}H NMR δ_H(DMSO-d_6) 10.56 (s, 1H, N^2H, exchanges with D_2O), 8.39 (s, 1H, H-8), 8.02 (d, J = 2.1, 1H, O_2NArH-2″), 7.78 (dd, J = 8.7, J′2.1, 1H, O_2NArH-6″), 7.55 (dd, J = 8.1, J′1.7, 2H, Ar-*o*), 7.46 (d, J = 8.7, 1H, O_2-NArH-5″), 7.41−7.35 (m, 3H, Ar-*m*, *p*), 6.35 (dd, J = 6.5, J′7.5, 1H, H-1′), 5.75 (d, J = 7.8, 1H, H-1‴), 5.61 (s, 2H, CH_2Ar), 5.50−5.41 (m, 2H, H-2‴ and H-3′), 5.22 (s, 2H, O_2NArCH_2), 5.15−5.08 (m, 2H, H-3‴,4‴), 4.74 (d, J = 9.8, 1H, H-5‴), 4.36−4.31 (m, 1H, H-4′), 4.26−4.22 (m, 2H, H-5′), 3.64 (s, 3H, CH_3), 3.29−3.20 (m, 1H, H-2′α), 2.54−52.51 (m, 1H, H-2′β), 2.09−1.98 (5s, 15H, 5 $COCH_3$); MS m/z 953.2 $[M + H]^+$, 975.1 $[M + Na]^+$; Anal. ($C_{42}H_{44}N_6O_{20}$) C, H, N.

***O*^{6}-Benzyl-*N*^{2}-[[[[4′-[(β-D-glucopyranuronosyl)oxy]-3′-nitrophenyl]methyl]oxy]carbonyl]guanine, Monosodium Salt (1).** To a ice-cooled solution of **8** (0.424 g, 0.49 mmol) in 10 mL of methanol was added 10 mL of an ice-cooled solution of 2 M sodium hydroxide, and the mixture was stirred at 0 °C for 30 min. The solution was neutralized with 10% acetic acid to produce a viscous suspension that was slowly suction filtered (Whatman #50 filter paper) and allowed to dry. The crude product was purified on a Sephadex LH-20 column eluted with H_2O:methanol (65:35) at a flow rate of 1 mL/min. UV absorption was continuously monitored at 254 nm, and fractions (10 mL) were collected. The product (**1**) eluted in fractions 40−50. Methanol was removed on a rotary evaporator at room temperature. The resulting aqueous solution was lyophilized to provide **1** as a white solid (0.210 g, 64%). UV [0.05 M phosphate buffer (pH7.0)] λ_{max}= 267 ($\epsilon = 1.32 \times 10^4$ $M^{-1}\cdot cm^{-1}$); ^{1}H NMR δ_H(D_2O, DSS internal standard) 8.00 (s, 1H, H-8), 7.49−7.06 (m, 8H, Ar), 5.36 (s, 2H, OCH_2Ar), 5.04 (d, J = 6.9, 1H, H-1″), 4.91 (s, 2H, O_2NArCH_2), 3.86 (d, J 9.2, 1H, H-5″), 3.68 − 3.61 (m, 3H, H-2″,3″, 4″); δ_H(DMSO-d_6) 10.34 (s, 1H, N^2H, exchanges with D_2O), 8.15 (s, 1H, H-8), 7.97 (d, J = 2.1, 1H, O_2NArH-2′), 7.71 (dd, J = 9.0, J = 2.1, 1H, O_2NArH-

6′), 7.57 (dd, J = 8.2, J'1.6, 2H, Ar-*o*), 7.45 (d, J = 8.7, 1H, O_2NArH-5′), 7.41−7.35 (m, 3H, Ar-*m*, *p*), 7.26 (broad s,1H, H-9, exchanges with D_2O), 5.59 (s, 2H, O_2NArCH_2), 5.23 (d, J = 4.6, 1H, OH-2″, exchanges with D_2O), 5.18 (s, 2H, CH_2Ar), 5.08 (d, J = 7.3, 1H, H-1″), 5.01 (d, J = 4.5, 1H, OH-3″), 3.47 (d, J = 10.0, 1H, H-5″), 3.26−3.11 (m, 4H, OH-4″ and H-2″,3″,4″); MS [LC (0.1% HCOOH)] *m/z* 613.2 [M(acid form) + H]$^+$, 635.2 [M(acid form) + Na]$^+$; Anal. ($C_{26}H_{23}N_6NaO_{12}\cdot 2H_2O$) C, H, N.

