Modulation of Melphalan Resistance in Glioma Cells with a Peripheral Benzodiazepine Receptor Ligand−Melphalan Conjugate

Lidia Kupczyk-Subotkowska,[†] Teruna J. Siahaan,[†] Anthony S. Basile,[‡] Henry S. Friedman,[§] Patricia E. Higgins,[▽] Di Song,[▽] and James M. Gallo*[,▽]

Department of Pharmaceutical Chemistry, Simons Laboratories, The University of Kansas, Lawrence, Kansas 66047, Laboratory of Neuroscience, NIDDK, National Institutes of Health, Bethesda, Maryland, Department of Pediatrics, Duke University Medical Center, Durham, North Carolina 27710, and Department of Medical Oncology, Fox Chase Cancer Center, 7701 Burholme Avenue, Philadelphia, Pennsylvania 19111

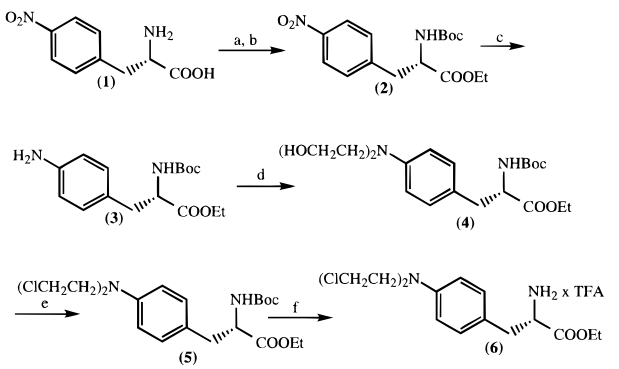
Received August 16, 1996[⊗]

Peripheral benzodiazepine receptors (PBRs) are located on the outer membrane of mitochondria, and their density is increased in brain tumors. Thus, they may serve as a unique intracellular and selective target for antineoplastic agents. A PBR ligand−melphalan conjugate (PBR−MEL) was synthesized and evaluated for cytotoxicity and affinity for PBRs. PBR−MEL (**9**) (*i.e.*, 670 amu) was synthesized by coupling of two key intermediates: 4-[bis(2-chloroethyl)-amino]-L-phenylalanine ethyl ester trifluoroacetate (**6**) and 1-(3′-carboxylpropyl)-7-chloro-1,3-dihydro-5-phenyl-2*H*-1,4-benzodiazepin-2-one (**8**). On the basis of receptor-binding displacement assays in rat brain and glioma cells, **9** had appreciable binding affinity and displaced a prototypical PBR ligand, Ro 5-4864, with IC_{50} values between 289 and 390 nM. **9** displayed differential cytotoxicity to a variety of rat and human brain tumor cell lines. In some of the cell lines tested including rat and human melphalan-resistant cell lines, **9** demonstrated appreciable cytotoxicity with IC_{50} values in the micromolar range, lower than that of melphalan alone. The enhanced activity of **9** may reflect increased membrane permeability, increased intracellular retention, or modulation of melphalan's mechanisms of resistance. The combined data support additional studies to determine how **9** may modulate melphalan resistance, its mechanisms of action, and if target selectivity can be achieved in *in vivo* glioma models.

Chemotherapy of malignant brain tumors remains an enormous therapeutic challenge. Treatment regimens are plagued by the inability to selectively deliver drugs to tumor cells and the emergence of drug resistance. The most recent advances in drug delivery to brain tumors have utilized regional implants of drug-loaded polymers that bypass the blood−brain barrier (BBB)[1,2] and gene therapy approaches.[3] Moreover, in attempts to circumvent mechanisms of drug resistance, modulators of alkylguanine transferase (*i.e.*, O^6-benzylguanine)[4] or glutathione (*i.e.*, buthionine sulfoximine)[5] have been applied. Presently, these approaches are experimental, and their ultimate place in the therapeutic arsenal will require further analyses.

One way to enhance the selective delivery of anticancer drugs to tumors is to target receptors that are overexpressed in tumors. The peripheral benzodiazepine receptor (PBR) is such a novel therapeutic target, as PBR expression is selectively increased in both experimental and human brain tumors compared to normal brain and peripheral tissues.[6−8] In fact, PBR expression by brain tumors correlates with the degree of malignancy.[7] The PBR is predominately expressed on the outer surface of mitochondria,[9−11] distinguishing it as an intracellular target, rather than the more common cell surface membrane targets. The identification of the PBRs in brain tumors has also served as a means to evaluate PBR ligands as diagnostic imaging agents.[7,8] Thus, the characteristics of PBRs in tumors suggest that PBR ligands can serve as receptor-mediated drug carriers to selectively target anticancer drugs to brain tumors.

Scheme 1[a]



[a] Reagents: (a) HCl_g, EtOH; (b) di-*tert*-butyl dicarbonate, TEA; (c) 10% Pd/C, H_2, MeOH; (d) ethylene oxide, CH_3COOH/H_2O; (e) CCl_4, Ph_3P/CH_2Cl_2; (f) TFA/anisole.

The goals of the current study were to synthesize a PBR ligand−melphalan conjugate able to bind to PBRs *in vitro* and to further evaluate its cytotoxicity in melphalan-sensitive and melphalan-resistant glioma cell lines.

