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ORIGINAL ARTICLE

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Targeted delivery of a peripheral benzodiazepine receptor ligand-gemcitabine conjugate to brain tumors in a xenograft model

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Abstract *Purpose*: Peripheral benzodiazepine receptors (PBRs) are overexpressed in brain tumors compared to normal brain, and could serve as a target to selectively increase anticancer drug delivery through a PBR ligand-drug conjugate system. We have previously synthesized PBR ligand-gemcitabine conjugates based on the model PBR ligand, PK11195. The goal of the current study was to examine this new drug delivery strategy in an intracerebral xenograft model by measurement of steady-state drug distribution following administration of gemcitabine (GEM) and PK11195-GEM. *Methods*: In vitro PBR receptor binding and cytotoxicity assays were used to screen three different PK11195-GEM conjugates (GG01, GG02, GG03) in human SF126 glioma cells. Based on these findings and the favorable chemical stability of GG01, here referred as PK11195-GEM, pharmacokinetic investigations of PK11195-GEM and GEM were conducted in male rats. These studies consisted of single-dose and steady-state dosing regimen studies, the latter to assess drug distribution in normal brain and brain tumors. PK11195-GEM and GEM were measured in blood and tissue samples by HPLC. *Results*: All PBR-GEM conjugates demonstrated appreciable receptor binding affinity and cytotoxicity with mean IC_{50} values ranging from 248 to 376 n*M* and 5.6 to 29.1 n*M*, respectively. The cytotoxicity of GEM was comparable with a mean IC_{50} value of 5.9 n*M*. Following administration of single 8 mg/kg doses of PK11195-GEM to rats ($n=4$), PK11195-GEM had a mean total clearance of 126.3 ± 29.6 ml/min per kg, and a volume of distribution at steady-state of 1261.9 ± 31.05 ml/kg that resulted in a very short elimination half-life of 16.1 ± 5.8 min. In comparison GEM had a similar volume of distribution (993.8 ± 131.6 ml/kg), reduced clearance (3.4 ± 0.8 ml/min per kg), and longer half-life (235.6 ± 26.7 min). In nude rats bearing intracerebral tumors, mean steady-state tumor/plasma, tumor/right brain, and tumor/left brain PK11195-GEM concentration ratios were 1.75 ± 0.46, 5.49 ± 5.2, and 9.96 ± 3.2, respectively. The analogous values following GEM administrations were 0.81 ± 0.5, 3.67 ± 1.57, and 5.21 ± 1.95, respectively. These values indicate a minimum twofold increase in tumor target selectivity for the conjugate delivery system compared to GEM treatment. *Conclusion*: Targeting intracellular PBRs is a new drug delivery strategy based on the use of low molecular weight drug conjugates that can be administered systemically. It was demonstrated under steady-state conditions that PK11195-GEM possessed a twofold enhancement in brain tumor selectivity compared to GEM alone. This type of target selectivity would allow higher tumor concentrations to be achieved in conjunction with lower drug concentrations in normal or non-target tissues.

Keywords Peripheral benzodiazepine receptor (PBR) · Gemcitabine · PK11195 · SF188 glioma cells · Brain tumor · Receptor-mediated drug delivery

Introduction

Chemotherapy of malignant brain tumors remains a formidable therapeutic challenge that is plagued by the inability to selectively deliver drugs to tumor cells and the emergence of drug resistance. Recent chemotherapeutic approaches to brain tumors consist of both regional and systemic techniques, such as drug-loaded polymeric implants [9, 10], interstitial clysis [11], and coadministration of O^6-benzylguanine, a modulator of alkylguanine transferase [16, 34]. Further investigations will determine their ultimate place in the therapeutic arsenal.

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We have proposed a new strategy to target cytotoxic drugs to brain tumors through the use of peripheral benzodiazepine receptor (PBR) ligand-drug conjugates. PBRs have been shown to be selectively increased in both experimental and human brain tumors compared to normal brain and peripheral tissues [6, 7, 22, 28, 36], and thus, could serve as a drug delivery target. In fact, the expression of PBRs in brain tumor has been suggested to correlate to the degree of malignancy [7, 17, 18, 27]. PBRs are predominantly expressed on the outer surface of mitochondria [2, 3, 26], distinguishing it as an intracellular target, rather than the more common cell membrane targets of drug delivery. The identification of PBRs in brain tumors has also served as a means to evaluate PBR ligands as diagnostic imaging agents [7, 22, 36]. Thus, the characteristics of PBRs in tumors suggest that PBR ligands could serve as receptor-mediated drug carriers to selectively target anticancer drugs to brain tumors.

A variety of chemical classes contain ligands that specifically bind to PBRs [23, 38]. Within the isoquinoline class is the prototypical PBR ligand, PK11195 (see Fig. 1), that has appreciable binding affinity to both rat and human brain [4, 28] and brain tumor PBRs [7, 22]. PK11195 was used as the basis to design PBR ligand-drug conjugates for this investigation. We chose gemcitabine (GEM, see Fig. 1) as the first PBR ligand-drug conjugate to be evaluated in vivo although other cytotoxic agents, such as certain alkylating agents might be viable choices [24]. GEM is approved for use in pancreatic cancer, and has shown activity against a wide spectrum of human solid tumors [1, 12, 19, 29]. As shown here, GEM has appreciable cytotoxicity to human glioma cells, and because of its hydrophilic nature and likely inability to cross the blood-brain barrier it might be a suitable candidate for attempting to selectively enhance its brain tumor uptake via a PBR ligand.

