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Development of High Affinity Camptothecin-Bombesin Conjugates That Have Targeted Cytotoxicity for Bombesin Receptor-containing Tumor Cells*

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Mammalian bombesin (BN) receptors are among those most frequently overexpressed by a number of common tumors including prostate, breast, lung, and colon cancers. The aim of this study was to develop a camptothecin-bombesin (CPT-BN) conjugate that interacts with all classes of BN receptors and possibly functions as a prodrug via a labile linker with site-specific cytotoxicity for cancer cells bearing these receptors. CPT was coupled to analogs of [D-Tyr^6,β-Ala^{11},Phe^{13},Nle^{14}]BN-(6–14) (BA0) using carbamate linkers (L1 and L2) with built-in nucleophile-assisted releasing groups for intracellular cleavage of free cytotoxic agents. One conjugate, CPT-L2-BA3, bound to all three BN receptor classes with high affinity and functioned as a full agonist at each. ^{125}I-CPT-L2-BA3 was rapidly internalized by cells expressing each BN receptor class and, using fluorescent imaging, was found to co-localize with BN receptors initially and later to be internalized in cytoplasmic compartments. HPLC analysis of internalized ligand showed that 40% was intact, 25% was metabolized by releasing free CPT, and 35% was metabolized to other breakdown products. CPT-L2-BA3 inhibited the growth of NCI-H1299 non-small cell lung cancer cells in 3-(4,5-dimethylthiazol-2-yl)-2.5-diphenyl-2H-tetrazolium bromide (MTT) and clonal growth assays. CPT-L2-BA3 was cytotoxic in an MTT assay for cells transfected with each class of BN receptor; however, it had significantly less effect in cells lacking BN receptors. These results indicate that CTP-L2-BA3 is a potent agonist that is cytotoxic for cells overexpressing any of the three BN receptor classes and functions as a prodrug for receptor-mediated cytotoxicity. It therefore should be a useful prototype to explore the effectiveness of tumor-specific cytotoxicity delivery using a receptor-mediated mechanism.

In order to enhance tumor cytotoxicity and decrease side effects, there has been increased interest in the development of prodrugs that improve site-specific delivery of cytotoxic anticancer agents (1, 2). Prodrug conjugates have been described utilizing antibodies directed against specific tumor-associated antigens, hydrophilic polymers, and peptide or steroid hormones that interact with receptors overexpressed or ectopically expressed on the tumor (1, 2). The goal of the prodrug is to deliver the therapeutic agent to the target cell, at which time a tumor-specific process (enzyme activity, specific cellular degradation, etc.) will site-specifically release the active drug (1). For peptide hormone receptor prodrugs, this requires coupling of the cytotoxic agent to a peptide hormone through a coupling mechanism that retains high affinity for the peptide hormone receptor and allows the cytotoxic drug to be released after receptor specific internalization and degradation (1, 3, 4). Peptide receptor ligands as vehicles to deliver cytotoxic agents are receiving considerable attention, because numerous studies using imaging methods or autoradiographic methods have shown many common malignant tumors either ectopically expressing large numbers of peptide receptors (4–9) or overexpressing them, thus allowing enhanced uptake of selective ligands for these receptors (6, 7).

The mammalian bombesin (BN)[1] family of receptors (gastrin-releasing peptide receptor (GRPR), neuromedin B receptor (NMBR), and the orphan receptor, bombesin receptor subtype 3 (BRS3)) are excellent candidates for possibly targeting cytotoxic agents to malignant neoplasms. Not only do many common tumors frequently possess and overexpress these receptors, but the BN family of receptors is one of the receptor families most frequently expressed by tumors, and the naturally occurring agonists for these receptors function as autocrine growth factors (7, 8, 10). BN receptors have been detected on 40–100% of prostate cancer, breast cancer, lung cancer, gastric cancer, malignant gliomas, colon cancer, and ovarian cancer (5).

Recently, a synthetic analog of BN has been described, [D-Tyr^6,β-Ala^{11},Phe^{13},Nle^{14}]BN-(6–14) (BA0), which functions as a universal ligand for all three mammalian BN receptors (11, 12). This analog binds with high affinity to each of the three BN receptor classes and is rapidly internalized by each class of BN receptors (11, 12). This is an important property because different tumors may possess different BN receptor

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[1] The abbreviations used are: BN, bombesin; CPT, camptothecin; GRP, gastrin-releasing peptide; BRS3, bombesin receptor subtype 3; hBRS3, human BRS3; GRPR, gastrin-releasing peptide receptor; hGRPR, human GRPR; NMBR, neuromedin B receptor; hNMBR, human NMBR; IP, inositol phosphate; NMB, neuromedin B; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; NSCLC, non-small cell lung cancer; BINAR, built-in nucleophile-assisted releasing; HA, hemagglutinin; TRITC, tetramethylrhodamine isothiocyanate; HPLC, high pressure liquid chromatography.