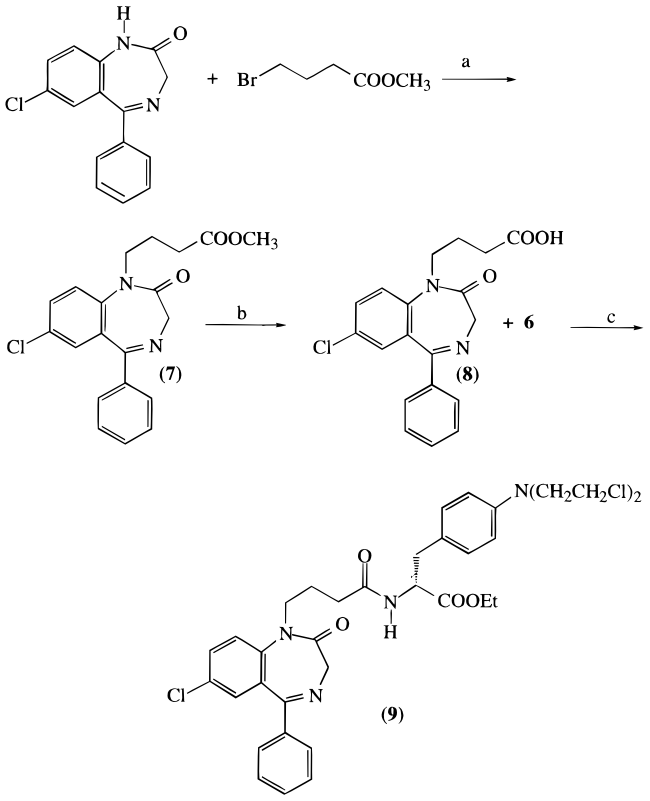
Results and Discussion

Synthesis of PBR−MEL (**9**) was accomplished by coupling **6** and **8** (Scheme 2). The synthesis of intermediate **6** was started by esterification of 4-nitrophenylalanine (**1**) with a mixture of ethanol and hydrogen chloride gas (Scheme 1). Next, the amino group was protected with *N*-*tert*-butyloxycarbonyl (*t*-Boc) group to

* To whom correspondence should be sent.
[†] The University of Kansas.
[‡] NIDDK.
[§] Duke University Medical Center.
[▽] Fox Chase Cancer Center.
[⊗] Abstract published in *Advance ACS Abstracts,* May 1, 1997.

S0022-2623(96)00592-4 CCC: $14.00

Scheme 2[a]



[a] Reagents: (a) NaH, DMF; (b) KOH, MeOH; (c) EDC, HOBt.

Table 1. Receptor-Binding Characteristics of Benzodiazepine−Melphalan Ethyl Ester (PBR−MEL, **9**), Nordiazepam, and Ro 5-4864 in Rat Brain and Glioma Cells

source	IC_{50} (nM)[a]	K_d (nM)[b]	B_{max} (pmol/mg of protein)
normal brain homogenate	327 520[c]	1.3 ± 0.1	0.29 ± 11
C6	289	4.0 ± 0.25	22 ± 1.5
RG-2	390	3.5 ± 0.64	33 ± 3.1

[a] Values indicate binding affinity of **9** unless otherwise indicated. [b] K_d and B_{max} are binding constants for Ro 5-4864. [c] Value for nordiazepam (desmethyldiazepam).

give compound **2**. Reduction of the nitro group in compound **2** was accomplished using 10% Pd/C with ammonium formate to yield compound **3**.[12] The aromatic amino group was reacted with ethylene oxide in acetic acid/water to give compound **4**. Treatment of compound **4** with triphenylphosphine, carbon tetrachloride, and dichloromethane gave compound **5**.[13] The *t*-Boc protecting group in **5** was removed by trifluoroacetic acid to give intermediate **6**. Intermediate **8** was synthesized by treatment of 7-chloro-1,3-dihydro-5-phenyl-2*H*-1,4-benzodiazepin-2-one[14] with methyl 4-bromobutyrate to generate compound **7** (Scheme 2). Basic hydrolysis of compound **7** gave the desired intermediate **8**. Intermediates **6** and **8** were coupled using EDC and HOBt to yield the target molecule **9**.

The receptor-binding assays (see Table 1) indicate that **9** binds to PBRs in rat brain and rat glioma cells (*i.e.*, C6 and RG-2) at nanomolar concentrations that should be achievable *in vivo*. The binding affinity of **9** in normal brain (*i.e.*, IC_{50} = 327 nM) was greater than that obtained with nordiazepam (*i.e.*, IC_{50} = 520 in normal brain), the benzodiazepine from which **9** is derived. This greater affinity exhibited by **9** is consistent with other reports where large alkyl substitutions

Table 2. Cytotoxicity of Melphalan (MEL), Benzodiazepine−Melphalan Ethyl Ester (PBR−MEL, **9**), and Peripheral Benzodiazepine Ligands in Glioma Cell Lines

cell line (species source)	IC_{50} (μM)[a]			
	MEL	PBR−MEL	Ro 5-4864	nordiazepam
C6 (rat)	11.0	43.4	>200	>200
C6-MR (rat)	74.9	74.4		
RG-2 (rat)	15.2	34.3	>100	>200
SF-126 (human)	103.0	21.0	>100	>100
SF-188 (human)	35.4	17.3	137.5	>100
D341 (human)	12.4	25.0	ND[b]	>200
D341 MR (human)	52.4	32.8	ND	>200
D283 (human)	6.8	72.3	ND	>200
D283 MR (human)	16.3	25.0	ND	>200
DAOY (human)	55.4	40.0	ND	>200
DAOY MR (human)	140.4	29.3	ND	>200

