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In Vitro and in Vivo Antitumor Effects of Cytotoxic Camptothecin-Bombesin Conjugates Are Mediated by Specific Interaction with Cellular Bombesin Receptors

Terry W. Moody, Li-Chun Sun, Samuel A. Mantey, Tapas Pradhan, L. Vienna Mackey, Nieves Gonzales, Joseph A. Fuselier, David H. Coy, and Robert T. Jensen

Department of Health and Human Services, Center for Cancer Research, National Cancer Institute (T.W.M.) and Digestive Diseases Branch, National Institute of Diabetes and Digestive and Kidney Diseases (S.A.M., T.P., N.G., R.T.J.), National Institutes of Health, Bethesda, Maryland; and Department of Medicine, Peptide Research Laboratories, Tulane Health Sciences Center, New Orleans, Louisiana (L.-C.S., L.V.M., J.A.F., D.H.C.)

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ABSTRACT

Most human tumors overexpress or ectopically express peptide hormone/neurotransmitter receptors, which are being increasingly studied as a means to selectively deliver cytotoxic agents. Although a number of peptide ligand-constructs demonstrate tumor cytotoxicity, the role of specific tumoral receptor interaction in its mediation is unclear. To address this question, we synthesized camptothecin (CPT) bombesin (Bn) analogs, in which CPT was coupled via a novel carbamate linker, L2 [*N*-(*N*-methyl-amino-ethyl)-glycine carbamate], that were chemically similar but differed markedly in their potency/affinity for human Bn receptors. We then examined their ability to interact with Bn receptors and cause in vitro and in vivo tumor cytotoxicity. CPT-L2-[D-Tyr^6,β-Ala^{11},D-Phe^{13},Nle^{14}] Bn (6–14) (BA3) bound with high affinity and had high potency for all three human Bn receptor subtypes, whereas CPT-L2-[D-Tyr^6,β-Ala^{11}, D-Phe^{13},Nle^{14}] Bn (6–14) [D-Phe-CPT-L2-BA3] had >1400-fold lower affinity/potency. ^{125}I-CPT-L2-BA3 but not ^{125}I-D-Phe-CPT-L2-BA3 was internalized by Bn receptor subtype-containing cells. CPT-L2-BA3 displayed significantly more cytotoxicity than D-Phe-CPT-L2-BA3 toward NCI-H1299 lung cancer cells in both 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide and clonogenic assays and more potently inhibited H1299 xenograft growth in nude mice. CPT-L2-BA3 was also metabolically more stable than its parent peptide and inhibited growth of a number of other tumor cell lines in vitro and in vivo. These results demonstrate that specific tumoral receptor interaction is important in mediating the ability of peptide ligand-cytotoxic constructs to cause cytotoxicity. Because many tumors overexpress Bn receptors, these results also demonstrate that CPT-L2-BA3 will be a useful agent for delivering receptor-mediated cytotoxicity to many different human tumors.

Recently, there has been increased interest in the development of cytotoxic antitumor agents that can be specifically targeted to tumors using cellular receptors highly expressed on the tumor or antigenic sites (Dubowchik and Walker, 1999; de Groot et al., 2001; Sun et al., 2002). Attempts at specific delivery have been sought by using antibodies directed against specific tumor antigens or by coupling cytotoxic agents to ligands that interact with receptors that are ectopically expressed or overexpressed by tumors (Dubowchik and Walker, 1999; Sun et al., 2000a). Peptide receptors have received considerable attention because numerous studies demonstrated that most benign as well as malignant human tumors ectopically express or overexpress one or more of these receptors (Virgolini, 1997; Jensen, 2000; Kwekkeboom et al., 2000; Hoffman et al., 2001; Reubi et al., 2002; Reubi, 2003; Schally and Nagy; 2003). Furthermore, ligands that function as agonists at these receptors are rapidly internalized, thus providing a mechanism for site-di-

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ABBREVIATIONS: Bn, bombesin; GRPR, gastrin-releasing peptide receptor; NMBR, neuromedin B receptor; BRS3, bombesin receptor subtype 3; L2, *N*-(*N*-methyl-amino-ethyl)-glycine carbamate; CPT, camptothecin; BA3, [D-Tyr^6,β-Ala^{11},Phe^{13},Nle^{14}] Bn (6–14); DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; FBS, fetal bovine serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide; hBRS3, human BRS3; hNMBR, human NMBR; GRP, gastrin-releasing peptide; hGRPR, human GRP receptor; IP, inositol phosphate; SIT medium, RPMI 1640 containing 3×10^{-8} M sodium selenite, 5 μg/ml bovine insulin and 10 μg/ml transferrin; RIA, radioimmunoassay; s.c., subcutaneous; i.p., intraperitoneal.

rected intracellular delivery of cytotoxic agents coupled to the agonist (Moody et al., 2004). In recent studies, receptor-mediated tumor localization or cytotoxicity has been reported with analogs of bombesin (Bn) (Carroll et al., 1999; Hoffman et al., 2001; Langer and Beck-Sickinger, 2001; Schally and Nagy; 2003; Moody et al., 2004), melanotropin (Langer and Beck-Sickinger, 2001), leutinizing hormone-releasing hormone (Langer and Beck-Sickinger, 2001; Schally and Nagy; 2003), vasoactive intestinal peptide (Virgolini, 1997), neuropeptide Y (Langer and Beck-Sickinger, 2001) ,and somatostatin (Kwekkeboom et al., 2000; Langer and Beck-Sickinger, 2001; Sun et al., 2002, 2004; Schally and Nagy; 2003; Moody et al., 2005).

The Bn family of receptors [gastrin-releasing peptide receptor (GRPR), neuromedin B receptor (NMBR), and the orphan receptor bombesin receptor subtype 3 (BRS3)] are particularly attractive for the development of possible tumor-directed cytotoxic agents because many common neoplasms including breast, prostate, gastric, colon, ovarian, small cell, and nonsmall cell lung cancers ectopically express or overexpress these receptors (Reubi et al., 2002). Although this approach shows promise in a number of studies to more specifically target cytotoxic agents to the tumor with lower toxicity (Kiaris et al., 1999; Schally and Nagy, 2003), the hypothesis that this specificity is due to tumor-specific targeting of the cytotoxic agent due to specific interaction with the tumor receptors has been difficult to prove, particularly with in vivo experiments.

In the present study, we have attempted to address this question pharmacologically by synthesizing active and inactive analogs of Bn that were physicochemically similar and were coupled to camptothecin by a novel carbamate linker with a built-in nucleophile-associated releasing group, L2 (Fuselier et al., 2003; Moody et al., 2004, 2005). Our results show that the active camptothecin (CPT)-Bn construct (CPT-L2-BA3), which is a potent, fully active agonist at each of the three human Bn receptor subtypes, binds with high affinity and is rapidly internalized by tumors or cells possessing each Bn receptor subtype, whereas the inactive analog (D-Phe-CPT-L2-BA3) is not. Furthermore, in vitro and in vivo cytotoxicity studies demonstrate the active CPT-Bn construct causes significantly greater inhibition of growth and inhibits the growth in vivo of a large range of human tumors. The active analog CPT-L2-BA3 had enhanced metabolic stability and caused cytotoxicity in a number of human tumors assessed by both in vitro and in vivo growth assays. These results provide direct evidence that interaction with tumor receptors is an important mechanism in mediating this enhanced cytotoxicity and show that this approach can deliver receptor-directed cytotoxicity to a number of tumors.