The objectives of the current study were to determine the in vitro cytotoxicity and PBR binding affinity of PK11195-GEM, and to evaluate its tumor targeting potential in an intracerebral xenograft model.

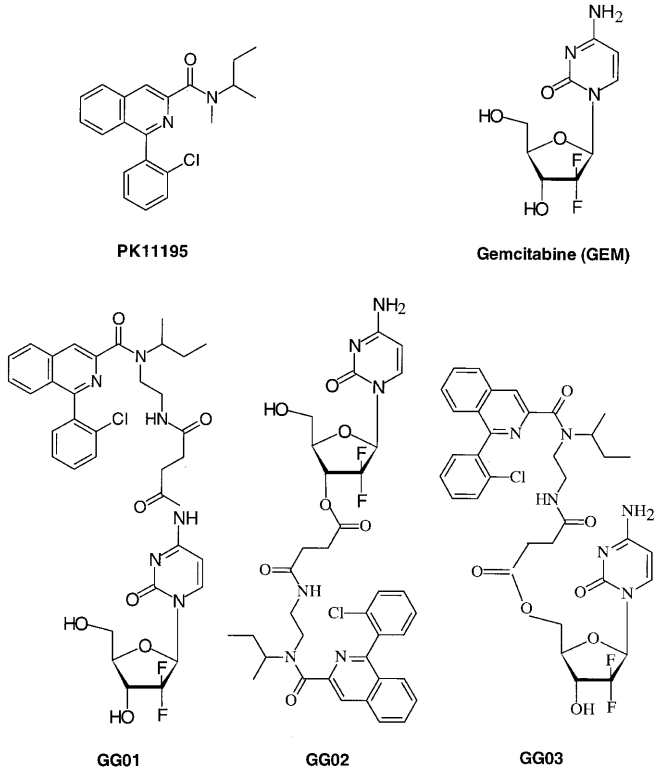

Fig. 1 Chemical structures of PK11195, gemcitabine (GEM) and peripheral benzodiazepine receptor (PBR) ligand-GEM conjugates (GG01, GG02, GG03)

Materials and methods

Drugs and chemicals

PK11195-GEM conjugates (GG01, GG02, GG03; see Fig. 1) were synthesized in our laboratory as previously described [21]. Gemcitabine hydrochloride was obtained from Eli Lily and Company (Indianapolis, Ind.). The PBR ligands, PK11195, and Ro 5-4864, were purchased from Research Biochemicals International (Natick, Mass.). ^{3}H-PK11195 (specific activity 86 Ci/mmol) was obtained from NEN Life Science Products (Boston, Mass.). All other chemicals were purchased from either Sigma Chemical Company (St. Louis, Mo.) or Fisher Chemicals (Fair Lawn, N.J.). Solvents of HPLC grade were purchased from Fisher Chemicals. Glass fiber filters were purchased from Schlecher & Schuell (Keene, N.H.).

In vitro studies

Cells and culture conditions

Human SF126 glioma cells were kindly provided by the Brain Tumor Research Center, University of California, San Francisco. The cells were cultured in DMEM supplemented with 10% FBS in a humidified atmosphere of 5% CO_2 in air at 37°C and grown as monolayers.

Receptor binding assay

Competitive binding displacement assays using [^{3}H]-PK11195, Ro5-4864, and the PK11195 gemcitabine conjugates (GG01, GG02, and GG03) were conducted with human glioma SF126 cells according to a previously described method [24]. Briefly, about 10^7 cells were collected by scraping and suspended in 25 ml 50 m*M* Tris-citrate (pH 7.4) and homogenized for 10 s (Artek Sonic Dismembrator, Farmingdale, N.Y.). The homogenate was centrifuged at 20,000 *g* for 20 min, and the resultant cell pellet was resuspended in 10 ml Tris-citrate buffer. The protein concentration was measured [8], and then adjusted to 8 μg/ml with a Tris-citrate buffer. A 125-μl aliquot of cell suspension was added to each assay tube containing 25 μl [^{3}H]-PK11195 to produce a final concentration of 1 n*M* (specific activity 86.0 Ci/mmol). Various concentrations (1 to 5000 n*M*) of PK11195-GEM prepared in 25 μl Tris-HCl buffer (50 m*M*) were added along with additional Tris-HCl buffer (50 m*M*) to bring the final volume to 250 μl. Nonspecific binding was determined by adding unlabeled Ro5-4864 at a final concentration of 10 μ*M*. The experiments were initiated by the addition of cells and terminated after 60 min incubation at 4°C by rapid filtration over glass fiber filters using a filtering manifold (Brandel Harvester, Biomedical Research & Development Laboratory, Gaithersburg, Md.). The filters were washed with three 4-ml aliquots of 50 m*M* Tris-HCl buffer (pH 7.4, 0–4°C). The radioactivity retained by the glass filters was measured using 5 ml of scintillation cocktail (Hionic-Fluor, Packard, Meriden, Ct.). Binding affinity (IC_{50}) was determined graphically from a plot of the percentage of

[^{3}H]-PK11195 bound versus concentrations of PBR-GEM conjugates compared to controls. All assays were performed in duplicate a minimum of three different times.