***O*^{6}-Benzyl-*N*^{2}-[[[[4″-[(β-D-glucopyranuronosyl)oxy]-3″-nitrophenyl]methyl]oxy]carbonyl]-2′-deoxyguanosine, Monosodium Salt (2).** Using the above procedure for **1**, compound **2**, which eluted from the Sephadex LH-20 column in fractions 35−48, was obtained in 64% yield. UV [0.05 M phosphate buffer (pH 7.0)] λ_{max} 267 ($\epsilon = 1.97 \times 10^4$ M$^{-1}\cdot$cm^{-1}); ^{1}H NMR δ_H(DMSO-d_6) 10.49 (s, 1H, N^{2}H, exchanges with D_2O), 8.40 (s, 1H, H-8), 7.97 (d, J = 2.1, 1H, O_2NArH-2″), 7.73 (dd, J = 8.8, J'2.2, 1H, O_2NArH-6″), 7.56 (dd, J = 8.2, J'1.7, 2H, Ar-*o*), 7.48 (d, J = 8.8, 1H, O_2NArH-5″), 7.41−7.35 (m, 3H, Ar-*m*, *p*), 7.19 (broad, 1H, OH-2‴, exchanges with D_2O), 6.31 (t, J = 7.1, 1H, H-1′), 5.60 (s, 2H, O_2NArCH_2), 5.43 (d, J = 3.9, 1H, OH-3′, exchanges in D_2O), 5.23 (d, J = 4.7, 1H, OH-3‴, exchanges with D_2O), 5.20 (s, 2H, CH_2Ar), 5.08 (d, J = 7.3, 1H, H-1‴), 5.01 (d, J = 4.8, 1H, OH-4‴, exchanges with D_2O), 4.89 (t, J = 5.4, 1H, OH-5′), 4.41−4.39 (m, 1H, H-3′), 3.87−3.84 (m, 1H, H-4′), 3.61−3.50 (m, 2H, H-5′), 3.47 (d, J = 9.9, 1H, H-5‴), 3.29−3.11 (m, 3H, H-2‴,3‴,4‴), 2.75−2.68 (m, 1H, H-2′α), 2.29−2.23 (m, 1H, H-2′β); MS [LC (H_2O/acetonitrile)] *m/z* 727.1[M − Na]$^-$; Anal. ($C_{31}H_{31}N_6NaO_{15}\cdot 1.5H_2O$) C, H, N.

Prodrug Stability and Enzyme Kinetic Analyses. Prodrug stability and purity was determined by HPLC on a Hewlett-Packard LC 1090 Series II system equipped with a Phenomenex 250 × 4 mm column (5 μm particle size) eluted isocratically at 1 mL/min with acetonitrile/0.1 M triethylammonium acetate (TEAA), pH 7.0, (3:7). Aliquots (100 μL) from prodrug solutions were withdrawn and diluted with 100 μL of a solution of *p*-nitrobenzyl alcohol (an internal standard) in acetonitrile/0.1 M TEAA (6:4). UV detection was at 254 and 280 nm. Retention times for **1**, **2**, *O*^{6}-benzylguanine, *O*^{6}-benzyl-2′-deoxyguanosine, and *p*-nitrobenzyl alcohol were 5.20, 4.97, 7.13, 7.18, and 8.64 min, respectively. All determinations were carried out in duplicate or triplicate.

The stability of prodrugs was determined in phosphate-buffered saline (pH 7.2) (Life Technologie, Inc), a Tris buffer containing 50 mM Tris-HCl (pH 7.5) (Life Technologies, Inc), 5 mM dithiothreitol, and 0.1 mM EDTA, a MOPS buffer (pH 7.0) containing 50 mM morpholinopropane sulfonic acid, 0.01% bovine serum albumin, and 0.01% NaCl, and a modified Dulbecco's medium prepared by combining 400 mL of Dulbecco's medium with 7 mL of 7.5% $NaHCO_3$, 4 mL of 15 mM glutamine, 2 mL of gentamicin (10 mg/mL), and 40 mL of fetal calf serum. Solutions were incubated at 37 °C, and prodrug concentrations were analyzed by HPLC at various times as indicated above.