Materials and Methods

The following cells and materials were obtained from the sources indicated: Balb 3T3 (mouse fibroblast) and NCI-H1299 (human nonsmall cell lung cancer) cells were from American Type Culture Collection (Rockville, MD); Dulbecco's modified Eagle's medium (DMEM), phosphate-buffered saline (PBS), RPMI 1640, trypsin-EDTA, and fetal bovine serum (FBS) were from Biofluids (Rockville, MD); G418 sulfate was from Life Technologies, Inc. (Grand Island, NY); $Na^{125}I$ (2200 Ci/mmol) and *myo*-[2-^{3}H]inositol (20 Ci/mmol) were from GE Healthcare (Little Chalfont, Buckinghamshire, UK); camptothecin, formic acid, ammonium formate, disodium tetraborate, 4-(dicyanomethylene)-2-methyl-6-(4-dimethylaminostyryl)-4-*H*-pyran, 2-dimethylaminoisopropyl chloride hydrochloride, 1-methyl-2-pyrrolidinone, dimethylaminopyridine, soybean trypsin inhibitor, bacitracin, leupeptin, 4-(2-aminoethyl)-benzenesulfonyl fluoride, poly-L-lysine, and Triton X-100 were from Sigma-Aldrich (St. Louis, MO); 1,2,4,6-tetrachloro-3α,6α-diphenylglycouril was from Pierce Chemical Co. (Rockford, IL); AG 1-X8 resin from Bio-Rad (Hercules, CA); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) and iodo-nitrotetrazolium violet were from Sigma-Aldrich; and agarose was purchased from FMC Corp. (Rockford, ME).

Cell Culture. NCI-H1299 cells were cultured in RPMI 1640 medium containing 10% heat-inactivated FBS. NCI-H1299 cells were split weekly 1/20 with trypsin-EDTA. Balb 3T3 cells stably expressing human BRS3 (hBRS3), human NMB receptors (hNMBRs), or human GRP receptors (hGRPRs) made as described previously (Benya et al., 1995) were grown in DMEM supplemented with 300 mg/l G418 sulfate. All the other cell lines were from American Type Culture Collection (Manassas, VA). Hs746T cells were grown in DMEM with 10% FBS. LNCaP cells were maintained in RPMI 1640 medium with 10% FBS, 1% sodium pyruvate, and 1% HEPES. DMS-53 cells were cultured in Waymouth's MB 752/1 medium with 10% FBS. U-87MG cells were grown in DMEM with 10% FBS, 1% sodium pyruvate, and 1% minimal essential medium nonessential amino acids. All other cells were cultured as described previously (Sun et al., 2004). Cells were mycoplasma free and were used when they were in exponential growth phase after incubation at 37°C in 5% CO_2 and 95% air.

Preparation of Peptides. The CPT-Bn conjugates were synthesized as described previously (Moody et al., 2004). To synthesize a possible inactive analog that was chemically and structurally very similar to the active conjugate CPT-L2-BA3, we analyzed previously published Bn structure-function studies (Rivier and Brown, 1978; Moody et al., 1982; Leban et al., 1993) that suggested that insertion of a D-Phe in position 13 for Phe would likely produce a Bn peptide with greatly reduced affinity and potency. CPT-Bn conjugates were purified to >97% purity by preparative high-performance liquid chromatography (C_{18} silica) and characterized by mass spectrometry and amino acid analysis. ^{125}I-CPT-L2-BA3, ^{125}I-BA3, and ^{125}I-CPT-D-Phe-BA3, with specific activities of 2200 Ci/mmol, were prepared as described previously (Moody et al., 2004). The radiolabeled peptides were separated using a C18 Sep-Pak (Waters Associates, Milford, MA) and further purified by reverse-phase high performance liquid chromatography on a C18 column. The fractions with the highest radioactivity were pooled, neutralized with 0.2 M Tris buffer, pH 9.5, and stored with 0.5% (w/v) bovine serum albumin at −20°C.

Binding of ^{125}I-Labeled Bn-Related Peptides. Binding was performed as described previously (Mantey et al., 1997). The standard binding buffer contained 24.5 mM HEPES, pH 7.4, 98 mM NaCl, 6 mM KCl, 5 mM $MgCl_2$, 2.5 mM NaH_2PO_4, 5 mM sodium pyruvate, 5 mM sodium fumarate, 0.01% (w/v) soybean trypsin inhibitor, 1% amino acid mixture, 0.2% (w/v) bovine serum albumin, and 0.05% (w/v) bacitracin. Balb 3T3 cells stably expressing hGRPR (0.3×10^6), hNMBR (0.03×10^6), hBRS3 (0.3×10^6), or NCI-H1299 cells were incubated with 0.05 nM ^{125}I-labeled ligand at 22°C for 60 min. Aliquots (100 μl) were removed and centrifuged through 300 μl of incubation buffer in 400-μl Microfuge tubes at 10,000*g* for 1 min using a Beckman microcentrifuge B. The pellets were washed twice with buffer and counted for radioactivity in a gamma counter. The nonsaturable binding was the amount of radioactivity associated with cells in incubations containing 50 pM radioligand (2200 Ci/mmol) and 1 μM unlabeled ligand. Nonsaturable binding was <10% of total binding in all the experiments. Inhibition constants (K_i) were determined using a least-square, curve-fitting program (KaleidaGraph; Synergy Software, Reading, PA) and the Cheng-Prusoff equation (Cheng and Prusoff, 1973).

Internalization. Balb 3T3 cells stably transfected with hGRPR, hNMBR, or hBRS3 were incubated with radioligands as stated un-

der *Materials and Methods* for binding. Internalization experiments were performed as described previously (Moody et al., 2004). In brief, after the indicated incubation times, 100-μl samples were added to a 1.5-ml Microfuge tube with 1 ml of 4°C acid-stripping solution containing 0.2 M acetic acid, pH 2.5, and 0.5 M NaCl to remove the surface bound radioligand. After 5 min, the cells were pelleted, the supernatant was removed, and the cells were washed twice with incubation buffer. In all cases, parallel incubations were conducted in the presence of 1 μM unlabeled ligand to determine changes in nonsaturable binding. Results are expressed as the percentage of saturable ^{125}I-ligand added that is surface bound (acid-stripped) or internalized (not acid-stripped).

Measurement of [^{3}H]Inositol Phosphate. Changes in total [^{3}H]inositol phosphates ([^{3}H]IP) were measured as described previously (Benya et al., 1995). hBRS3-, hGRPR-, or hNMBR-transfected Balb 3T3 cells were cultured into 24-well plates (5×10^4 cells/well) in media and then incubated for 24 h at 37°C in a 5% CO_2 atmosphere. The cells were then incubated with 3 μCi/ml *myo*-[2-^{3}H] inositol in media supplemented with 2% FBS for an additional 24 h. The 24-well plates were washed by incubating for 30 min at 37°C with 1 ml/well PBS, pH 7.0, containing 20 mM lithium chloride. The wash buffer was aspirated and replaced with 500 μl of IP assay buffer containing 135 mM sodium chloride, 20 mM HEPES, pH 7.4, 2 mM calcium chloride, 1.2 mM magnesium sulfate, 1 mM EGTA, 20 mM lithium chloride, 11.1 mM glucose, and 0.05% (w/v) bovine serum albumin and incubated with or without any of the peptides studied. After 60 min of incubation at 37°C, the experiments were terminated by the addition of 1 ml of ice-cold 1% (v/v) hydrochloric acid in methanol. Samples were loaded onto Dowex AG1-X8 anion exchange resin columns (Bio-Rad), washed with 5 ml of distilled water to remove free [^{3}H]inositol, then washed with 2 ml of 5 mM disodium tetraborate/60 mM sodium formate solution to remove [^{3}H]glycerophosphorylinositol. Two milliliters of 1 mM ammonium formate/100 mM formic acid solution was added to the columns to elute total [^{3}H]IP. Each eluate was mixed with scintillation cocktail and measured for radioactivity in a scintillation counter.