Cytotoxicity assay

Cytotoxicity of PBR-GEM conjugates and GEM against human SF126 glioma cells was evaluated by a standard SRB assay [35]. Briefly, cells were harvested by trypsinization and plated into 96-well plates (2000 cells/well) overnight followed by the addition of various concentrations of drugs. After 96 h drug exposure, experiments were terminated by removal of the medium and addition of 100 µl 10% trichloroacetic acid. Cells were fixed by trichloroacetic acid for 1 h at 4°C, then washed five or six times with water followed by an incubation for a minimum of 30 min at room temperature in 50 µl 0.4% SRB (sulforhodamine B, Sigma) in 1% acetic acid. Free SRB was then washed away with 1% acetic acid and completely dissolved in 150 µl 10 m*M* Tris-HCl buffer. Following incubation for at least 4 h at room temperature, plates were read at 590 nm with a microplate reader (Bio-Rad, Hercules, Calif.). Cell survival was calculated from the absorbance at each drug concentration relative to the control.

In vivo studies

Animals

Adult male Sprague-Dawley rats were used for initial pharmacokinetic studies, whereas male nude rats (NIH rnu/rnu; Taconic Farms, Gemantown, N.Y.) bearing intracerebral tumors were used for the drug distribution studies.

Pharmacokinetic studies in normal rats

Normal Sprague-Dawley rats (170–250 g) were used to determine the pharmacokinetic properties of GEM and PK11195-GEM so that steady-state dosing regimens could be designed for tumor-bearing nude rats.

Rats were anesthetized with a 3:2:1 (v/v/v) mixture of ketamine hydrochloride (100 mg/ml), acepromazine maleate (10 mg/ml) and xylazine hydrochloride (20 mg/ml) at a dose of 1 ml/kg. The right common carotid artery and jugular vein were exposed and two PE50 cannulas were implanted with the tips directed towards the heart. Arterial cannulas were used for drug administrations and jugular vein cannulas were used for blood sampling. The cannulas were tied into place, and exteriorized at the back of the neck. Animals were allowed to recover for at least 12 h before entering the pharmacokinetic studies.

Four rats were each given 8 mg/kg PK11195-GEM (GG01) dissolved in DMSO/methanol/water (18:18:80, v/v/v) intraarterially (i.a.) over 1 min. Serial blood samples were collected at 2, 5, 10, 15, 30, 45, 60, 90, 120, and 150 min after drug administration. Another three rats were each given 3.2 mg/kg GEM (dissolved in 0.9% saline) i.a. over 1 min with blood samples collected at 5, 15, 30, 60, 90, 120, 180, 240, 360, 480, and 720 min after drug administration. For all studies, plasma was separated from blood and stored at –80°C until analyzed by HPLC as described below.

The resultant plasma drug concentration-time data were analyzed by noncompartmental methods to yield estimates of total drug clearance (CL) and the volume of distribution at steady-state (V_{ss}). These parameters were then used to calculate steady-state regimens for both GEM and PK11195-GEM in the brain tumor-bearing animals. These regimens consisted of an i.a. loading dose and constant rate infusion.

Drug distribution studies in brain tumor-bearing rats

Vascular endothelial growth factor (VEGF) overexpressing human glioma cells (SF188/VEGF$^+$) were previously established in our laboratory [25], and grown to about 50% confluency in DMEM with 10% fetal bovine serum at 37°C in an atmosphere containing 5% CO_2. The medium was removed and the cells were trypsinized with 0.04% trypsin/EDTA, centrifuged at 1000 rpm for 10 min and resuspended in DMEM at a concentration of 1×10^8 cells/ml for implantation.

Male nude rats (200–330 g) were anesthetized as described above, and placed in a rodent stereotaxic device (Stoelting Physiology Research Instruments, Wood Dale, Ill.) equipped with a microinjector (David Kopf Instruments, Tujunga, Calif.). The scalp was cleaned with 70% alcohol, and the bregma carefully exposed. A small burr hole was drilled in the right hemisphere 2 mm posterior and 2 mm lateral to the bregma. A microsyringe containing SF188/VEGF$^+$ cells (0.7×10^6 cells) was lowered to a depth of 4.5 mm and 7 µl of the cell suspension slowly injected over 1 min. The needle was slowly removed after 1 min, and then the hole was sealed with bone wax. The incision was sutured and the animals returned to their cage where they were provided with food and water ad libitum.

Approximately 3∼4 weeks after implantation, when the rats became symptomatic (lethargy, arched back, ruffled fur and unsteady gait), they were anesthetized as described above. An incision was made in the neck region right of the midline and the external carotid artery, occipital and the pterygopalatine arteries were isolated and ligated. A small cut was made in the external carotid artery caudal to the initial ligation and a PE10 cannula with sodium heparin (50 IU/ml; Elkins-Sinn, Cherry Hill, N.J.) was inserted directed towards the heart with the cannula tip placed at the bifurcation of the internal and external carotid arteries. The PE10 cannula was used for drug administration. A PE50 cannula was implanted into the jugular vein for blood sampling. The cannulas were tied into place, exteriorized at the back of the neck and the incision sutured.