[a] Data are expressed as mean of two separate experiments except for SF-126, SF-188, DAOY, and DAOY MR in which three separate studies were completed. [b] ND = not determined.

at the N-4 position were inserted.[15] Using the model PBR ligand Ro 5-4864, the binding affinity (*i.e.*, K_d) and receptor density (*i.e.*, B_{max}) in the rat glioma cells compared favorably to values previously reported with either Ro 5-4864 or PK-11195 in C6 and RG-2 tumors *in vivo*.[6−8] The *in vitro* binding affinities were about 1.5−2-fold greater, whereas the PBR densities were about 20-fold greater than those reported *in vivo*. The higher B_{max} values obtained *in vitro* may in part be due to the greater specificity of the assay since contamination from normal adjacent brain is not possible. The higher density of PBRs in brain tumors compared to normal brain is the basis of using PBR ligand−drug conjugates to selectively target brain tumors.

The prototypical isoquinoline ligand PK-11195 has appreciable binding to both rat and human brain PBRs[16,17] and brain tumors.[7,8] The Ro 5-4864 PBR ligand and **9**, both based on a benzodiazepine structure, specifically bind only to rat PBRs. Thus, a pertinent direction to follow for targeting human PBRs will be the development of isoquinoline-based drug conjugates.

Cytotoxicity assays of MEL, **9**, and the PBR ligands nordiazepam and Ro 5-4864 were conducted in various rat and human brain tumor cell lines using an SRB assay[18] for adherent cells or the MTS assay[19] for suspended cells (*i.e.*, D341, D341 MR, D283, D283 MR) (see Table 2). The SRB assay has been adopted for routine use by the NCI.[20] It can be seen that **9** has greater activity in five cell lines with the greatest difference being a 5-fold enhancement in cytotoxicity in the human glioma SF-126 cell line. MEL was more active in the other five cell lines. The two benzodiazepines, nordiazepam and Ro 5-4864, did not demonstrate appreciable cytotoxicity, and in only one cell line, SF-188, was an IC_{50} value obtained.

Cytotoxicity assays (*i.e.*, SRB assay) were also conducted in RG-2 cells with MEL and **9** in the presence of two PBR ligands, nordiazepam (a weak ligand) and Ro 5-4864 (a strong ligand), at concentrations from 70 nM to 70 μM, to determine if cytotoxicity was attenuated by competitive inhibition of PBR binding. For both MEL and **9**, there was no change in the IC_{50} values obtained in the absence and presence of the PBR ligands. In the presence of the PBR ligands, MEL's IC_{50} values ranged from 12 to 15 μM (compared to 15.2 μM without binding inhibitors), and for **9** the values ranged from 32 to 38 μM (compared to 34.3 μM without binding inhibitors).

Table 3. Glutathione *S*-Transferase (GST) Activity and Glutathione (GSH) Concentrations in Human Medulloblastoma Cells[a]

cell line	GST (nmol/min/mg of protein)	GSH (μmol/g)
D341	212.6 ± 21.7	17.5 ± 3.6
D341 MR	327.9 ± 43.6	33.2 ± 8.5
D283	150.6 ± 35.8	18.4 ± 4.5
D283 MR	417.3 ± 65.6	50.7 ± 16.1
DAOY	88.7 ± 7.1	11.4 ± 2.4
DAOY MR	84.0 ± 10.9	10.8 ± 1.5

[a] Data are expressed as mean of three to four experiments.

Since there is a species dependence in PBR ligand binding,[16,17] enhanced cytotoxicity of **9** may have been anticipated in a rat glioma cell line, such as RG-2, in which Ro 5-4867 and analogues have greater binding affinity than in human glioma cell lines. However, there was no direct relationship between *in vitro* cytotoxicity and binding to PBRs. Although this disjunction may cause concern, it may, in fact, be an advantage to separate receptor binding and cytotoxicity processes. The ability of **9** to bind to PBR located on the outer membrane of mitochondria is a mechanism that may control the selective distribution of **9** to brain tumors *in vivo*, as demonstrated with PBR ligands.[6−8] Following initial distribution to PBRs, **9** may exert its cytotoxic action via multiple pathways upon dissociation from PBRs. Either MEL, generated from the conjugate, or the conjugate itself can interact with DNA forming lethal adducts. If PBR binding and cytotoxicity were correlated, it would suggest a single pathway of cytotoxicity via interaction of **9** with mitochondrial DNA, contingent upon internalization of **9** into mitochondria. Thus, the strategy of PBR−drug conjugates is to attain target (brain tumor) selectivity via binding to PBRs followed by subsequent cytotoxic actions, most likely directed at genomic DNA. *In vivo* tissue distribution studies will be needed to demonstrate the feasibility of this strategy.

It was of interest to contrast cytotoxicity of MEL and **9** in sensitive and MEL-resistant cell lines. Mechanisms of resistance to MEL can vary depending on the cell type and normally consist of membrane transport defects, inactivation via glutathione, and enhanced repair of DNA adducts.[21−23] In two of the human-derived MEL-resistant clones, D283 MR and D341 MR, glutathione *S*-transferase (GST) activity, an enzyme that catalyzes the formation of glutathione conjugates,[22] and glutathione (GSH) concentrations were elevated (see Table 3). MEL's activity is reduced about 4-fold in these lines compared to the parental lines, whereas **9**'s activity is either unchanged or enhanced. In the DAOY and DAOY MR lines, MEL is approximately 3-fold less toxic to the MR line, whereas **9** is equally toxic, with IC_{50}s less than 40 μM. Since **9** exhibits analogous cytotoxicity to the MR lines as the parental lines, unlike MEL, it is not susceptible to the mechanism of MEL resistance in these cell lines. In contrasting the cytotoxicity of MEL and **9** in the rat C6 and C6 MR cell lines, it is again shown that **9** is not nearly as influenced by the mechanisms that control resistance to MEL. On the basis of the combined data in both rat and human MR cell lines, **9** may modulate the formation of inactive drug−GSH conjugates and possibly bypass membrane transport defects. It will be important to extend these preliminary findings and characterize how **9** and other analogues may modulate MEL's mechanisms of resistance.