classes (5), and this ligand would still interact with high affinity with each. Furthermore, recently, a new novel carbamate linker system (3) has been described that allows the conjugation of a peptide to either the topoisomerase I inhibitor, camptothecin (CPT), or the tubulin-binding agent, combretastatin. This novel linker system (3) contains a built-in nucleophile-assisted releasing (BINAR) group that enables fine timing of intracellular cleavage rates of free cytotoxic agents containing reactive hydroxyl groups such as CPT or combretastatin (3).

In the present study, we have synthesized a number of CPT analogs coupled to universal bombesin agonists through different carbamate linkers (L1 and L2) to identify a potential general BN receptor cytotoxic prodrug. We have identified one analog, CPT-L2-BA3, which shows high affinity for all three mammalian BN receptors, is fully biologically active at each receptor, and is rapidly internalized by each receptor subtype. Furthermore, CPT-L2-BA3 is cytotoxic for NCI-H1299 non-small cell lung cancer (NSCLC) cells, which possess native GRP receptors (13) and demonstrate greater cytoxicity for Balb/c 3T3 cells containing GRP receptors than those lacking these receptors, suggesting site-selective cytotoxicity.

EXPERIMENTAL PROCEDURES

Materials—The following cells and materials were obtained from the sources indicated: Balb/c 3T3 (mouse fibroblast), NCI-H345 (human small cell lung cancer), NCI-H1299 (human NSCLC), and HEK 293 (human embryonic kidney) cells from ATCC (Manassas, VA); Dulbecco's minimum essential medium, phosphate-buffered saline, RPMI 1640, trypsin-EDTA, and fetal bovine serum from Biofluids (Rockville, MD); G418 sulfate from Invitrogen; $Na^{125}I$ (2200 Ci/mmol) and myo-[2-^{3}H]-inositol (20 Ci/mmol) from Amersham Biosciences; 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, protease inhibitor mixture, and Triton X-100 from Sigma; 1,2,4,6-tetrachloro-3α,6α-diphenylglycouril (IODO-GEN) from Pierce; Fura-2/AM from Calbiochem; AG 1-X8 resin from Bio-Rad; BN, GRP, NMB, and [Tyr^4]BN from Bachem (Torrence, CA); paraformaldehyde (16% stock solution) from Electron Microscopy Sciences (Ft. Washington, PA); bovine serum albumin from ICN Pharmaceutical Inc. (Aurora, OH); HA antibody from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); secondary antibodies and donkey serum from Jackson ImmunoResearch Laboratories, West Grove, PA; and Vectorshield from Vector (Burlingame, CA). The mammalian expression vector, pcDNA3, custom primers, and restriction endonucleases (BamHI, HindIII, XbaI, and EcoRI) were from Invitrogen. The ExSite PCR-based site-directed mutagenesis kit was from Stratagene (La Jolla, CA).

Cell Culture—NCI-H345 and NCI-H1299 cells were cultured in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum (Invitrogen). NCI-H1299 cells were split weekly 1:20 with trypsin-EDTA. NCI-H345 cells were diluted 1:1 into new media. Balb/c 3T3 cells stably expressing human BRS3 receptors (hBRS3), human NMB receptors (hNMBR), or human GRP receptors (hGRPR) made as described previously (11, 14, 15) were grown in Dulbecco's modified Eagle's cell medium supplemented with 300 mg/liter G418 sulfate. HEK 293 cells were cultured in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. The cells (Balb/c 3T3 (mouse fibroblast), NCI-H345 (human SCLC), NCI-H1299 (human NSCLC), and HEK 293 (human embryonic kidney) cells were from ATCC, were mycoplasma-free, and were used when they were in the exponential growth phase after incubation at 37 °C in 5% CO_2, 95% air.