Nude rats ($n=5$) bearing intracerebral tumors were given a loading dose of 0.43 mg/kg of PK11195-GEM (GG01) and a constant rate infusion of 0.08 mg/kg per min for 30 min. Another group of nude rats ($n=5$) bearing brain tumors were given a loading dose of 1.8 mg/kg of GEM and a constant rate infusion of 0.0055 mg/kg per min for 30 min. Blood was collected at 10, 20 and 30 min during the infusions following which the animals were killed by decapitation. Pieces of normal brain tissue and the brain tumor were collected, immediately frozen with an ethanol-dry ice mixture, and then stored at –80°C until analysis.

Drug concentration analyses

High-performance liquid chromatography (HPLC) was used to quantify PK11195-GEM and GEM in plasma and brain tissue.

PK11195-GEM

Brain tissue homogenates were prepared in water at a ratio 1:4 (g:ml) using a mechanical homogenizer (Tekmar Company, Cincinnati, Ohio). Homogenate (200 µl) was mixed with 900 µl methanol by vortex for 1 min. An internal standard (GG03) was added (100 µl, 3 µg/ml) and the mixture was centrifuged at 15,000 rpm for 5 min. The resultant supernatant was diluted with 2 ml water and then applied to a Bond Elut C2 cartridge (Varian Sample Preparation Products, 1 ml/100 mg) preconditioned with 1 ml each of methanol and water. The cartridge was washed with 2 ml water and then 200 µl of an acetonitrile/ammonium acetate (3:1) solution was used to elute the solutes. Aliquots (50 µl) of the eluents were injected onto the HPLC system.

A similar preparation method was used for measurement of PK11195-GEM in plasma. Plasma (200 µl) was mixed with 600 µl methanol by vortexing for 1 min. An internal standard (GG03, 100 µl, 3 µg/ml) was added followed by centrifugation at 15,000 rpm for 5 min. The supernatant was diluted with 1.8 ml water and then applied to the C2 cartridge. All others procedures were as described for brain tissue.

PK11195-GEM was separated on an ODS analytical column (Hypersil,100×4.6 mm, 5 µm particle size, Hewlett-Packard) using

a mobile phase of 2 m*M* ammonium acetate/acetonitrile (60/40, v/v) pumped at a flow rate of 1 ml/min. Solutes were detected at a wavelength of 236 nm with retention times of 5.8 min (PK11195-GEM) and 6.5 min (internal standard, GG03). Complete run times were 23 min because of the retention of endogenous substances in both plasma and brain tissue. The limits of quantitation were 55 and 72 ng/ml for plasma and brain tissue, respectively.

For the measurement of GEM following administration of PK11195-GEM a solid phase extraction method was developed to avoid ex vivo conversion to GEM caused by the acid protein precipitation method used for GEM administrations (see below). A 15-µl aliquot of internal standard solution (5-iodouracil, 0.08 mg/ml in methanol) was added to either 150 µl brain homogenate or plasma. Brain homogenate was vortexed for 30 s and then centrifuged at 15,000 rpm for 5 min. The resultant supernatant or plasma was applied to a Bond-Elut C18 cartridge (Varian Sample Preparation Products, 1 ml/100 mg) preconditioned with 1 ml each of methanol and water. The cartridge was dried under vacuum. The cartridge was then eluted with 150 µl 25% acetonitrile in water. Perchloric acid (10 µl) was added to the eluent. Following centrifugation at 15,000 rpm for 5 min, an aliquot (50 µl) of the supernatant was injected onto the HPLC system described for GEM (see below). The limit of quantitation was 220 ng/ml for plasma and brain tissue.

Gemcitabine

A protein precipitation method was used to extract GEM from both plasma and tissue homogenates. To 150 µl of either plasma or tissue homogenate (1:4, g:ml), was added 10 µl perchloric acid followed by vortexing for 30 s. Following centrifugation at 15,000 rpm for 5 min, 50-µl aliquots were injected onto the HPLC system.

GEM was separated on an ODS analytical column (Adsorbosphere HS 250×4.6 mm, 5 µm particle size, Alltech) maintained at 40°C. The mobile phase consisted of solution A (50:50 methanol/water with 50 m*M* KH_2PO_4, 40 m*M* hexanesulfonic acid, pH 2.5) and solution B (water with 50 m*M* KH_2PO_4, 40 m*M* hexanesulfonic acid, pH 2.5) operated at a linear gradient of 0% to 60% solution A for the first 15 min and then switched to 100% solution B from 15 min to 22 min. At a flow rate of 1.5 ml/min, GEM was detected at 272 nm with a retention time of 11.9 min. The limit of quantitation was 220 ng/ml for plasma and brain tissue.

All assays were validated based on intra- and interday replicates of calibration curves and quality control samples. In all cases, calibration curves were linear with correlation coefficients greater than 0.99, and coefficients of variation less than 15% on all quality control samples.

Drug distribution

Steady-state (30 min) tissue and plasma PK11195-GEM and GEM concentrations were used to evaluate drug distribution. Drug concentration ratios, calculated as the ratio of the brain tumor concentration to the non-target concentration, indicated drug target selectivity. These values were determined for both PK11195-GEM and GEM in which non-target concentrations represented either plasma, normal right brain hemisphere or normal left brain hemisphere concentrations. All drug concentrations are expressed as nanomoles per milliliter assuming a tissue density of 1. Drug concentration and concentration ratios are summarized as the means ± SD. The Wilcoxon nonparametric test was used to compare drug concentration ratios for the drug treatments with a *P*-values < 0.05 considered statistically significant.