Proliferation Assays. Growth studies were performed using the MTT assay (Moody et al., 2004). CFPAC-1, DMS-53, DU-145, Hs746T, HT-29, IMR32, LNCaP, MCF-7, MOLT-4, NCI-H69, NCI-H1299, PC-3, SKNSH, or U-87MG human cancer cells (10^4/well) were placed in SIT medium (100 μl), and various concentrations of CPT-L2-BA3 or D-Phe-CPT-L2-BA3 were added. After 4 days, 15 μl (1 mg/ml) of MTT was added. After 4 h, 150 μl of dimethyl sulfoxide was added. After 16 h, the optical density at 570 nm was determined using an enzyme-linked immunosorbent assay reader. The proliferation rates were calculated from the optical density readings with various concentrations of CPT-L2-BA3 using the untreated cells as 100.

Clonogenic Assays. The effects of CPT-BN conjugates on the growth of NCI-H1299 cells were investigated using a clonogenic assay (Mahmoud et al., 1991). The base layer consisted of 3 ml of 0.5% agarose in SIT medium containing 5% fetal bovine serum in six-well plates. The top layer consisted of 3 ml of SIT medium in 0.3% agarose, CPT-BN conjugates, and 5×10^4 NCI-H1299 nonsmall cell lung cancer cells. Triplicate wells were plated; after 2 weeks, 1 ml of 0.1% *p*-iodonitrotetrazolium violet was added, and after 16 h at 37°C, the plates were screened for colony formation; the number of colonies larger than 50 μM in diameter were counted using an Omnicon image analysis system (BioLogics, Inc., Manassas, VA).

Nude Mouse Xenografts. The ability of the CPT-L2-BA3 or D-Phe-CPT-L2-BA3 to inhibit CFPAC-1, NCI-H69, NCI-H1299, or PC-3 tumor proliferation was investigated in vivo (Mahmoud et al., 1991). Female athymic BALB/c nude mice (Taconic Farms, Germantown, NY), 4 to 5 weeks old, were housed in a pathogen-free temperature-controlled isolation room with a diet consisting of autoclaved rodent chow and autoclaved water given ad libitum. NCI-H1299 cells (1×10^7) were injected into the right flank of each mouse by s.c. injection. Palpable tumors were observed in approximately 90% of the mice after 1 week. CPT-L2-BA3 and D-Phe-CPT-L2-BA3 (1 nmol in 100 μl of PBS) were injected daily s.c. adjacent to the tumor or, in the case of the studies shown in Table 2, in the flank opposite the tumor or i.p. as indicated. The tumor volume (height $\times$ width $\times$ depth) was determined twice weekly by calipers and recorded. The animal studies were approved by National Institutes of Health or Tulane University animal care and use committees.

Stability of CPT-L2-BA3. The stability of CPT-L2-BA3 in mouse plasma was investigated by radioimmunoassay. Mouse blood was obtained from nude mice, and after centrifugation of the red cells, the mouse plasma was added to 100 μM CPT-L2-BA3. As a function of time after addition of mouse plasma, 0.2 N HCl was added and the sample frozen and lyophilized. The sample was resuspended in radioimmunoassay (RIA) buffer (0.25% bovine serum albumin in PBS), and the amount of immunoreactive CPT-L2-BA3 was determined. The radioimmunoassay was performed using a rabbit antibody elicited against BA3 linked to keyhole limpet hemocyanin with carbodiimide. The BA3-carbodiimide-KLH conjugate was initially injected s.c. into rabbits, in Freund's complete adjuvant, followed by monthly boosts in Freund's incomplete adjuvant. Plasma samples were incubated with rabbit anti-BA3 sera (1:1000) and ^{125}I-CPT-L2-BA3 (10,000 cpm) added in RIA buffer. After incubation at 4°C for 16 h, free ^{125}I-CPT-L2-BA3 was removed by charcoal addition. The sample was centrifuged at 1000*g* for 5 min, and the ^{125}I-CPT-L2-BA3 bound to the antibody remained in solution and was counted in a LKB gamma counter (GE Healthcare).

Results

BA3, CPT-L2-BA3, and D-Phe-CPT-L2-BA3 Binding and Internalization. ^{125}I-CPT-L2-BA3 and ^{125}I-D-Phe-CPT-L2-BA3 were examined for their ability to interact with cells stably transfected with human Bn receptors (i.e., hGRPR, hNMBR, hBRS3) (Fig. 1). For each of these three human Bn receptor subtypes, ^{125}I-CPT-L2-BA3 but not ^{125}I-D-Phe-CPT-L2-BA3 demonstrated saturable binding (Fig. 1, top). Saturable binding of ^{125}I-CPT-L2-BA3 was time-dependent and half-maximal after approximately 3 min and maximal by 30 min in each Bn receptor subtype (Fig. 1, top).

The ability of human Bn receptor containing cells to internalize ^{125}I-CPT-L2-BA3 and ^{125}I-D-Phe-CPT-L2-BA3 was investigated. Approximately 40% of the added ^{125}I-CPT-L2-BA3 was internalized by cells containing hGRPR, hNMBR, or hBRS3 after 10 min (Fig. 1, bottom). In all cases, however, the amount of ligand internalized after a 30-min incubation was greater than the amount internalized after a 10-min incubation (Fig. 1, bottom). In contrast, there was no significant internalization of ^{125}I-D-Phe-CPT-L2-BA3 in cells containing hGRPR, hNMBR, or hBRS3 at 10 or 30 min (Fig. 1, bottom). The results indicate that ^{125}I-CPT-L2-BA3 but not ^{125}I-D-Phe-CPT-L2-BA3 internalizes significantly in cells containing each of the different human Bn receptor subtypes.

To determine the affinities of the various BA3 analogs for each human Bn receptor subtype, dose-inhibition curves were performed with each BA3 analog and ^{125}I-BA3 for each of the three human Bn receptor subtypes (Fig. 2). Specific ^{125}I-BA3 binding to cells containing hGRPR was inhibited in a dose-dependent manner by BA-3, CPT-L2-BA3, and D-Phe-CPT-L2-BA3 (Fig. 2, top, left). The IC_{50} values for BA-3, CPT-L2-BA3, and D-Phe-CPT-L2-BA3 were 0.17 $\pm$ 0.01, 0.16 $\pm$ 0.01, and 603 $\pm$ 16 nM, respectively. Similar IC_{50} values were obtained using cells containing hNMBR (Fig. 2, top, middle); however, cells containing hBRS3 (Fig. 3, top, right) bound BA-3, CPT-L2-BA3, and D-Phe-CPT-L2-BA3