The **9** moiety is a unique drug conjugate, not only due to its intracellular target but also because of its low molecular weight (*i.e.*, MW = 670 DA). **9** is formed through an amide linkage between a benzodiazepine moiety and melphalan ethyl ester that is not susceptible to acid or base hydrolysis in biological fluids. Other amide linkages of melphalan conjugates have been shown to be stable intracellularly,[24] and thus, cleavage of the amide bond of **9** will likely require intracellular endopeptidases. This potentially enhanced stability may have consequences for its pharmacological action, since generation of free MEL may not be required to initiate cytotoxic events.

The lipophilicity of **9**, imparted by the benzodiazepine moiety, may enable the conjugate to pass through cell membranes by passive diffusion rather than by facilitated transport via the large neutral amino acid carrier as for MEL.[25] This could result in higher intracellular concentrations of **9** compared to those obtained by MEL alone. In addition, the presence of the ethyl ester group, as opposed to the carboxylic acid group of MEL, could also facilitate membrane uptake and cellular retention following conversion to the free acid.

In summary, the novel design features of **9** may lead to enhanced membrane transport, intracellular stability, and retention. In addition, the interaction of **9** with PBRs may serve as a selective target for *in vivo* delivery. Development of this class of agents should be pursued.

Experimental Section

Equipment. All reagents are commercially available. Melting points were determined on a Fisher-Johns melting point apparatus and are uncorrected. NMR spectra were determined in $CDCl_3$ on a QE (300 MHz) spectrometer. High-resolution mass spectra were obtained using a VG Analytical ZAB double-focusing spectrometer. A Polytron (Brinkmann Instruments, Westbury, NY) tissue homogenizer was used. Glass fiber filters (#32) were obtained from Schleicher & Schuell (Keene, NH). A Brandel (Brandel Instruments, Gaithersburg, MD) M-24R filtering manifold was used for receptor-binding assays. Radioactivity was measured in a liquid scintillation spectrometer (model LS 5801, Beckman Instruments, Fullerton, CA).

Synthesis. *N*-(*tert*-Butyloxycarbonyl)-4-nitro-L-phenylalanine Ethyl Ester (2). 4-Nitro-L-phenylalanine (**1**) was prepared as described in the literature[26] and converted into its ethyl ester hydrochloride.[27] To a suspension of 4-nitro-L-phenylalanine ethyl ester hydrochloride (5.49 g, 0.02 mol) in anhydrous dichloromethane (60 mL) were added triethylamine (3.5 mL) followed by di-*tert*-butyl dicarbonate (4.36 g, 0.02 mol), and the reaction mixture was stirred at room temperature for 4 h. TLC (silica gel, chloroform:petroleum ether, 7:3) indicated complete reaction. Dichloromethane (200 mL) was added, and the reaction mixture was washed successively with a 1 N solution of HCl, water, saturated $NaHCO_3$ solution, water, and brine and then dried ($MgSO_4$). Solvent was evaporated, and product was crystallized from a diethyl ether:hexane mixture to give 5.40 g (0.016 mol, 80%) of **2**, homogeneous by TLC (chloroform:petroleum ether, 7:3): mp 64−65 °C; ^{1}H NMR ($CDCl_3$, δ) 8.15 (d, J = 8.1 Hz, 2H), 7.3 (d, J = 8.4 Hz, 2H), 5.0 (bd, 1H), 4.5−4.4 (m, 1H), 4.2 (q, J = 7.0 Hz, 2H), 3.3−3.1 (m, 2H), 1.4 (s, 9H), 1.25 (t, 3H); FAB HRMS [M + H] calcd for ($C_{16}H_{22}N_2O_6$) 339.1556, found 339.1574.

***N*-(*tert*-Butyloxycarbonyl)-4-amino-L-phenylalanine Ethyl Ester (3).** To a solution of **2** (5.40 g, 0.016 mol) in anhydrous methanol (100 mL) were added 10% Pd/C catalyst (100 mg) followed by anhydrous ammonium formate (5.1 g,

0.08 mol). The reaction mixture was stirred at room temperature for 1 h and TLC (silica gel, diethyl ether) indicated complete reduction. The reaction mixture was filtered through a Celite 545 pad and washed with methanol, and combined methanol solutions were evaporated. The solid residue was triturated with water and the product extracted into diethyl ether. The ether solution was dried ($MgSO_4$) and evaporated to give an oily product, homogeneous by TLC (diethyl ether) and resulting in 4.89 g (0.016 mol, 100%) of **3**: ^{1}H NMR ($CDCl_3$, δ) 6.9 (d, J = 8.1 Hz, 2H), 6.6 (d, J = 8.4 Hz, 2H), 4.95 (bd, 1H), 4.5−4.4 (m, 1H), 4.15 (q, J = 7.0 Hz, 2H), 2.95 (d, J = 7.0 Hz, 2H), 1.4 (s, 9H), 1.2 (t, 3H); FAB HRMS [M^+] calcd for $C_{16}H_{24}N_2O_4$ 308.1736, found 308.1740.