Results

In vitro evaluation of PBR-GEM conjugates

The cytotoxicity of the three PBR-GEM conjugates and GEM against human SF126 glioma cells is shown in Table 1. All three GEM conjugates and GEM showed appreciable cytotoxicity against these cells with IC_{50} values in the low nanomolar concentration range.

The receptor binding affinities of the PBR-GEM conjugates, expressed as the IC_{50} values for displacement of PK11195, are also shown in Table 1. All the conjugates had significant binding affinity with IC_{50} values ranging from 248 to 377 n*M*. As expected, GEM had no binding affinity for PBRs.

Pharmacokinetics in normal rats

The plasma concentrations of PK11195-GEM and GEM following administration to normal rats are shown in Fig. 2. Concentrations of PK11195-GEM were below the quantitation limit after 60 min for two of the four rats and only one animal's plasma concentration remained detectable until 120 min. In contrast, concentrations of GEM were quantitated in all rats over the entire sampling period (720 min). A comparative summary of the pharmacokinetic parameters of each drug is shown in Table 2.

PK11195-GEM was rapidly cleared with a mean systemic clearance of 126.3 ± 29.6 ml/min per kg. PK11195-GEM had a mean volume of distribution at steady-state of 1261.9 ± 31.1 ml/kg that was comparable to that of GEM (993.8 ± 131.6 ml/kg). Given the much greater clearance of PK11195-GEM compared to GEM (3.38 ± 0.8 ml/min per kg) and the similar volumes of distribution, PK11195-GEM had a much shorter elimination half-life (16.1 ± 5.8 min) compared to GEM (235.6 ± 26.7 min).

Tissue distribution in rats bearing brain tumors

Steady-state plasma, brain and brain tumor concentrations of PK11195-GEM and GEM are shown in Fig. 3. These values along with the tissue concentration ratios of PK11195-GEM and GEM are given in Table 3. Absolute brain tumor concentrations of PK11195-GEM were slightly less than those attained for GEM (4.54 ± 0.39 nmol/ml vs 5.84 ± 3.89 nmol/ml, respectively), yet the interanimal variability was much greater for GEM than for PK11195-GEM (%CV 66.6% vs 8.6%). Since both the PK11195-GEM and GEM dosing

Table 1 Cytotoxicity and receptor binding affinity of PBR-GEM conjugates and GEM in human SF126 glioma cells. Values are means ± SD derived from at least three individual experiments, each carried out in duplicate (*ND* not detected)

Compounds	Cytotoxicity (IC_{50} n*M*)	Binding affinity (IC_{50} n*M*)
PK11195-GEM (GG01)	5.6 ± 1.1	376.6 ± 138.5
GG02	29.1 ± 11.4	343.3 ± 55.9
GG03	12.3 ± 3.3	248.2 ± 103.9
GEM	5.9 ± 1.0	ND

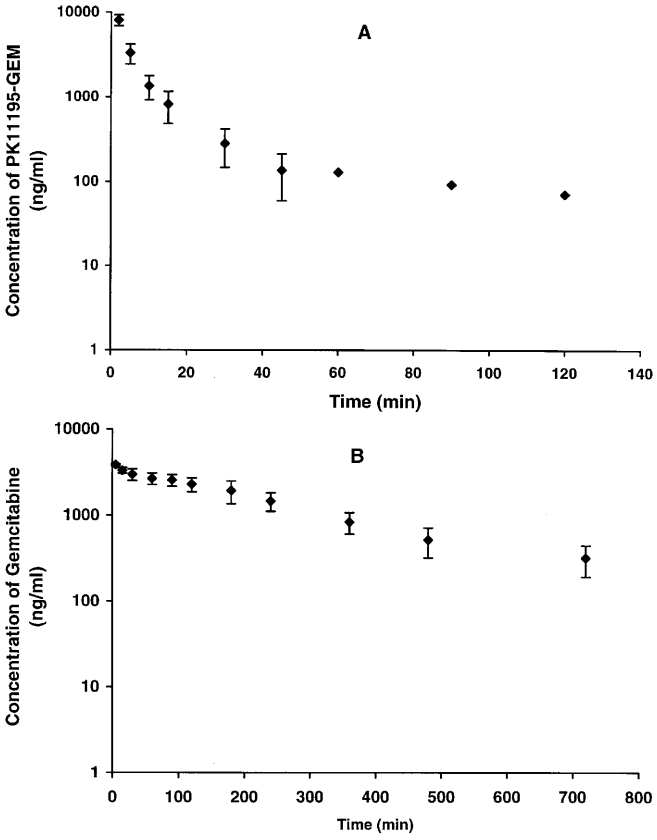

Fig. 2A, B Drug plasma concentration-time profiles of PK11195-GEM (**A**; values are the means ± SD of four, except $n = 2$ at 60 min, and $n = 1$ at 90 and 120 min) and GEM (**B**; values are the means ± SD of three) after administration of 8 mg/kg PK11195-GEM (GG01) and 3.2 mg/kg GEM, respectively