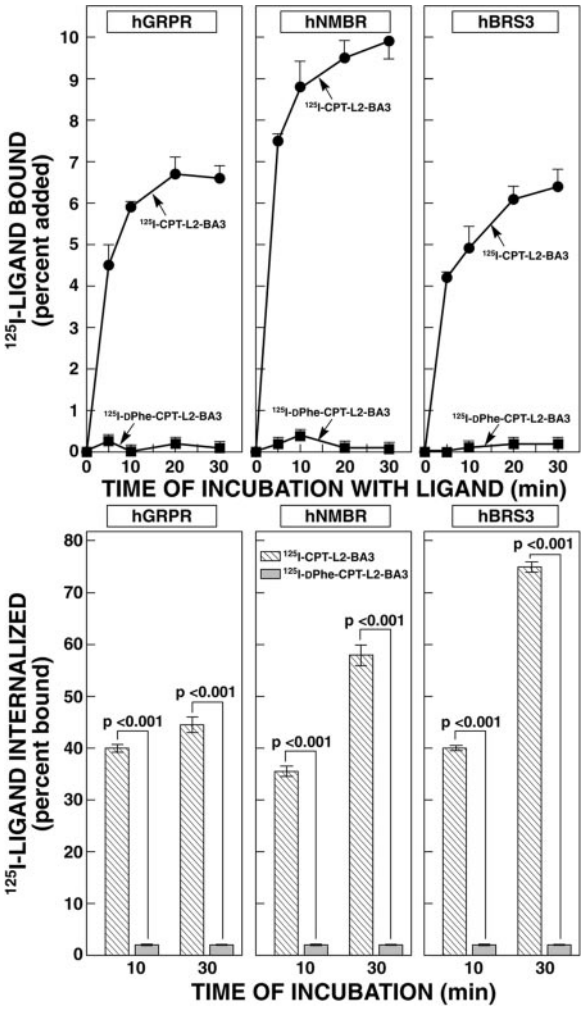

Fig. 1. Top, time course of binding of ^{125}I-CPT-L2-BA3 and ^{125}I-D-Phe-CPT-L2-BA3 to cells containing human Bn receptors. The ability of ^{125}I-CPT-L2-BA3 and ^{125}I-D-Phe-CPT-L2-BA3 to bind Balb 3T3 cells stably transfected with hGRPR, hNMBR, or hBRS3 (0.3×10^6 cells/ml) as a function of time was determined. Results are means ± S.E.M. from at least three experiments, and in each experiment, each data point was repeated in duplicate. Bottom, ability of ^{125}I-CPT-L2-BA3 and ^{125}I-D-Phe-CPT-L2-BA3 to be internalized by cells containing human Bn receptor subtypes. Balb/3T3 cells stably transfected with hGRPR, hNMBR, or hBRS3 were incubated for 10 or 30 min at 37°C with 0.05 nM ^{125}I-CPT-L2-BA3 or ^{125}I-D-Phe-CPT-L2-BA3 in the presence or absence of 1000 nM CPT-L2-BA3 or D-Phe-CPT-L2-BA3, respectively. Results are expressed as the percentage of the saturably bound radiolabeled peptide internalized. Results are mean ± S.E.M. from at least three experiments, and in each experiment, each data point was determined in duplicate.

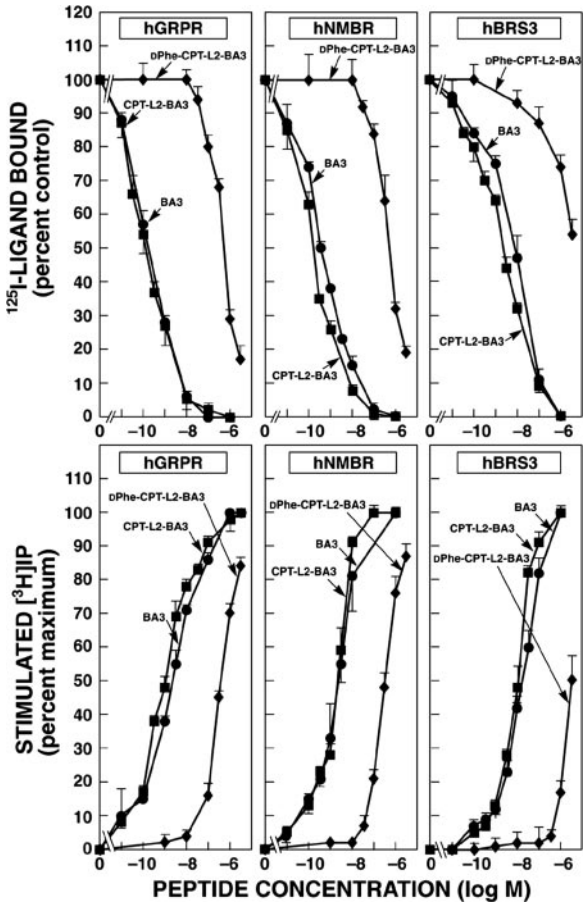

Fig. 2. Top, relative abilities of BA3, CPT-L2-BA3, and D-Phe-CPT-L2-BA3 to inhibit binding to cells containing human Bn receptor subtypes. Specific binding of ^{125}I-BA3 to cells containing hGRPR, hNMBR, or hBRS3 was determined as a function of unlabeled BA3, CPT-L2-BA3, or D-Phe-CPT-L2-BA3 concentration. From the dose-inhibition curves, the IC_{50} was determined using the curve-fitting program, KaleidaGraph, and the K_i was calculated using the Cheng-Prusoff equation. Results are means ± S.E.M. from at least three experiments, and in each experiment, each data point was determined in duplicate. Bottom, ability of CPT-L2-BA3 and D-Phe-CPT-L2-BA3 to stimulate accumulation of IP in cells containing human Bn receptors. hGRPR-, hNMBR-, and hBRS3-transfected Balb/3T3 cells in 24-well plates were labeled with myo-2[^{3}H] inositol as described under *Materials and Methods*. The cells were incubated for 60 min at 37°C with the indicated concentrations of BA3, CPT-L2-BA3, and D-Phe-CPT-L2-BA3 in IP assay buffer, and total [^{3}H]IP was determined as described under *Materials and Methods*. Results are expressed as the percentage of stimulation caused by a maximally effective concentration of agonist (i.e., 1 μM BA3). Control and 1 μM BA3-stimulated values were: hGRPR, 396 ± 50 and 6386 ± 818 cpm, respectively; hNMBR cells, 2566 ± 191 and 63076 ± 2636 cpm, respectively; and hBRS3 cells, 3076 ± 493 and 14618 ± 432 cpm, respectively. Results are means ± S.E.M. of at least three experiments, and in each experiment, each value was determined in duplicate.

with lower affinity (IC_{50} values of 8.1 ± 0.3, 2.1 ± 0.1, and >3000 nM). These results indicate that for each of the three human Bn receptor subtypes, CPT-L2-BA3 had approximately equal high affinity to BA3, whereas D-Phe-CPT-L2-BA3 had a 400- to 3000-fold lower affinity.

BA3, CPT-L2-BA3, and D-Phe-CPT-L2-BA3 Function as Full Agonists. To determine the biological activity of BA3, CPT-L2-BA3, and D-Phe-CPT-L2-BA3, their ability to stimulate phospholipase C was assessed because activation of each of the human Bn receptor subtypes is known to stimulate this intracellular cascade (Mantey et al., 1997). To assess phospholipase C activation, dose-response curves for BA3, CPT-L2-BA3, and D-Phe-CPT-L2-BA3 to stimulate turnover of [^{3}H]inositol phosphates was examined (Fig. 2, bottom). BA3, CPT-L2-BA3, and D-Phe-CPT-L2-BA3 were equally efficacious agonists stimulating a 16.1 ± 2.1-, 24.1 ± 1.4-, and 4.7 ± 0.7-fold increase in [^{3}H]IP in cells containing hGRPR, hNMBR, and hBRS3, respectively (Fig. 2, bottom). The EC_{50} values for BA3, CPT-L2-BA3, and D-Phe-CPT-L2-BA3 were 2.3 ± 0.1, 1.0 ± 0.1, and 417 ± 29 nM, respectively, in cells containing hGRPR (Fig. 2, bottom, left). Similar results were obtained in cells containing hNMBR (Fig. 2, bottom, middle). In contrast, in cells containing hBRS3, the EC_{50} values for BA3, CPT-L2-BA3, and D-Phe-CPT-L2-BA3 were 15.1 ± 2.0, 11.1 ± 0.8, and 3260 ± 210 nM, respectively (Fig. 2, bottom, right). These results indicate that BA3 and