***N*-(*tert*-Butyloxycarbonyl)-4-[bis(2-hydroxyethyl)amino]-L-phenylalanine Ethyl Ester (4).** Solution of **3** (3.84 g, 0.0124 mol) in water:glacial acetic acid mixture (50 mL:34 mL) was cooled to 0 °C. Ethylene oxide (8.5 mL) was then added and the reaction mixture left at room temperature for 24 h. Water and acetic acid were evaporated, and the product, after neutralization with saturated Na_2CO_3 solution, was extracted into ethyl acetate. The organic layer was washed three times with water followed by brine and dried ($MgSO_4$). Ethyl acetate solution was evaporated and the product purified by column chromatography on silica gel (70−230 mesh) with ethyl acetate as eluent to give 4.27 g (0.0108 mol, 87%) of oily **4**, homogeneous by TLC (ethyl acetate): ^{1}H NMR ($CDCl_3$, δ) 6.95 (d, J = 8.1 Hz, 2H), 6.6 (d, J = 8.4 Hz, 2H), 5.0 (bd, 1H), 4.55−4.45 (m, 1H), 4.15 (q, J = 7.0 Hz, 2H), 3.8 (t, 6H), 3.5 (t, 4H), 3.0−2.95 (m, 2H), 1.4 (s, 9H), 1.2 (t, 3H); FAB HRMS [M + H] calcd for $C_{20}H_{33}N_2O_6$ 397.23339, found 397.2357.

***N*-(*tert*-Butyloxycarbonyl)-4-[bis(2-chloroethyl)amino]-L-phenylalanine Ethyl Ester (5).** To the stirred and 0 °C cooled solution of **4** (4.27 g, 0.011 mol) in anhydrous dichloromethane (100 mL) was added anhydrous carbon tetrachloride (10 mL) following addition of 1 g portions of triphenylphosphine (8.20 g, 0.031 mol). After addition was completed, the reaction mixture was stirred at room temperature overnight. Solvent was evaporated and the product purified by column chromatography on silica gel (70−230 mesh) with dichloromethane followed by diethyl ether as eluent to give 3.10 g (0.007 mol, 66%) of oily **5**, homogeneous by TLC (diethyl ether): ^{1}H NMR ($CDCl_3$, δ) 7.0 (d, J = 8.1 Hz, 2H), 6.6 (d, J = 8.4 Hz, 2H), 4.95 (bd, 1H), 4.55−4.45 (m, 1H), 4.15 (q, J = 7.0 Hz, 2H), 3.75−3.55 (m, 8H), 3.05−2.95 (m, 2H), 1.4 (s, 9H), 1.25 (t, 3H); FAB HRMS [M + H] calcd for $C_{20}H_{31}N_2O_4Cl_2$ 433.1661, found 433.1670.

4-[Bis(2-chloroethyl)amino]-L-phenylalanine Ethyl Ester Trifluoroacetate (6). A solution of **5** (3.10 g, 0.007 mol) in trifluoroacetic acid (50 mL) containing anisole (1 mL) was stirred at room temperature for 1 h. TFA was evaporated and diethyl ether was added to precipitate the product that resulted in 2.60 g (0.0058 mol, 83%) of **6**: mp 138−139 °C; ^{1}H NMR ($CDCl_3$, δ) 7.1 (d, J = 8.1 Hz, 2H), 6.6 (d, J = 8.4 Hz, 2H), 4.25−4.05 (m, 3H), 3.8−3.55 (m, 8H), 3.15 (d, J = 7.0 Hz, 2H), 1.25 (t, 3H); FAB HRMS [M + H] calcd for $C_{15}H_{23}N_2O_2Cl_2$ 333.1137, found 333.1145.

1-(3′-Carbomethoxypropyl)-7-chloro-1,3-dihydro-5-phenyl-2*H*-1,4-benzodiazepin-2-one (7). 7-Chloro-1,3-dihydro-5-phenyl-2*H*-1,4-benzodiazepin-2-one (1.35 g, 0.005 mol) was dissolved in anhydrous dimethylformamide (10 mL), and sodium hydride (0.40 g) was added. The reaction mixture was stirred at room temperature for 30 min, and then 4-bromobutyric acid methyl ester (1.80 g, 0.010 mol) was added dropwise. After overnight stirring at room temperature, solvent was evaporated and the residue treated with an ethyl acetate:water mixture. The organic layer was separated, washed with water and brine, and dried (Na_2SO_4). Solvent was evaporated, and the thick oily product was used for the next step to obtain 1.27 g (0.003 mol, 60%) of crude **7**: ^{1}H NMR ($CDCl_3$, δ) 7.6−7.25 (m, 8H), 7.1 (d, 2H), 4.8 (d, 1H), 4.3 (q, 1H), 3.75 (d, 1H), 3.55 (s, 1H), 3.7−3.6 (m, 1H), 2.3−1.7 (m, 4H); FAB HRMS [M + H] calcd for $C_{20}H_{20}N_2O_3Cl$ 371.1162, found 371.1141.