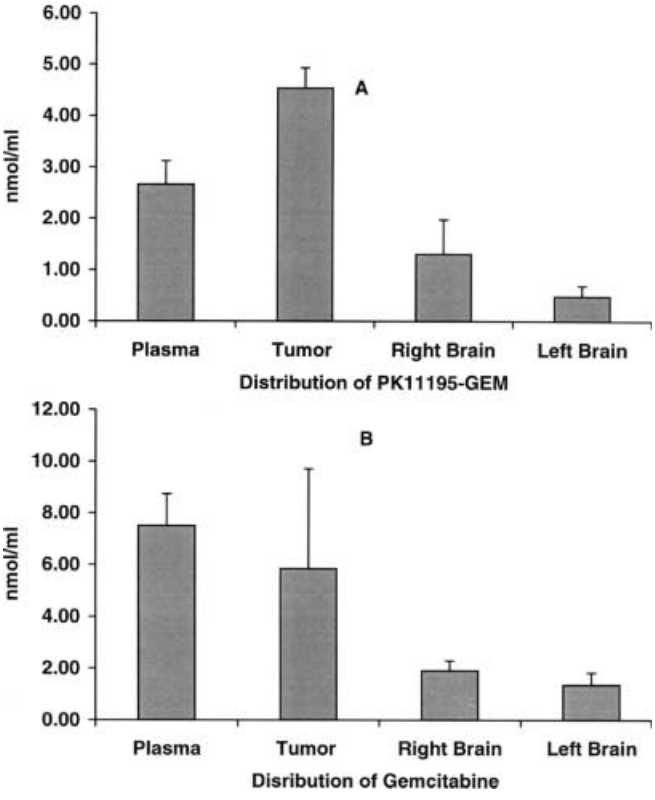

Fig. 3A, B Steady-state concentrations of PK11195-GEM (**A**) and GEM (**B**) in plasma and brain tissues at 30 min in nude rats bearing intracerebral tumors. The steady-state dosing regimen of PK11195-GEM consisted of a loading dose of 0.43 mg/kg and a 0.08 mg/kg per min constant rate infusion. The steady-state dosing regimen of GEM consisted of a 1.8 mg/kg loading dose and a 0.0055 mg/kg per min constant rate infusion

Table 2 Pharmacokinetic parameters of PK11195-GEM and GEM in normal rats after a single i.a. bolus doses of either 8 mg/kg PK11195-GEM or 3.2 mg/kg GEM. Values are means ± SD; $n = 4$ for PK11195-GEM, $n = 3$ for GEM (*CL* total systemic clearance, *Vss* volume of distribution at steady state, $T_{1/2(terminal)}$ terminal elimination half-life)

Parameter	PK11195-GEM	GEM
CL (ml/min per kg)	126.3 ± 29.6	3.38 ± 0.8
Vss (ml/kg)	1261.9 ± 31.05	993.8 ± 131.6
$T_{1/2(terminal)}$ (min)	16.1 ± 5.79	235.6 ± 26.7

Table 3 Steady-state concentrations (nmol/ml) and concentration ratios of PK11195-GEM and GEM in plasma and tissue following administration to nude rats bearing human SF188/VEGF$^+$ intracerebral tumors (concentration ratios: *T*/*P* tumor to plasma, *T*/*RB* tumor to right brain, *T*/*LB* tumor to left brain)

	PK11195-GEM	GEM
Plasma	2.66 ± 0.46 ($n = 5$)	7.51 ± 1.23 ($n = 5$)
Tumor	4.54 ± 0.39 ($n = 5$)	5.84 ± 3.89 ($n = 5$)
Right brain	1.30 ± 0.67 ($n = 4$)	1.91 ± 0.38 ($n = 4$)
Left brain	0.49 ± 0.20 ($n = 3$)	1.36 ± 0.46 ($n = 3$)
T/P	1.75 ± 0.40 ($n = 5$)*	0.81 ± 0.50 ($n = 5$)
T/RB	5.49 ± 5.17 ($n = 4$)	3.67 ± 1.58 ($n = 4$)
T/LB	9.96 ± 3.25 ($n = 3$)*	5.21 ± 1.95 ($n = 3$)

*$P < 0.05$ vs GEM

regimens were designed to produce analogous 8 μ*M* plasma concentrations, it was unexpected that PK11195-GEM concentrations were appreciably less (mean 2.66 μ*M*). However, values were constant throughout the 30-min infusion period (see Fig. 4). Steady-state GEM plasma concentration did approximate the 8 μ*M* target (see Fig. 4). Normal right (1.30 ± 0.67 nmol/ml) and left brain (0.49 ± 0.20 nmol/ml) PK11195-GEM concentrations were less than the corresponding values obtained for GEM, being 1.91 ± 0.38 nmol/ml and 1.36 ± 0.46 nmol/ml, respectively.

PK11195-GEM concentrations in brain tumor were a mean of 1.75-fold higher than the concentrations in plasma whereas GEM brain tumor concentrations were lower than those in plasma with a mean tumor to plasma ratio (T/P) of 0.81. This difference in T/P ratio was significant ($P < 0.02$). PK11195-GEM brain tumor concentrations were more that 5-fold greater than the ipsilateral right brain concentration (mean T/RB ratio 5.49 ± 5.2), and 10-fold greater than those in the contralateral left brain (mean T/LB ratio 9.96 ± 3.2). The analogous T/RB and T/LB ratios for GEM were lower,