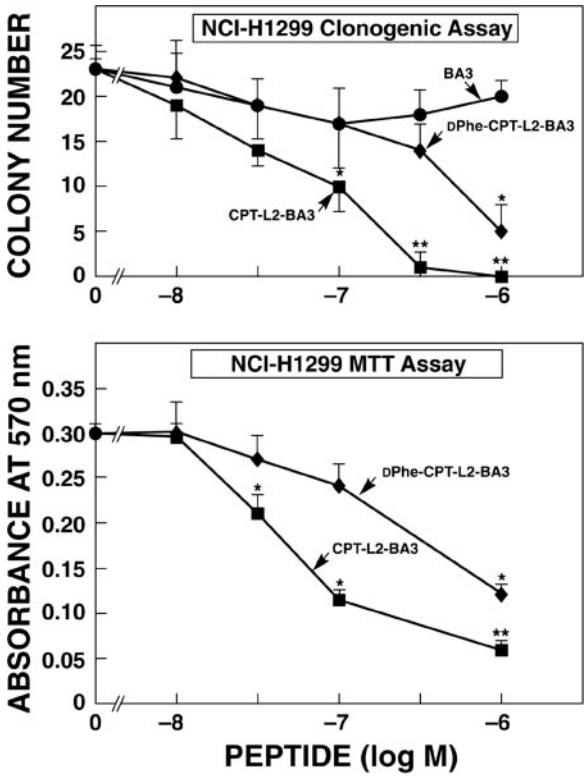

Fig. 3. Comparison of the growth effects of CPT-L2-BA3 and D-Phe-CPT-L2-BA3 in vitro using lung cancer cell line NCI-H1299. Top, effects of BA3, CPT-L2-BA3, and D-Phe-CPT-L2-BA3 on the proliferation of NCI-H1299 cells were investigated using the clonogenic assay. Results are means ± S.E.M. of at three determinations. Bottom, effects of D-Phe-CPT-L2-BA3 and CPT-L2-BA3 on NCI-H1299 cells were investigated using the MTT assay. Results are the means ± S.E.M. of eight determinations. Each of these experiments is representative of four others, $p < 0.05$; *, $p < 0.01$; **, compared with control using the Student's t test.

CPT-L2-BA3 both had high potency for activating each of the three human Bn receptors and were >200-fold more potent that D-Phe-CPT-L2-BA3 for each receptor subtype.

Ability of BA3, CPT-L2-BA3, and D-Phe-CPT-L2-BA3 to Inhibit Cancer Cell Growth in Vitro. The ability of BA3, CPT-L2-BA3, and D-Phe-CPT-L2-BA3 to alter cancer cell proliferation in vitro was investigated using human cancer cell lines. CPT-L2-BA3 and D-Phe-CPT-L2-BA3 decreased NCI-H1299 lung cancer tumor growth in a dose-dependent manner (Fig. 3). In the clonogenic assay, CPT-L2-BA3 and D-Phe-CPT-L2-BA3 inhibited NCI-H1299 colony formation with IC_{50} values of 70 and 500 nM, respectively (Fig. 3, top) In contrast, 1000 nM BA3 had no significant effect on proliferation. CPT-L2-BA3 and D-Phe-CPT-L2-BA3 inhibited the proliferation of NCI-H1299 lung cancer cells assessed using the MTT assay with IC_{50} values of 70 and 600 nM (Fig. 3, bottom). The ability of CPT-L2-BA3 to inhibit the proliferation of a number of different human cancer cell lines was assessed. Using the MTT assay, CPT-L2-BA3 was found to inhibit the proliferation of breast cancer (MCF-7), colon cancer (HT-29), gastric cancer (Hs746T), glioblastoma (U-87MG), leukemia (MOLT-4), lung cancer (NCI-H69), neuroblastoma (IMR32, SKNSH), pancreatic cancer (CFPAC-1), and prostate cancer (PC-3, DU-145, LNCaP) cell lines with IC_{50} values ranging from 33 to 2269 nM (Table 1). Cell lines with low IC_{50} values such as NCI-H1299 (100 nM, Fig. 3) had a higher density of ^{125}I-BAO binding sites than did cells with a high IC_{50} value such as NCI-H69 (1923 nM, Table 1). These results demonstrate that a CPT-Bn conjugate can inhibit the proliferation of numerous human cancer cell lines and support the conclusion that the inhibition is mediated by a specific Bn receptor-mediated mechanism.

TABLE 1
Ability of CPT-L2-BA3 to inhibit the growth of various human cancer cell lines

Cell Line	Cancer Type	IC_{50}
		nM
CFPAC-1	Pancreatic cancer	625
DMS-53	Lung cancer	828
DU-145	Prostate cancer	240
Hs746T	Gastric cancer	821
HT-29	Colon cancer	2269
IMR32	Neuroblastoma	90
LNCaP	Prostate cancer	92
MCF-7	Breast cancer	1705
MOLT-4	Leukemia	33
NCI-H69	Lung cancer	1923
PC-3	Prostate cancer	198
SKNSH	Neuroblastoma1610	
U-87MG	Glioblastoma	842

The ability of CPT-L2-BA3 to inhibit the proliferation of the human lung cancer cells was determined by the MTT assay. The mean value of eight determinations, IC_{50} (nanomolar), is shown. The S.E.M. was less than 10%.

Ability of CPT-L2-BA3 and D-Phe-CPT-L2-BA3 to Inhibit Cancer Cell Growth in Vivo. To assess the ability of CPT-L2-BA3 and D-Phe-CPT-L2-BA3 to inhibit tumor growth in vivo, their ability to inhibit NCI-H1299 xenograft proliferation in nude mice was determined (Fig. 4). Palpable NCI-H1299 tumors were present 2.5 weeks after the injection of NCI-H1299 cells s.c. (Fig. 4). CPT-L2-BA3, D-Phe-CPT-L2-BA3 (0.8 mg/kg), and the PBS control were then injected s.c. into nude mice three times a week. At 4.5 weeks, animals injected with CPT-L2-BA3 had significantly smaller tumors (679 ± 141 mm^3) than did animals receiving D-Phe-CPT-L2-BA3 (1088 ± 161 mm^3) or PBS (1188 ± 227). In addition, at 4 weeks, animals injected with CPT-L2-BA3 had significantly smaller tumors than did animals receiving D-Phe-CPT-L2-BA3 or PBS (Fig. 4). In contrast, animals injected with CPT-L2-BA3 had a similar body weight (23.3 ± 0.5 g) relative to animals, which received D-Phe-CPT-L2-BA3

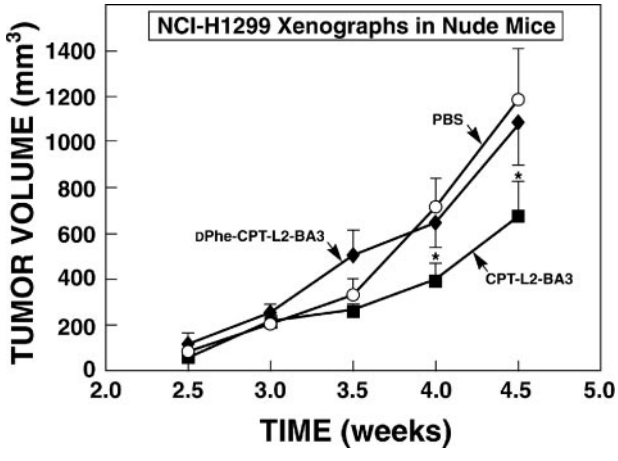

Fig. 4. Growth effects of CPT-L2-BA3 and D-Phe-CPT-L2-BA3 in vivo. The effects of CPT-L2-BA3, D-Phe-CPT-L2-BA3, and PBS on the proliferation of NCI-H1299 tumors in nude mice were investigated. Results are means ± S.E.M. of eight determinations. This experiment was representative of two others, $p < 0.05$; *, using analysis of variance.