Enzymatic Cleavage by β-Glucuronidase. Prodrugs and *p*-nitrobenzyl alcohol (HPLC internal standard) were dissolved in the MOPS buffer (pH 7.0) at 37 °C. Enzymatic cleavage was initiated by adding 0.2 Fishman units of *E. coli* β-glucuronidase (Sigma type IX-A) (42.8 units/mg of protein) or 10 Fishman units of bovine liver β-glucuronidase (Sigma type B-10) (10.2 units/mg of protein) to the incubation buffer. Aliquots (100 μL) of the reaction mixture were withdrawn at various times and were mixed with 100 μL of acetonitrile/0.1 M TEAA (6:4) to quench the enzymatic reaction. Prodrug and product concentrations were determined by HPLC. For determination of enzyme kinetic parameters, prodrug solutions at concentrations between 5 and 300 μM were incubated with a fixed amount of enzyme. Aliquots were withdrawn at time intervals varying from 1 to 20 min and the reactions were quenched as described above. Initial reaction velocities were determined at each substrate concentration. Nonlinear regression methods were used to determine K_M and V_{max} values. Data were processed with Prism 3.0 software.

In Vitro Alkyltransferase Activity Assay. 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 for 30 min at 37 °C. For experiments involving β-glucuronidase, the bovine liver protein, prodrugs, and alkyltransferase were incubated together in the above hemocyanin-containing buffer for 30 min at 37 °C. The remaining alkyltransferase activity was determined after incubation with a [^{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. 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.

Cell Culture Cytotoxicity Assay. HT29 cells were grown in RPMI 1640 medium 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. Cells were plated at a density of 10^6 in 25 cm^2 flasks and 24 h later were incubated with different concentrations of prodrugs for the time indicated before exposure to 40 mM BCNU for 2 h. For experiments involving β-glucuronidase, the bovine liver protein was added to the cell cultures at 20 units/mL of medium and incubated along with the prodrug. BCNU was dissolved in absolute ethanol at a concentration of 8 mM. It was diluted with the same volume of ice-cold phosphate-buffered saline and was immediately administered to cells. After 2 h, the medium was replaced with fresh medium and the cells were left to grow for an additional 16−18 h. The cells were then replated at densities of 250 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. The plating efficiency of cells not treated with drugs was about 50%.

References

(1) Sinkula, A. A.; Yalkowsky, S. H. Rationale for design of biologically reversible drug derivatives: prodrugs. *J. Pharm. Sci.* **1975**, *64*, 181−210.

(2) Stella, V. J.; Himmelstein, K. J. Prodrugs and site-specific drug delivery. *J. Med. Chem.* **1980**, *23*, 1276−1282.

(3) Bagshawe, K. D. Antibody directed enzymes revive anticancer prodrugs concept. *Br. J. Cancer* **1987**, *56*, 531−532.

(4) Bagshawe, K. D.; Springer, C. J.; Searle, F.; Antoniw, P.; Sharma, S. K.; Melton, R. G.; Sherwood, R. F. A cytotoxic agent can be generated selectively at cancer sites. *Br. J. Cancer* **1988**, *58*, 700−703.

(5) Bosslet, K.; Czech, J.; Hoffmann, D. A novel one-step tumor-selective prodrug activation system. *Tumor Targeting* **1995**, *1*, 45−50.

(6) de Groot, F. M. H.; Damen, E. W. P.; Scheeren, H. W. Anticancer prodrugs for application in monotherapy: Targeting hypoxia, tumor-associated enzymes, and receptors. *Curr. Med. Chem.* **2001**, *8*, 1093−1122.

(7) Bosslet, K.; Straub, R.; Blumrich, M.; Czech, J.; Gerken, M.; Sperker, B.; Kroemer, H. K.; Gesson, J.-P.; Koch, M.; Monneret, C. Elucidation of the mechanism enabling tumor selective prodrug monotherapy. *Cancer Res.* **1998**, *58*, 1195−1201.

(8) de Graaf, M.; Boven, E.; Scheeren, H. W.; Haisma, H. J.; Pinedo, H. M. Beta-glucuronidase-mediated drug release. *Curr. Pharm. Des.* **2002**, *8*, 1391−1403.

(9) Leenders, R. G. G.; Damen, E. W. P.; Bijsterveld, E. J. A.; Scheeren, H. W.; Houba, P. H. J.; van der Meulen-Muileman, I. H.; Boven, E.; Hidde, J. H. Novel anthracycline-spacer-beta-glucuronide, -beta-glucoside, and -beta-galactoside prodrugs for application in selective chemotherapy. *Bioorg. Med. Chem.* **1999**, 7, 1597−1610.

(10) Houba, P. H. J.; Boven, E.; van der Meulen-Muileman, I. H.; Leenders, R. G. G.; Scheeren, J. W.; Pinedo, H. M.; Haisma, H. J. A novel doxorubicin-glucuronide prodrug DOX-GA3 for tumour-selective chemotherapy: distribution and efficacy in experimental human ovarian cancer. *Br. J. Cancer* **2001**, *84*, 550−557.