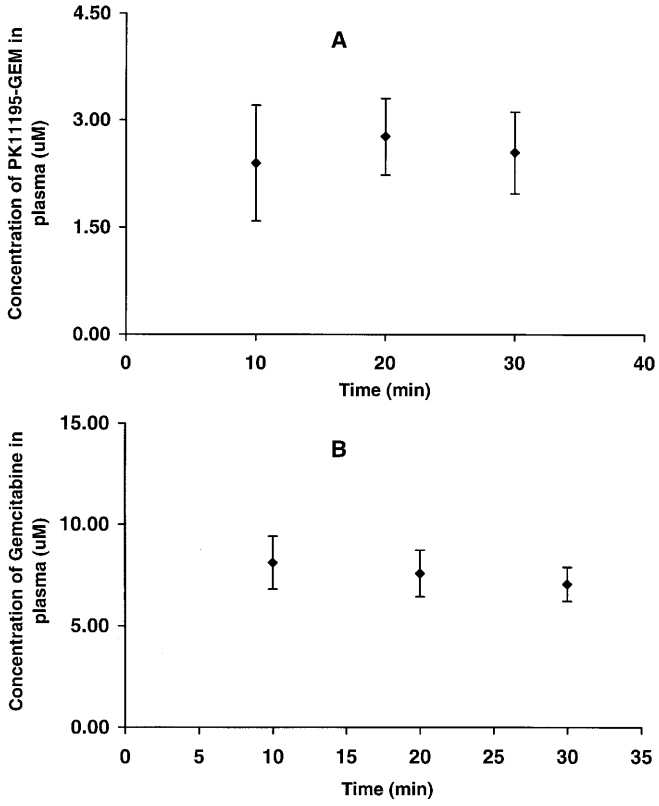

Fig. 4A, B Steady-state plasma concentrations of PK11195-GEM (**A**) and GEM (**B**) in nude rats bearing intracerebral tumors. The steady-state dosing regimen of PK11195-GEM consisted of a loading dose of 0.43 mg/kg and a 0.08 mg/kg per min constant rate infusion. The steady-state dosing regimen of GEM consisted of a 1.8 mg/kg loading dose and a 0.0055 mg/kg per min constant rate infusion

being 3.67 ± 1.57 and 5.21 ± 1.95, with only the T/LB ratios significantly different ($P < 0.05$).

GEM was not detectable in brain following administration of PK11195-GEM. Three of five animals had measurable concentrations of GEM in plasma at 30 min with a mean of 0.46 ± 0.01 nmol/ml.

Discussion

Possibly the biggest hurdle to successful cancer chemotherapy is the ability to selectively target tumors as opposed to normal tissues. This formidable challenge is particularly pertinent to the treatment of brain tumors that reside within normal brain parenchyma. Greater drug selectivity for the tumor permits greater dose escalation and may retard the development of drug resistance. It is known that the normal blood-brain barrier (BBB) in brain tumors or blood-tumor barrier (BTB) may be compromised, creating a more permissive environment to the uptake of drugs and particulate drug delivery systems [20, 30, 31, 37]. Although the BTB is generally more permissive to drug uptake than the intact BBB, there are significant regional heterogeneities of blood flow and membrane permeability in brain tumors that contribute to regional differences in drug concentrations, and the elusive goal of tumor selectivity.

Drug delivery techniques for brain tumors have utilized a vast array of regional and systemic systems [33]. Regional drug delivery approaches bypass whatever barrier the BBB provides, yet may be unable to achieve lethal drug concentrations throughout the tumor. Systemic drug delivery systems for brain tumors offer ease of administration, and the potential to reach remote tumor cells, but these systems have remained largely in the preclinical domain. There have been clinical trials using osmotic BBB disruption in conjunction with systemic chemotherapy [14], yet this has not been adopted as a standard therapy for malignant gliomas. Given the dismal life expectancy of malignant brain tumor patients, and the limited successes of targeted drug delivery, new drug delivery strategies for brain tumors are needed.

PBRs have been found to be overexpressed in both preclinical models of brain tumors as well as in patients with brain tumors [6, 7, 22, 36]. The past focus was to use PBR ligands, such as PK11195, as diagnostic imaging tools for brain tumors. Overexpression of PBRs has also been found in other tumor types, and their expression correlates with the degree of malignancy [7, 17, 18, 27]. PBRs reside on the outer membrane of mitochondria, although their role in tumor cell behavior is open to speculation [5]. We considered that PBRs could serve as a unique intracellular drug delivery target, and embarked on a chemical synthesis program to design PBR-drug conjugates [21, 24]. The current study represents the first investigation of the in vivo drug disposition characteristics of a PBR-drug conjugate.

The in vitro cytotoxicity and binding affinity studies indicated that all three PBR-GEM conjugates possessed suitable pharmacological properties to be further evaluated. In consideration of conducting pharmacokinetic studies, it was found that both conjugates (i.e. GG02 and GG03, see Fig. 1) using ester linkages between the PK11195 moiety and the sugar portion of GEM underwent rapid hydrolysis, with in vitro half-lives in plasma of less than 5 min. This property would likely limit their ability to target PBRs intact. PK11195-GEM has an amide bond between the PK11195 component and the cytidine base portion of GEM that conferred relatively greater in vitro stability in plasma with a half-life of more than 30 min.