Preparation of Peptides—The peptide portions of the peptide-cytotoxin conjugates were synthesized by solid phase methods using the standard Fmoc (*N*-(9-fluorenyl)methoxycarbonyl) protection strategy (3). Sequences were assembled on Rink amide resin (Advanced ChemTech, Louisville, KY), and Trt group protection was employed for the imidazole NH of His, Boc for the indole NH of Trp, and *tert*-butyl group for the OH groups of Ser and Tyr. Bromoacetic acid was coupled to the N terminus of the protected peptide, which was then reacted with a 5 M excess of tertiary butyloxycarbonyl-1-*N*-methylamino,2-aminoethane (2 h). CPT (Roche Applied Science) was activated at its position 20 OH group by treating a suspension in anhydrous dichloromethane with dimethyaminopyridine (4 eq) and a 20% solution of phosgene in toluene (4 eq) (4 h) followed by removal of excess solvent and phosgene *in vacuo*. A 3 M excess of the resulting yellow oil was added to the above resin in anhydrous dichloromethane and allowed to react overnight. After washing and drying of the resin, the camptothecin-peptide conjugate was cleaved using the standard acid mixture, trifluoroacetic acid/H_2O/ethanedithiol/triisopropylsilane, 95:2:2:1 (2 h), followed by evaporation of the trifluoroacetic acid and precipitation and washing of the peptide with ether. Conjugates were purified to >97% purity by preparative HPLC (C_{18} silica) and characterized by mass spectrometry and amino acid analysis. For the compounds shown in Fig. 1, BA0 had a calculated mass of 1133.3 and was found to have a mass of 1132.7; CPT-L1-BA1 had a calculated mass of 1661.8 and was found to have a mass of 1666.3; CPT-L2-BA1 had a calculated mass of 1675.5 and was found to have a mass of 1676.1; CPT-L1-BA2 (mixture of polymers) had an average mass of 3000; and CPT-L2-BA3 had a calculated mass of 1709.9 and was found to have a mass of 1709.1.

Preparation of ^{125}I-BA0, ^{125}I-L2-BA3, ^{125}I-CPT-L2-BA3, ^{125}I-[D-Tyr^0]NMB, and ^{125}I-[Tyr^4]BN—These radioligands, with specific activities of 2200 Ci/mmol, were prepared as previously described (11, 12) or as described below. Briefly, 0.8 μg of IODO-GEN solution (0.01 μg/μl in chloroform) was added to a 5-ml plastic test tube, dried under nitrogen, and washed with 100 μl of 0.5 M potassium phosphate solution (pH 7.4 except for ^{125}I-L2-BA3 and ^{125}I-CPT-L2-BA3, which required pH 7.0). To this tube, 20 μl of potassium phosphate of the appropriate pH, 8 μg of peptide in 4 μl of water, and 2 mCi (20 μl) of $Na^{125}I$ were added and incubated for 6 min at room temperature ($Na^{125}I$ (2200 Ci/mmol) and myo-[2-^{3}H]inositol (20 Ci/mmol) were from Amersham Biosciences). The incubation was stopped with 300 μl of water. For ^{125}I-[D-Tyr^0]NMB and ^{125}I-[Tyr^4]BN, which have COOH-terminal methionine groups, 300 μl of 1.5 M dithiothreitol was added and heated for 1 h at 80 °C. 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 and binding were neutralized with 0.2 M Tris buffer (pH 9.5) and stored with 0.5% bovine serum albumin (w/v) at −20 °C.

Binding of ^{125}I-Labeled BN-related Peptides to Various Cells—Binding was performed as described previously (11, 12, 14). 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). Balb/c 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 50 pM ^{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 $10{,}000 \times g$ for 1 min using a Beckman Microfuge B. The pellets were washed twice with buffer and counted for radioactivity in a γ 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. Receptor affinities (K_i) were determined using a least-square, curve-fitting program (KaleidaGraph) and the Cheng-Prusoff equation (16).

Internalization—Balb/c 3T3 cells stably transfected with hGRPR, hNMBR, or hBRS3 were incubated with radioligands as stated under "Experimental Procedures." Internalization experiments were performed as described previously (14, 15). Briefly, 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). The internalization of ^{125}I-CPT-BN conjugates was investigated in human NCI-H1299 NSCLC cells that naturally possess hGRPR (13). ^{125}I-CPT-L2-BA3 (50 pM) was incubated with NCI-H1299 NSCLC cells for 2 h at 4 °C. Two sets of cells were washed three times in receptor binding buffer to remove free peptide. One set was then treated with 0.5 M acetic acid and 0.15 M NaCl for 5 min at 4 °C to remove peptide bound to the cell surface. The supernatant was counted in a γ counter. A second set of cells was treated with 0.2 N NaOH to determine total binding at 4 °C. Also, two sets of cells were incubated at 37 °C for 5 min and washed

three times in receptor binding buffer to remove free peptide. One set was then treated with 0.5 M acetic acid and 0.15 M NaCl for 5 min at 4 °C to remove peptide bound to the cell surface. The supernatant was counted in a γ counter. A second set of cells was treated with 0.2 N NaOH to determine total binding at 37 °C.