(11) Schmidt, F.; Florent, J. C.; Monneret, C.; Straub, R.; Czech, J.; Gerken, M.; Bosslet, K. Glucuronide prodrugs of hydroxy compounds for antibody directed enzyme prodrug therapy (ADEPT): A phenol nitrogen mustard carbamate. *Bioorg. Med. Chem. Lett.* **1997**, 7, 1071−1076.

(12) Lougerstay-Madec, R.; Florent, J. C.; Monneret, C.; Nemati, F.; Poupon, M. F. Synthesis of self-immolative glucuronide-based prodrugs of a phenol mustard. *Anti-Cancer Drug Des.* **1998**, *13*, 995−1007.
(13) Florent, J. C.; Dong, X.; Gaudel, G.; Mitaku, S.; Monneret, C.; Gesson, J. P.; Jaquesy, J. C.; Mondon, M.; Renoux, B.; Andrianomenjanahary, S.; Michel, S.; Koch, M.; Tillequin, F.; Gerken, M.; Czech, J.; Straub, R.; Bosslet, K. Prodrugs of anthracyclines for use in antibody-directed enzyme prodrug therapy. *J. Med. Chem.* **1998**, *41*, 3572−3581.
(14) Madec-Lougerstay, R.; Florent, J. C.; Monneret, C. Synthesis of self-immolative glucuronide spacers based on aminomethylcarbamate. Application to 5-fluorouracil prodrugs for antibody-directed enzyme prodrug therapy. *J. Chem. Soc., Perkin Trans. 1* **1999**, 1369−1375.
(15) Leu, Y. L.; Roffler, S. R.; Chern, J. W. Design and synthesis of water-soluble glucuronide derivatives of camptothecin for cancer prodrug monotherapy and antibody- directed enzyme prodrug therapy (ADEPT). *J. Med. Chem.* **1999**, *42*, 3623−3628.
(16) Schmidt, F.; Monneret, A. C. Prodrug mono therapy: Synthesis and biological evaluation of an etoposide glucuronide-prodrug. *Bioorg. Med. Chem.* **2003**, *11*, 2277−2283.
(17) Pegg, A. E.; Dolan, M. E.; Moschel, R. C. Structure, function, and inhibition of O^6-alkylguanine-DNA alkyltransferase. *Prog. Nucleic Acid Res. Mol. Biol.* **1995,** *51*, 167−223.
(18) Moschel, R. C.; McDougall, M. G.; Dolan, M. E.; Stine, L.; Pegg, A. E. Structural features of substituted purine derivatives compatible with depletion of human O^6-alkylguanine-DNA alkyltransferase. *J. Med. Chem.* **1992**, *35*, 4486-4491.
(19) Kokkinakis, D. M.; Moschel, R. C.; Pegg, A. E.; Schold, S. C. Eradication of human medulloblastoma tumor xenografts with a combination of O^6-benzyl-2 ′-deoxyguanosine and 1,3-bis(2-chloroethyl)-1-nitrosourea. *Clin. Cancer Res.* **1999**, *5*, 3676−3681.
(20) Dolan, M. E.; Pegg, A. E.; Moschel, R. C.; Grindey, G. B. Effect of O^6-benzylguanine on the sensitivity of human colon tumor xenografts to 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU). *Biochem. Pharmacol.* **1993**, *46*, 285−290.
(21) Felker, G. M.; Friedman, H. S.; Dolan, M. E.; Moschel, R. C.; Schold, C. Treatment of subcutaneous and intracranial brain tumor xenografts with O^6-benzylguanine and 1,3-bis(2-chloroethyl)-1-nitrosourea. *Cancer Chemother. Pharmacol.* **1993**, *32*, 471−476.
(22) Schold, S. C.; Kokkinakis, D. M.; Rudy, J. L.; Moschel, R. C.; Pegg, A. E. Treatment of human brain tumor xenografts with O^6-benzyl-2′-deoxyguanosine and BCNU. *Cancer Res.* **1996**, *56*, 2076−2081.
(23) Kurpad, S. N.; Dolan, M. E.; McLendon, R. E.; Archer, G. E.; Moschel, R. C.; Pegg, A. E.; Bigner, D. D.; Friedman, H. S. Intraarterial O^6-benzylguanine enables the specific therapy of nitrosourea-resistant intracranial human glioma xenografts in athymic rats with 1,3-bis(2-chloroethyl)-1-nitrosourea. *Cancer Chemother. Pharmacol.* **1997**, *39*, 307−316.