(22.9 ± 0.5 g) or PBS (22.5 + 0.7 g). Likewise, high doses of CPT-L2-BA3 (8 mg/kg twice a week) injected into nude mice resulted in a 84% inhibition of the growth of pancreatic CFPAC-I tumors (Table 2). When CPT-L2-BA3 was injected i.p. into nude mice (8 mg/kg twice a week), CFPAC-I, NCI-H69, and PC-3 tumor growth was inhibited by 55, 56, and 68%, respectively, relative to control mice injected with PBS. These results indicate that the CPT-Bn conjugate can inhibit xenograft growth in vivo of a number of different human cancers.

Stability of CPT-L2-BA3. To assess the stability of CPT-L2-BA3, we used a newly developed RIA with a rabbit antibody that was formed against BRS3. Figure 5, top, demonstrates that the RIA had high specificity for BA3 and CPT-L2-BA3 (IC_{50} = 0.5 ± 0.1 and 2.1 ± 0.6 pmol, respectively). Incubation in mouse plasma led to a progressive loss of CPT-L2-BA3 immunoreactivity with a half-time of CPT-L2-BA3 metabolism of 30 min (Fig. 5, bottom). By radioimmunoassay, peak blood concentrations (10 nM) of CPT-L2-BA3 were obtained 30 min after injection into nude mice (T. Moody, unpublished data).

Discussion

One of the principal problems limiting novel treatments (antisense, chemotherapeutic agents with limited solubility, tumor-sensitizing agents, etc.) of many common tumors is the inability to selectively target the therapy to the cancer cell (Dyba et al., 2004). Because many human tumors overexpress or ectopically express at least one peptide hormone/neurotransmitter growth factor receptor and in many cases multiple receptors, this is receiving increasing attention as a useful approach for localizing tumors by imaging modalities and/or allowing selective delivery of cytotoxic compounds (Schally and Nagy, 1999, 2003; Langer and Beck-Sickinger, 2001; Reubi et al., 2003). A central premise of these latter studies is that the cytotoxic agent is being delivered specifically to the tumor by a receptor-mediated mechanism. Studies with radiolabel octreotide for imaging of breast cancer in vivo showed imaging results correlated with the expression of somatostatin receptors with high affinity for octreotide on the tumor (i.e., sst2 and sst5) (Schulz et al., 2002), and studies with neuroblastomas showed a similar correlation (Orlando et al., 2001). Studies using somatostatin receptor knockout mice (Hofland et al., 2003) demonstrated decreases in ^{111}In-penetreotide uptake in tissues that normally possess higher densities of somatostatin receptors. Conversely, overexpression of somatostatin receptor subtype 2 with an adenoviral vectors in human small cell lung tumors increased uptake of radiolabeled somatostatin in the tumor implanted in mice (Rogers et al., 2002). Although these results provide evidence for the importance of a receptor-mediated mechanism for radionuclide-conjugated peptide ligand uptake for imaging, similar evidence is lacking for the possible involvement of this mechanism in mediating tumor cytotoxicity with peptide receptor agonist chemotherapeutic constructs.

TABLE 2

Inhibition of tumor proliferation in vivo

Nude mice bearing xenografts of the human tumor lines were injected twice a week with the indicated dose of CPT-L2-BA3. Subcutaneous injections were made in the flank opposite to the tumor implant. The tumor volume for animals treated with CPT-L2-BA3 was compared with that of animals injected with PBS. The mean value ± S.E.M. of 11 determinations is shown.

Xenograft	Injection Dose	Tumor Growth Inhibition
		%
CFPAC-1	8 mg/kg s.c	84 ± 16
CFPAC-1	8 mg/kg i.p.	55 ± 14
NCI-H69	8 mg/kg i.p.	56 ± 14
PC-3	8 mg/kg i.p.	68 ± 17

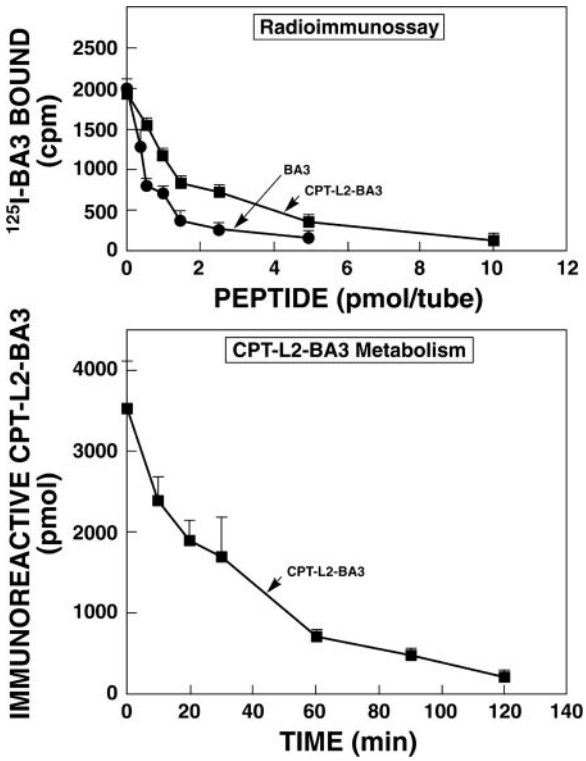

Fig. 5. CPT-L2-BA3 quantitation by radioimmunoassay. Top, ability of unlabeled BA3 or CPT-L2-BA3 to compete for ^{125}I-BA3 binding to rabbit antisera (1:1000) generated against BA3 was investigated. Bottom, CPT-L2-BA3 was added to mouse plasma for the indicated amount of time at 37°C. The sample was treated with boiling 2 N acetic acid and lyophilized. The sample was resuspended and tested in the radioimmunoassay. The mean value ± S.E.M. of three determinations is indicated. This experiment is representative of four others.

In the present study, we addressed this issue using a pharmacological approach coupled with in vitro and in vivo cytotoxicity studies. Numerous studies demonstrate that many common tumors overexpress or ectopically express Bn receptor subtypes, including 44 to 100% of small cell or non-small cell lung cancers (Guinee et al., 1994; Siegfried et al., 1999), 57% of gastric cancers (Preston et al., 1993), 72% of breast cancers (Reubi et al., 2002), 100% of prostate cancers (Reubi et al., 2002), 100% of head and neck squamous cell tumors (Lango et al., 2002), 40 to 80% of colon cancer (Guinee et al., 1994; Carroll et al., 1999), 100% of ovarian cancer (Sun et al., 2000b; Lango et al., 2002), and 80% of glioblastomas/gliomas (Reubi et al., 2002). Second, many studies also demonstrate that radiolabeled analogs of Bn-related peptides can target tumors or tissues containing or overexpressing Bn-receptor subtypes and that these analogs, if agonists, are rapidly internalized by the tumors (Moody et al., 2004). Third, synthetic Bn analogs coupled to various chemotherapeutic agents (i.e., doxorubicin, camptothecin) have been shown to cause cytotoxicity in a number of human tumors (Kiaris et al., 1999; Schally and Nagy, 2003; Moody et al.,

2004). Fourth, each of the three subtypes of human Bn receptors (hGRPR, hNMBR, hBRS3) can be overexpressed by different tumors; however, each Bn receptor subtype usually has markedly different affinities for different Bn analogs (Mantey et al., 1997). We have recently discovered a synthetic Bn analog BA3 that interacts with high affinity with each of the three human Bn receptor subtypes (Mantey et al., 1997); thus, if a tumor expresses any of the three Bn receptor subtypes, it will bind with high affinity. Furthermore, radiolabeled analogs of this peptide interact with high affinity with Bn receptors on many human tumors. Fifth, we have recently coupled this universal Bn receptor ligand, BA3, through a built-in nucleophile-associated releasing group linker (L2) to the topoisomerase 1 inhibitor, CPT, and the resultant CPT-L2-BA3 peptide not only retained high affinity for each Bn receptor subtype but functioned as a fully efficacious agonist at each receptor subtype, was rapidly internalized, and was cytotoxic for NCI-H1299 lung cancer cells expressing Bn receptors in both an MTT and clonogenic growth assay (Moody et al., 2004).