1-(3′-Carboxylpropyl)-7-chloro-1,3-dihydro-5-phenyl-2*H*-1,4-benzodiazepin-2-one (8). A solution of **7** (1.27 g, 0.003 mol) in methanol (50 mL) containing a 1 M solution of potassium hydroxide (4 mL) was stirred at room temperature for 2 h. TLC (silica gel, ethyl acetate) indicated complete reaction. Methanol was evaporated, water was added to the residue, and the solution was acidified with 1 N HCl. Product was extracted into ethyl acetate and dried over anhydrous sodium sulfate. The solution was dried (Na_2SO_4) and solvent evaporated. Crystallization from an ethyl ether:petroleum ether mixture gave 0.91 g (0.0025 mol, 85%) of **8**, homogeneous by TLC (ethyl acetate): mp 160−163 °C; ^{1}H NMR ($CDCl_3$, δ) 7.6−7.2 (m, 8H), 4.8 (d, 1H), 4.35 (q, 1H), 3.75 (d, 1H), 3.75−3.6 (m, 1H), 2.3−1.6 (m, 4H); FAB HRMS [M + H] calcd for $C_{19}H_{18}N_2O_3Cl$ 357.1006, found 357.1002.

1-[4-Butanoyl-[*p*-bis(2-chloroethyl)amino]-L-phenylalanine ethyl ester]-7-chloro-1,3-dihydro-5-phenyl-2*H*-1,4-benzodiazepin-2-one (9). To a stirred and 0 °C cooled solution of **8** (0.36 g, 0.001 mol) in dimethylformamide (20 mL) were added 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC) (0.20 g) and hydroxybenzotriazole (0.14 g). The reaction mixture was stirred for 30 min; then compound **6** (0.45 g, 0.001 mol) followed by triethylamine (0.2 mL) were added. After overnight stirring at room temperature, the solvent was evaporated and the residue treated with an ethyl acetate:water mixture. The organic layer was separated, washed successively with citric acid solution, water, saturated sodium bicarbonate solution, water, sodium chloride solution, water, saturated $NaHCO_3$ solution, water, and brine, and then dried (Na_2SO_4). Solvent was evaporated and product purified by column chromatography on silica gel (60−200 mesh) with ethyl acetate as eluent. Crystallization from an ethyl acetate:petroleum ether mixture gave 0.21 g (0.00031 mol, 31%) of **9**, homogeneous by TLC (ethyl acetate): mp 70−72 °C; ^{1}H NMR ($CDCl_3$, δ) 7.65−7.35 (m, 8H), 6.9 (dd, 2H), 6.55 (dd, 2H), 6.05 (bd, 1H), 5.8 (bd, 1H), 4.85−4.6 (m, 2H), 4.5−4.1 (m, 3H), 3.8−3.55 (m, 9H), 3.05−2.85 (m, 2H), 2.1−1.6 (m, 4H), 1.3 (t, 3H); FAB HRMS [M + H] calcd for $C_{34}H_{38}N_4O_4Cl_3$ 671.1959, found 671.1959.

Receptor-Binding Assay. Competitive binding assays using Ro 5-4864, [^{3}H]Ro 5-4864, and **9** were performed with rat glioma cells and rat brain homogenates as previously described.[28] For whole brain homogenates, rats were sacrificed (80% CO_2, 20% O_2 prior to decapitation) and the brains removed and placed into ice-cold 0.32 M sucrose. The intact brain (1.5−2 g) was homogenized in 50 vol of 50 mM Tris-HCl (pH 7.4, 0−4 °C) and then centrifuged once at 20 000*g* for 20 min. The pellet was retained and resuspended in 100 vol of 50 mM Tris-HCl buffer. For RG-2 and C6 glioma cells, 6−8 × 10^6 cells were suspended in 25 mL of 50 mM Tris-citrate buffer (pH 7.4) and homogenized for 10 s. The homogenate was centrifuged at 20 000*g* for 20 min, and the resultant pellet was resuspended in 16 mL of Tris-citrate buffer. Aliquots of either cell suspension (125 μL, 4 μg of protein) or tissue suspension (250 μL of brain, ∼200 μg of protein) were added to each assay tube containing 25 μL (for cells) or 50 μL (for tissues) of [^{3}H]Ro 5-4864 (final concentration = 1 nM, specific activity = 86.9 Ci/mmol; DuPont/New England Nuclear, Boston, MA). Nonspecific binding was determined using unlabeled PK-11195 (final concentration = 10 μM). Sufficient buffer was added to bring the final volume to either 250 μL (for cells) or 500 μL (for tissues). All assays were performed in duplicate. The assay was initiated by the addition of cells or tissues and terminated after 60 min of incubation at 0−4 °C by rapid filtration over glass fiber filters using a filtering manifold. The samples were washed with two 5 mL aliquots of 50 mM Tris-HCl buffer (pH 7.4, 0−4 °C). The radioactivity retained by the filters was measured using 4 mL of scintillation cocktail (Cytoscint, ICN Biomedical, Aurora, OH). Nonlinear regression analysis of the data was performed using Prism II (GraphPad Software, San Diego, CA).

Cytotoxicity Assays. The standard SRB (sulforhodamine B) assay, used in the NCI screen, was used to determine cytotoxicity for monolayer cell lines.[18,19] For suspension cell lines (*i.e.*, D283, D283 MR, D341, and D341 MR), a commercially available kit[29] was used for the MTS (3-(4,5-dimethylthiazol-2-yl)-5-[3-(carboxymethoxy)phenyl]-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt) assay. C6 glioma cells were obtained from American Type Culture Collection (ATCC, Rockville, MD); SF126 and SF188 were kindly provided by the

Brain Tumor Research Center, University of California, San Francisco, CA. Development of a C6 MEL-resistant cell line (*i.e.*, C6 MR) was achieved by exposure of C6 cells to increasing MEL concentrations, starting at 10 μM, at weekly intervals. In each MEL treatment cycle, MEL would be exposed to cells for 24 h, then removed, and split before addition of the next higher MEL concentration. All other cell lines were supplied by Dr. Darrell Bigner, Duke University Medical Center, Durham, NC. For the three human melphalan-resistant cell lines (*i.e.*, D282 MR, D41 MR, and DAOY MR), resistance was maintained by exposure to melphalan (between 70 and 78 μM) for 24 h every third passage. After the 24 h exposure period, melphalan and media were removed and then resuspended in fresh medium prior to use in the cytotoxicity assays.