(24) Quinn, J. A.; Pluda, J.; Dolan, M. E.; Delaney, S.; Kaplan, R.; Rich, J. N.; Friedman, A. H.; Reardon, D. A.; Sampson, J. H.; Colvin, O. M.; Haglund, M. M.; Pegg, A. E.; Moschel, R. C.; McLendon, R. E.; Provenzale, J. M.; Sridharan, G.; Tourt-Uhlig, S.; Herndon, J. E.; Bigner, D. D.; Friedman, H. S. Phase II trial of carmustine plus O^6-benzylguanine for patients with nitrosourea-resistant recurrent or progressive malignant glioma. *J. Clin. Oncol.* **2002**, *20*, 2277−2283.
(25) Dolan, M. E.; Roy, S. K.; Garbiras, B. J.; Helft, P.; Paras, P.; Chae, M.-Y.; Moschel, R. C.; Pegg, A. E. O^6-Alkylguanine-DNA alkyltransferase inactivation by ester prodrugs of O^6-benzylguanine derivatives and their rate of hydrolysis by cellular esterases. *Biochem. Pharmacol.* **1998**, *55*, 1701−1709.
(26) Friedman, H. S.; Kokkinakis, D. M.; Pluda, J.; Friedman, A. H.; Cokgor, I.; Haglund, M. M.; Ashley, D. M.; Rich, J.; Dolan, M. E.; Pegg, A. E.; Moschel, R. C.; McLendon, R. E.; Kerby, T.; Herndon, J. E.; Bigner, D. D.; Schold, S. C. Phase I trial of O^6-benzylguanine for patients undergoing surgery for malignant glioma. *J. Clin. Oncol.* **1998**, *16*, 3570−3575.
(27) Friedman, H. S.; Pluda, J.; Quinn, J. A.; Ewesuedo, R. B.; Long, L.; Friedman, A. H.; Cokgor, I.; Colvin, O. M.; Haglund, M. M.; Ashley, D. M.; Rich, J. N.; Sampson, J.; Pegg, A. E.; Moschel, R. C.; McLendon, R. E.; Provenzale, J. M.; Stewart, E. S.; Tourt-Uhlig, S.; Garcia-Turner, A. M.; Herndon, J. E.; Bigner, D. D.; Dolan, M. E. Phase I trial of carmustine plus O^6-benzylguanine for patients with recurrent or progressive malignant glioma. *J. Clin. Oncol.* **2000**, *18*, 3522−3528.
(28) Carl, P. L.; Chakravarty, P. K.; Katzenellenbogen, J. A. A novel connector linkage applicable in prodrug design. *J. Med. Chem.* **1981**, *24*, 479−480.
(29) Chae, M. Y.; McDougall, M. G.; Dolan, M. E.; Swenn, K.; Pegg, A. E.; Moschel, R. C. Substituted O^6-benzylguanine derivatives and their inactivation of human O^6-alkylguanine-DNA alkyltransferase. *J. Med. Chem.* **1994**, *37*, 342−347.
(30) Zajc, B.; Lakshman, M. K.; Sayer, J. M.; Jerina, D. M. Epoxide and diol epoxide adducts of polycyclic aromatic hydrocarbons at the exocyclic amino group of deoxyguanosine. *Tetrahedron Lett.* **1992**, *33*, 3409−3412.
(31) Houba, P. H. J.; Leenders, R. G. G.; Boven, E.; Scheeren, J. W.; Pinedo, H. M.; Haisman, H. J. Characterization of novel anthracycline prodrugs activated by human beta-glucuronidase for use in antibody-directed enzyme prodrug therapy. *Biochem. Pharmacol.* **1996**, *52*, 455−463.
(32) Haisma, H. J.; Boven, E.; Vanmuijen, M.; Dejong, J.; Vandervijgh, W. J. F.; Pinedo, H. H. A monoclonal antibody-beta-glucuronidase conjugate as activator of the prodrug epirubicin-glucuronide for specific treatment of cancer. *Br. J. Cancer* **1992**, *66*, 474−478.
(33) Schaller, H. W. G.; Lerch, B.; and Khorana, H. G. Studies on polynucleotides. XXIV. The stepwise synthesis of specific deoxyribopolynucleotides (4). Protected derivatives of deoxyribonucleosides and new syntheses of deoxyribonucleoside-3′ phosphates. *J. Am. Chem. Soc.* **1963**, *85*, 3821−3827.

JM0493865