A Bn analog was synthesized that was chemically similar to the active CPT-Bn conjugate (CPT-L2-BA3) but was inactive or had much reduced Bn receptor affinity. Previous Bn structure-function studies demonstrated the importance of the penultimate COOH-terminal amino acid (i.e., position 13 in Bn) for high-affinity receptor interaction of high potency (Rivier and Brown., 1978; Moody et al., 1982; Leban et al., 1993). These studies demonstrated that insertion of a DLeu^{13}, replacement of the Leu^{13} with a rigid amino acid such as proline, or alteration of the Leu^{13}-Met^{14} peptide bond all resulted in greatly reduced receptor affinity and loss of or reduced potency. [D-Tyr^{6},β-Ala^{11},D-Phe^{13},Nle^{14}] Bn (6–14), in which a D-Phe was substituted for the leucine in position 13, reduced potency but had identical chemical properties (solubility, lipophilicity, electrochemical charge) to the active peptide, BA3. ^{125}I-D-Phe-CPT-L2-BA3 demonstrated no saturable binding to cells expressing any of the three human Bn receptor subtypes, was not internalized, and in competition assays, D-Phe-CPT-L2-BA3 had a >1400-fold lower affinity for each of the Bn receptor subtypes than the active analog, CPT-L2-BA3 (i.e., 3400-fold less for hGRPR and hNMBR and >1400 fold less for hBRS3).

A number of our results support the conclusion that specific interaction with Bn receptor subtypes is important for mediating the tumor cytotoxicity of the active analog, CPT-L2-BA3. First, in both a clonogenic growth assay and in the MTT growth assay, CPT-L2-BA3 demonstrated significantly greater cytotoxicity and was significantly more potent at causing cytotoxicity than D-Phe-CPT-L2-BA3. Second, CPT-L2-BA3 was significantly more potent than the inactive analog, D-Phe-CPT-L2-BA3, at inhibiting the growth of NCI-H1299 lung cancer xenografts in nude mice. Because CPT-L2-BA3 and D-Phe-CPT-L2-BA3 differ principally in their ability to interact with high affinity with human Bn receptor subtypes, these results support the conclusion that this interaction is important for the ability of CPT-L2-BA3 to cause tumoral cytotoxicity in vitro and in vivo. Because D-Phe-CPT-L2-BA3 had weak cytotoxicity, however, for NCI-H1299 cells, it may slowly diffuse across the plasma membrane, as does the hydrophobic CPT, and cause cancer cell apoptosis. CPT, which could only be dissolved in dimethyl sulfoxide, was cytotoxic for NCI-H1299 cells but could not be tested in vivo.

CPT-L2-BA3 specifically interacts with high affinity with all human Bn receptor subtypes, thus increasing the possibility of a specific delivery of CPT to tumoral receptor sites containing any of the three human Bn receptor subtypes. GRP, which has an identical heptapeptide COOH terminus to Bn (Bunnett, 1994), has a half-life of only 1.4 min in the circulation in the pig (Knuhtsen et al., 1984) and <5 min in mouse plasma (Marquez et al., 2000) while being degraded primarily by cleavage at the His^{12}-Leu^{13} peptide bond and also at the Trp^{8}-Ala^{9} peptide bond at the COOH terminus (Shipp et al., 1991). CPT-L2-BA3 had a half-life of 30 min in mouse plasma, which exceeded the value for GRP of <5 min, demonstrating its enhanced stability in plasma. This enhanced stability could be due to the substitution of Phe^{13} for Leu^{13}, which might interfere with the degradation at the His^{12}Leu^{13} peptide bond that occurs in GRP or to the coupling of a moiety (i.e., CPT-L2) to the NH_2 terminus, which can effect degradation of Bn-related peptides (Shipp et al., 1991). Secondly, we found that CPT-L2-BA3 inhibited the proliferation of a large number of human cancer cell lines in vitro using the MTT assay and including cancers of the breast, colon, lung, stomach, pancreas, and prostate as well as leukemia and glioblastoma cancer cell lines. Furthermore, we also demonstrated that CPT-L2-BA3 inhibited the growth of pancreatic, lung, and prostate cell lines in vivo in nude mice and was devoid of observed toxicity at relatively high doses.

In conclusion, our results demonstrate that D-Phe-CPT-L2-BA3, which is chemically closely related to CPT-L2-BA3, has a greatly reduced ability to interact with human Bn receptors because it does not interact with high affinity or activate with high potency any of the three mammalian Bn receptor subtypes and is not internalized by any Bn receptor subtype. Compared with the active analog CPT-L2-BA3, D-Phe-CPT-L2-BA3 had a much-reduced cytotoxic effect both in vitro and in vivo, providing support for the conclusion that specific interaction with Bn receptor subtypes is important for mediating its tumor cytotoxicity effect.

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References

Benya RV, Kusui T, Pradhan TK, Battey JF, and Jensen RT (1995) Expression and characterization of cloned human bombesin receptors. *Mol Pharmacol* **47:**10–20.

Bunnett N (1994) Gastrin-releasing peptide, in *Gut Peptides* (Walsh JH, Dockray GJ eds) pp 423–445, Raven Press, Ltd., New York.

Carroll RE, Matkowskyj KA, Chakrabarti S, McDonald TJ, and Benya RV (1999) Abberrant expression of gastrin-releasing peptide and its receptor by well-differentiated colon cancers in humans. *Am J Physiol* **276:**G655–G665.

Cheng Y and Prusoff WH (1973) Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 percent inhibition (IC_{50}) of an enzymatic reaction. *Biochem Pharmacol* **22:**3099–3108.

de Groot FM, Damen EW, and Scheeren HW (2001) Anticancer prodrugs for application in monotherapy: targeting hypoxia, tumor-associated enzymes, and receptors. *Curr Med Chem* **8:**1093–1122.

Dubowchik GM and Walker MA (1999) Receptor-mediated and enzyme-dependent targeting of cytotoxic anticancer drugs. *Pharmacol Ther* **83:**67–123.

Dyba M, Tarasova NI, and Michejda CJ (2004) Small molecule toxins targeting tumor receptors. *Curr Pharm Des* **10:**2311–2334.

Fuselier JA, Sun L, Woltering SN, Murphy WA, Vasilevich N, and Coy DH (2003) An adjustable release rate linking strategy for cytotoxin-Peptide conjugates. *Bioorg Med Chem Lett* **13:**799–803.

Guinee DG Jr, Fishback NF, Koss MN, Abbondanzo SL, and Travis WD (1994) The spectrum of immunohistochemical staining of small-cell lung carcinoma in specimens from transbronchial and open-lung biopsies. *Am J Clin Pathol* **102:**406–414.

Hoffman TJ, Quinn TP, and Volkert WA (2001) Radiometallated receptor-avid peptide conjugates for specific *in vivo* targeting of cancer cells. *Nucl Med Biol* **28:**527–539.