For monolayer cells (*i.e.*, SRB assay), cell suspensions prepared in their established culture medium were added to 96-well microtiter plates (200 μL/well at 10−20% confluency) and incubated overnight at 37 °C in 95% CO_2, 5% air. Solutions of melphalan, **9**, nordiazepam, and Ro 5-4864 were added to the cells either as single agents or in combination (*i.e.*, **9** + Ro 5-4864) and incubated for 48 h at 37 °C. Suspension cells (*i.e.*, MTS assay) were prepared in their established culture medium and added to 96-well microtiter plates (100 μL/well at 10−20% confluency) followed by the addition of drug solutions. Melphalan solutions were prepared in normal saline, whereas all other compounds were prepared in ethanol. The volume of ethanol added to each well did not exceed 1% of the total volume. To avoid drug degradation, in which melphalan is particularly susceptible, all drug solutions were freshly prepared and rapidly added to the cells.

For the SRB assay, following the 48 h incubation period, the medium was removed, and the cells were washed with phosphate-buffered saline. Next, 10% trichloroacetic acid (TCA) was added, and the plates were incubated for 60 min at 4 °C. The TCA was removed, and the cells were washed five times with distilled water followed by addition of 100 μL of SRB solution to each well. The plates were then incubated for 30 min at room temperature, the dye was removed, and then the cells were washed four times with 1% acetic acid followed by addition of 100 μL of 10 mM Tris buffer to each well. Following a minimum incubation of 60 min, the absorbance at 560 nm of each well was measured in a microplate reader. The absorbance values were converted to percent of control cell growth, and the IC_{50} was calculated from an algebraic equation.

For the MTS assay, following the 48 h incubation, 20 μL of an MTS/PBS solution was added to each well. The background absorbance was immediately determined, and then the plates were incubated at 37 °C. The absorbance was continually monitored every 30 min until a maximum was achieved.

GST activity and GSH concentrations in the medulloblastoma cell lines were determined as reported previously.[21]

References

(1) Brem, H.; Mahaley, M. S., Jr.; Vick, N. A.; Black, K. L.; Schold, S. C., Jr.; Burger, P. C.; Freidman, H.; Ciric, I. S.; Cozzens, T. W.; Kenealy, J. N. Interstitial chemotherapy with drug polymer implants for the treatment of recurrent gliomas. *J. Neurosurg.* **1991**, *74*, 441−446.

(2) Brem, H.; Piantadosi, S.; Burger, P. C.; Group, P. B. T. T. Intraoperative chemotherapy using biodegradable polymers: safety and effectiveness for recurrent glioma evaluated by a prospective, multi-institutional placebo-controlled clinical trial. *Lancet* **1995**, *345*, 1008−1012.

(3) Weller, M.; Malipiero, U.; Rensing-Ehl, A.; Barr, P. J.; Fontana, A. Fas/APO-1 gene transfer for human malignant glioma. *Cancer Res.* **1995**, *55*, 2936−2944.

(4) Felker, G. M.; Friedman, H. S.; Dolan, M. E.; Moschel, R. C.; Schold, S. C., Jr. Treatment of subcutaneous and intracranial brain tumor xenografts with O6-benzylguanine and 1,3-bis(2-chloroethyl)-1-nitrosourea. *Cancer Chemother. Pharmacol.* **1993**, *32*, 471−476.

(5) Skapek, S. X.; Colvin, O. M.; Griffith, O. W.; Groothius, D. R.; Colapinto, E. V.; Lee, Y.; Hilton, J.; Elion, G. B.; Bigner, D. D.; Friedman, H. S. Buthionine sulfoximine-mediated depletion of glutathione in intracranial human glioma-derived xenografts. *Biochem. Pharmacol.* **1988**, *37*, 4313−4317.

(6) Black, K. L.; Ikezaki, K.; Toga, A. W. Imaging of brain tumors using peripheral benzodiazepine receptor ligands. *J. Neurosurg.* **1989**, *71*, 113−118.

(7) Black, K. L.; Ikezaki, K.; Santori, E.; Becker, D. P.; Vinters, H. V. Specific high-affinity binding of peripheral benzodiazepine receptor ligands to brain tumors in rat and man. *Cancer* **1990**, *65*, 93−97.

(8) Ikezaki, K.; Black, K. L.; Toga, A. W.; Santori, E. M.; Becker, D. P.; Smith, M. L. Imaging peripheral benzodiazepine receptors in brain tumors in rats: in vitro binding characteristics. *J. Cerebral Blood Flow Metab.* **1990**, *10*, 580−587.

(9) Marangos, P. J.; Patel, J.; Boulenger, J.-P.; Clark-Rosenberg, R. Characterization of peripheral-type benzodiazepine binding sites in brain using [3H]Ro 5-4864. *Mol. Pharmacol.* **1982**, *22*, 26−32.

(10) Basile, A. S.; Skolnick, P. Subcellular localization of peripheral-type binding sites for benzodiazepine in rat brain. *J. Neurochem.* **1986**, *46*, 305−308.