The pharmacokinetic characteristics of PK1195-GEM indicate it possesses a large volume of distribution consistent with high protein binding in tissues and its lipophilic nature. Total clearance of PK11195-GEM was also very high, and greater than standard estimates of either rat kidney or liver blood flow [13] suggesting that elimination of PK11195-GEM could involve both renal and hepatic mechanisms. Given the relatively rapid hydrolysis of PK11195-GEM in plasma determined in vitro, it is likely that direct plasma hydrolytic clearance

contributes to the overall clearance of PK11195-GEM. The relatively low GEM plasma concentrations following PK11195-GEM administration are most consistent with rapid distribution into tissues (the volume of distribution of GEM was high, 952 ml/kg) given the lower systemic clearance of GEM. Rapid tissue uptake of GEM would seemingly result in measurable brain concentrations of GEM, yet this was not the case following PK11195-GEM administration. Further studies incorporating a broad dose-range are required to fully characterize the clearance and metabolic mechanisms of PK11195-GEM.

The pharmacokinetic parameters of GEM are within the range of values previously reported in rats [15, 32]. Compared to PK11195-GEM, GEM has a reduced clearance consistent with its longer half-life (16 min vs 260 min, respectively).

In the xenograft model, steady-state dosing regimens were employed because they facilitated a parsimonious use of both animals and the limited supply of PK11195-GEM. Single drug doses (i.e. non-steady-state) would require a serial killing study design, and approximately 40 animals (4 animals at ten time points) to obtain meaningful tissue drug concentrations. Such a design would provide an estimate of the area-under-the drug concentration-time curve (i.e. AUC) which is an analogous measure of drug exposure as the steady-state concentration.

It was found that steady-state plasma PK11195-GEM concentrations were about threefold lower than the observed steady-state plasma concentrations of GEM and the intended target concentrations of 8 μM. Other than attributing this to a difference in PK11195-GEM disposition in animal models (normal Sprague-Dawley vs nude intracerebral tumor-bearing rats) the explanation is unknown. Nonetheless, steady-state was still achieved for PK11195-GEM as 10 min, 20 min and 30 min plasma concentration were analogous (see Fig. 4). The consequence of the difference in PK11195-GEM and GEM steady-state plasma concentrations requires an extrapolation to compare tissue concentrations that may have been achieved at equal plasma concentrations. Under the assumption of linear drug distribution, tissue concentrations would change in proportion to the plasma concentrations. Simple multiplication of the mean PK11195-GEM plasma and tissue values by 3, the factor that normalizes the plasma concentrations to those of GEM, results in brain tumor, right brain and left brain PK11195-GEM concentrations of 13.5, 3.9, and 1.5 nmol/ml, respectively. The analogous observed GEM concentrations were 5.84, 1.91, and 1.36 nmol/ml (see Table 3). Of course, of equal importance is what fraction of PK11195-GEM in the tumor is converted to GEM. At 100% conversion, equimolar GEM would be produced from PK1195-GEM, and thus, it can be predicted that at equivalent PK11195-GEM and GEM plasma concentrations, brain tumor concentrations of GEM would be two- to threefold greater following PK11195-GEM administration than following GEM administration. Further studies will be needed to identify the metabolic pathways of PK11195-GEM.

It is most instructive to assess tumor or target selectivity, as opposed to absolute drug concentrations in tumors, from the drug concentration ratios provided in Table 3. Based on the concentration ratios (i.e. T/P, T/RB, T/LB) obtained for PK11195-GEM and GEM, PK1195-GEM produced a significant twofold increase, indicative of superior target selectivity. The highest ratios for both PK11195-GEM and GEM were the T/LB values, consistent with first-pass drug extraction in the right brain hemisphere, the side of drug administrations. Both the T/RB and T/LB values reflect the more permeable BTB that presumably does not discriminate between GEM and PK11195-GEM. The small molecular weight difference between GEM and PK11195-GEM (299.66 and 727.2, respectively), and the fact that colloid-based drug delivery systems can traverse the BTB supports a non-discriminating anatomically compromised BTB.

Although not proved definitively, it can be inferred that PK11195-GEM did achieve greater tumor selectivity due to receptor-mediated drug delivery as opposed to non-specific enhancements in tissue uptake due to its greater lipophilicity. Without a receptor-mediated mechanism, the brain concentration ratios (see Table 3) for PK11195-GEM would be equal to or lower than those observed for GEM, since increased lipophilicity would more likely enhance normal BBB penetration, thus increasing right and left brain concentrations. As illustrated above, multiplication of PK11195-GEM brain concentrations threefold to compare brain distribution at equivalent steady-state plasma concentrations resulted in greater brain tumor and normal right brain, and analogous left brain concentrations compared to those observed for GEM. This would suggest that the brain uptake of PK11195-GEM is not simply a function of its lipophilicity. The lipophilic nature of the PK11195-GEM conjugate would facilitate its transport across the BBB enabling it to reach the intracellular PBR target. The lipophilicity of the PBR ligands, like PK11195, offers new opportunities to design conjugates with hydrophilic drugs such as GEM that would not appreciably cross the BBB. Thus, it is proposed that PBR-drug conjugates can accumulate in brain tumors through their ability to cross the BBB and bind to PBRs.

In conclusion, targeting intracellular PBRs is a new drug delivery strategy based on the use of low molecular weight drug conjugates that are able to bind intracellular PBRs. It was demonstrated under steady-state conditions that PK11195-GEM possessed a minimum twofold enhancement in tumor selectivity compared to GEM alone. This type of target selectivity would allow higher tumor concentrations in conjunction with lower drug concentrations in normal or non-target tissues. Based on this first proof-of-principle study, further investigations are warranted and would include drug metabolism and tumor efficacy studies, and implementation of a drug

design program to identify PBR-drug conjugates with improved pharmacological properties.

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