Hofland LJ, Lamberts SW, van Hagen PM, Teubi JC, Schaeffer J, Wooijers M, von Koetsveld PM, Srinivasan A, Krenning EP, and Breaven WA (2003) Crucial role

for somatostatin receptor subtype 2 in determining the uptake of [111In-DTPA-D-Phe1]octreotide in somatostatin receptor-positive organs. *J Nucl Med* **44:**1315–1321.
Jensen RT (2000) Somatostatin receptor based scintigraphy and anti-tumor treatment: an expanding vista? *J Clin Endocrinol Metab* **85:**3507–3508.
Kiaris H, Schally AV, Nagy A, Sun B, Armatis P, and Szepeshazi K (1999) Targeted cytotoxic analogue of bombesin/gastrin-releasing peptide inhibits the growth of H-69 human small-cell lung carcinoma in nude mice. *Br J Cancer* **81:**966–971.
Knuhtsen S, Holst JJ, Knigge U, Olesen M, and Nielsen OV (1984) Radioimmunoassay, pharmacokinetics, and neuronal release of gastrin-releasing peptide in anesthetized pigs. *Gastroenterology* **87:**372–378.
Kwekkeboom D, Krenning EP, and deJong M (2000) Peptide receptor imaging and therapy. *J Nucl Med* **41:**1704–1713.
Langer M and Beck-Sickinger AG (2001) Peptides as carrier for tumor diagnosis and treatment. *Curr Med Chem Anticancer Agents* **1:**71–93.
Lango MN, Dyer KF, Lui VW, Gooding WE, Gubish C, Siegfried JM, and Grandis JR (2002) Gastrin-releasing peptide receptor-mediated autocrine growth in squamous cell carcinoma of the head and neck. *J Natl Cancer Inst* **94:**375–383.
Leban JJ, Kull FC Jr, Landavazo A, Stockstill B, and McDermed JD (1993) Development of potent gastrin-releasing peptide antagonists having a D-Pro-psi (CH2NH)-Phe-NH_2 C terminus. *Proc Natl Acad Sci USA* **90:**1922–1926.
Mahmoud S, Staley J, Taylor J, Bogden A, Moreau JP, Coy D, Avis I, Cuttitta F, Mulshine J, and Moody TW (1991) [Psi 13, 14] bombesin analogues inhibit growth of small cell lung cancer in vitro and in vivo. *Cancer Res* **51:**1798–1802.
Mantey SA, Weber HC, Sainz E, Akeson M, Ryan RR, Pradhan T, Searles RP, Spindel ER, Battey JF, Coy DH, et al. (1997) Discovery of a high affinity radioligand for the human orphan receptor, bombesin receptor subtype 3, which demonstrates it has a unique pharmacology compared with other mammalian bombesin receptors. *J Biol Chem* **272:**26062–26071.
Marquez C, Treston A, Moody E, Jakowlew S, and Moody TW (2000) The metabolism of BW2258U89, a GRP receptor antagonist. *Neuropeptides* **34:**108–115.
Moody TW, Crawley JN, and Jensen RT (1982) Pharmacology and neurochemistry of bombesin-like peptides. *Peptides* **3:**559–563.
Moody TW, Fuselier J, Coy DH, Mantey S, Pradhan T, Nakagawa T, and Jensen RT (2005) Camptothecin-somatostatin conjugates inhibit the growth of small cell lung cancer cells. *Peptides* **26:**1560–1566.
Moody TW, Mantey SA, Pradhan TK, Schumann R, Nakagawa T, Martinez A, Fusilier J, Coy DH, and Jensen RT (2004) Development of high affinity camptothecin-bombesin conjugates that have targeted cytotoxicity for bombesin receptor-containing tumor cells. *J Biol Chem* **279:**23580–23589.
Orlando C, Raggi CC, Bagnoni L, Sestini R, Briganti V, LaCara G, Bernini G, Tonini GP, Pazzagli M, Serio M, et al. (2001) Somatostatin receptor type 2 gene expression in neuroblastoma, measured by competitive RT-PCR, is related to patient survival and to somatostatin receptor imaging by indium-111-pentetreotide. *Med Pediatr Oncol* **36:**224–226.
Preston SR, Woodhouse LF, Jones-Blackett S, Wyatt JI, and Primrose JN (1993) High affinity binding sites for gastrin releasing peptide on human gastric cancer and Menetrier's mucosa. *Cancer Res* **53:**5090–5092.
Reubi JC (2003) Peptide receptors as molecular targets for cancer diagnosis and therapy. *Endocr Rev* **24:**389–427.
Reubi JC, Wenger S, Schumuckli-Maurer J, Schaer JC, and Gugger M (2002) Bombesin receptor subtypes in human cancers: detection with the universal radioligand (125)I-[D-TYR(6), beta-ALA(11),PHE(13), NLE(14)] bombesin(6–14). *Clin Cancer Res* **8:**1139–1146.
Rivier JE and Brown MR (1978) Bombesin, bombesin analogues and related peptides: effects on thermo regulation. *Biochemistry* **17:**1766–1771.
Rogers BE, Zinn KR, Lin CY, Chaudhuri TR, and Buchsbaum DJ (2002) Targeted radiotherapy with [(90)Y]-SMT 487 in mice bearing human nonsmall cell lung tumor xenografts induced to express human somatostatin receptor subtype 2 with an adenoviral vector. *Cancer* **94:**1298–1305.
Schally AV and Nagy A (1999) Cancer chemotherapy based on targeting of cytotoxic peptide conjugates to their receptors on tumors. *Eur J Endocrinol* **141:**1–14.
Schally AV and Nagy A (2003) New approaches to treatment of various cancers based on cytotoxic analogs of LHRH, somatostatin and bombesin. *Life Sci* **72:**2305–2320.
Schulz S, Helmholz T, Schmitt J, Franke K, Otto HJ, and Weise W (2002) True positive somatostatin receptor scintigraphy in primary breast cancer correlates with expression of sst2A and sst5. *Breast Cancer Res Treat* **72:**221–226.
Shipp MA, Tarr GE, Chen CY, Switzer SN, Hersh LB, Stein H, Sunday ME, and Reinherz EL (1991) CD10/neutral endopeptidase 24.11 hydrolyzes bombesin-like peptides and regulates the growth of small cell carcinomas of the lung. *Proc Natl Acad Sci USA* **88:**10662–10666.
Siegfried JM, Krishnamachary N, Davis AG, Gubish C, Hunt JD, and Shriver SP (1999) Evidence for autocrine actions of neuromedin B and gastrin-releasing peptide in non-small cell lung cancer. *Pulm Pharmacol Ther* **12:**291–302.
Sun L, Fuselier JA, and Coy DH (2004) Effects of camptothecin conjugated to a somatostatin analog vector on growth of tumor cell lines in culture and related tumors in rodents. *Drug Deliv* **11:**231–238.
Sun L, Fuselier JA, Murphy WA, and Coy DH (2002) Antisense peptide nucleic acids conjugated to somatostatin analogs and targeted at the n-myc oncogene display enhanced cytotoxicity to human neuroblastoma IMR32 cells expressing somatostatin receptors. *Peptides* **23:**1557–1565.
Sun B, Halmos G, Schally AV, Wang X, and Martinez M (2000a) Presence of receptors for bombesin/gastrin-releasing peptide and mRNA for three receptor subtypes in human prostate cancers. *Prostate* **42:**295–303.
Sun B, Schally AV, and Halmos G (2000b) The presence of receptors for bombesin/GRP and mRNA for three receptor subtypes in human ovarian epithelial cancers. *Regul Pept* **90:**77–84.
Virgolini I (1997) Receptor nuclear medicine: vasointestinal peptide and somatostatin receptor scintigraphy for diagnosis and treatment of tumour patients. *Eur J Clin Investig* **27:**793–800.

Address correspondence to: Dr. Terry W. Moody, Department of Health and Human Services/National Institutes of Health, National Cancer Institute, Office of the Director, Building 31, Room 4A48, 31 Center Drive, Bethesda, MD 20892. E-mail: moodyt@mail.nih.gov