(11) Anholt, R. R. H.; Pedersen, P.; De Souza, E.; Snyder, S. The peripheral-type benzodiazepine receptor: localization to the mitochondrial outer membrane. *J. Biol. Chem.* **1986**, *261*, 576−583.

(12) Ram, S.; Ehrenkaufer, R. E. A General Procedure for Mild and rapid Reduction of Aliphatic and aromatic Nitro Compounds Using Ammonium Formate as a Catalytic Hydrogen Transfer Agent. *Tetrahedron Lett.* **1984**, *25*, 3415−3418.

(13) Appel, R. Tertiary Phosphane/Tetrachloromethane, a Versatile Reagent For Chlorination, Dehydration, and P-N Linkage. *Angew. Chem., Int. Ed. Engl.* **1975**, *14*, 801−811.

(14) Sternback, L. H.; Fryer, R. I.; Metlesics, W.; Reeder, E.; Sach, G.; Saucy, G.; Stempel, A. Quinazolines and 1,4-Benzodiazepines VI. Halo-, Methyl-, and Methoxy-substituted 1,3-Dihydro-5-phenyl-2H-1,4-benzodiazepin-2-ones. *J. Org. Chem.* **1962**, *27*, 3788−3796.

(15) Wang, J. K. T.; Taniguchi, T.; Spector, S. Structural requirements for the binding of benzodiazepines to their peripheral-type sites. *Mol. Pharmacol.* **1984**, *25*, 349−351.

(16) Olson, J. M. M.; Junck, L.; Young, A. B.; Penney, J. B.; Mancini, W. R. Isoquinoline and peripheral-type benzodiazepine binding in gliomas: Implications for diagnostic imaging. *Cancer Res.* **1988**, *48*, 5837−5841.

(17) Basile, A. S.; Klein, D. C.; Skolnick, P. Characterization of benzodiazepine receptors in the bovine pineal gland: Evidence for the presence of an atypical binding site. *Mol. Brain Res.* **1986**, *1*, 127−135.

(18) Skehan, P.; Storeng, R.; Scudiero, D.; Monks, A.; McMahon, J.; Vistica, D.; Warren, J. T.; Bokesch, H.; Kenney, S.; Boyd, M. R. New colorimetric cytotoxicity assay for anticancer-drug screening. *J. Natl. Cancer Inst.* **1990**, *82*, 1107−1112.

(19) Buttke, T. M.; McCubrey, J. A.; Owen, T. C. Use of an aqueous soluble tetrazolium/formazan assay to measure viability and proliferation of lymphokine-dependent cell lines. *J. Immunol. Methods* **1993**, *157*, 233−340.

(20) Rubinstein, L. V.; Shoemaker, R. H.; Paull, K. D.; Simon, R. M.; Tosini, K.; Skehan, P.; Scudiero, D. A.; Monks, A.; Boyd, M. R. Comparison of *in vitro* anticancer-drug-screening data generated with a tetrazolium assay versus a protein assay against a diverse panel of human tumor cell lines. *J. Natl. Cancer Inst.* **1990**, *82*, 1113−1118.

(21) Rosenberg, M. C.; Colvin, O. M.; Griffith, O. W.; Bigner, S. H.; Elion, G. B.; Horton, J. K.; Lilley, E.; Bigner, D. D.; Friedman, H. S. Establishment of a melphalan-resistant rhabdomyosarcoma xenograft with cross-resistance to vincristine and enhanced sensitivity following buthionine sulfoximine-mediated glutathione depletion. *Cancer Res.* **1989**, *49*, 6917−6922.

(22) Phillips, P. C. Antineoplastic drug resistance in brain tumors. *Neurol. Clin.* **1991**, *9*, 383−404.

(23) Friedman, H. S.; Dolan, M. E.; Kaufmann, S. H.; Colvin, O. M.; Griffith, O. W.; Moschel, R. C.; Schold, S. C.; Bigner, D. D.; Ali-Osman, F. Elevated DNA polymerase a, DNA polymerase B, and DNA topoisomerase II in a melphalan-resistant rhabdomyosarcoma xenograft that is cross-resistant to nitrosourease and topotecan. *Cancer Res.* **1994**, *54*, 3487−3493.

(24) Kupczyk-Subotkowska, L.; Tamura, K.; Pal, D.; Sakaeda, T.; Siahaan, T. J.; Stella, V. J.; Borchardt, R. T. Derivative of melphalan designed to enhance drug accumulation in cancer cells. *J. Drug Targeting*, accepted for publication.

(25) Greig, N. H.; Momma, S.; Sweeney, D. J.; Rapoport, S. I. Facilitated transport of melphalan at the blood-brain barrier by the large neutral amino acid carrier system. *Cancer Res.* **1987**, *47*, 1571−1576.

(26) Erlenmeyer, E.; Lipp, A. Synthese des Tyrosins. *Ann. Chem.* **1883**, *219*, 161−233.

(27) Bergel, F.; Stock, J. A. Cyto-active Amino-acid and Peptide Derivatives. Part I. Substituted phenylalanines. *J. Chem. Soc.* **1954**, 2409−2417.

(28) Basile, A. S.; Skolnick, P. Tissue specific regulation of "peripheral-type" benzodiazepine receptor density after chemcial sympathectomy. *Life Sci.* **1988**, *42*, 273−283.

(29) *CellTiter 96 AQueous Assay*; Promega: Madison, WI, 1994.

JM960592P