Characterization of Internalized Radioactivity—Balb/c 3T3 cells with no transfected bombesin receptors and Balb/c 3T3 cells stably transfected with hGRPR (2×10^6/ml) were incubated at 37 °C for 15 min with 0.4 nM ^{125}I-CPT-L2-BA3 in standard binding buffer containing 25 μg/ml leupeptin, 0.02% (w/v) bacitracin, 10 μg/ml antipain, 50 μg/ml 4-(2-aminoethyl)-benzenesulfonyl fluoride, and 2 μl/ml of protease inhibitor mixture (Sigma). Cells were then washed with standard binding buffer with protease inhibitors at 4 °C and underwent acid stripping to remove surface-bound ligand performed for 5 min at 4 °C with 0.2 M acetic acid (pH 2.5) and 0.5 M NaCl, as described under "Internalization." After centrifugation, cells were resuspended in 1 ml of standard binding buffer with protease inhibitors containing 1% Triton X-100 (pH 6), and the resuspension was sonicated to solubilize the radioactivity. Nine hundred μl of the sonicated mixture was injected on an HPLC (Waters model 510, Milford, MA) equipped with a C-18 column and eluted with a linear gradient increasing at 0.8% acetonitrile in trifluoroacetic acid (0.1%) per min starting at 12% acetonitrile and ending at 80%. One-ml fractions were collected, and radioactivity in each fraction was determined by counting in a Packard γ counter.

Measurement of [^{3}H]Inositol Phosphates ([^{3}H]IP)—Changes in total [^{3}H]IP were measured as described previously (14, 15). Briefly, hBRS3-, hGRPR-, or hNMBR-transfected Balb/c 3T3 cells were subcultured into 24-well plates (5×10^4 cells/well) in regular propagation medium 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 growth medium supplemented with 2% fetal bovine serum for an additional 24 h. Before assay, the 24-well plates were washed by incubating for 30 min at 37 °C with 1 ml/well of phosphate-buffered saline (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, 0.05% bovine serum albumin (w/v) 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. Total [^{3}H]IP was isolated by anion exchange chromatography as described previously (14, 15). Briefly, samples were loaded onto Dowex AG1-X8 anion exchange resin columns, washed with 5 ml of distilled water to remove free [^{3}H]inositol, and then washed with 2 ml of 5 mM disodium tetraborate, 60 mM sodium formate solution to remove [^{3}H]glycerophosphorylinositol. Two ml of 1 mM ammonium formate, 100 mM formic acid solution were added to the columns to elute total [^{3}H]IP. Each eluate was mixed with scintillation mixture and measured for radioactivity in a scintillation counter.

Cytosolic Calcium [Ca^{2+}]$_i$ Measurement—The ability of the CPT-BN conjugates to alter cytosolic [Ca^{2+}]$_i$ was investigated as described previously (17). NCI-H1299 cells and NCI-H345 cells were harvested (2.5×10^6/ml) and incubated with 5 μM Fura-2/AM at 37 °C for 30 min. The cells, which contained loaded Fura-2, were centrifuged at $1500 \times g$ for 10 min and resuspended at the same concentration in new SIT medium (RPMI 1640 containing 30 nM sodium selenite, 5 μg/ml bovine insulin, and 10 μg/ml transferrin). After two washes with the same medium, cells were placed in a Delta PTI Scan 1 spectrofluorometer (Photon Technology International, South Brunswick, NJ) equipped with a magnetic stirring mechanism and temperature (37 °C)-regulated cuvette holder. The fluorescence intensity was continuously monitored at dual excitation wavelengths of 340 and 380 nm, using an emission wavelength of 510 nm prior to and after the addition of BN-like peptides.

Proliferation Assays—Growth studies were performed using the 3-(4,5-dimethylthiazol-2-yl)-2.5-diphenyl-2H-tetrazolium bromide (MTT) assay. Growth studies *in vitro* were conducted using MTT (Sigma) colorimetric assays. NCI-H1299 cells, hGRPR-transfected Balb/c 3T3 cells, or Balb/c 3T3 cells (10^4/well) were placed in SIT medium (100 μl), and various concentrations of CPT-L2-BA3 were added. After 4 days, 15 μl (1 mg/ml) of MTT was added, and after another 4 h, 150 μl of Me_2SO was added. After 16 h, the optical density at 570 nm was determined using an ELISA reader. The proliferation rates were calculated from the OD readings with various concentrations of CPT-L2-BA3 using the untreated cells as 100%.

Clonogenic Assays—The effects of CPT-BN-containing peptides on the growth of NCI-H1299 and NCI-H345 cells were investigated using a clonogenic assay (18). The base layer consisted of 3 ml of 0.5% agarose in SIT medium containing 5% fetal bovine serum in 6-well plates. The top layer consisted of 3 ml of SIT medium in 0.3% agarose (FMC Corp., Rockford, ME), CPT-BN conjugates, and 5×10^4 NCI-H1299 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.

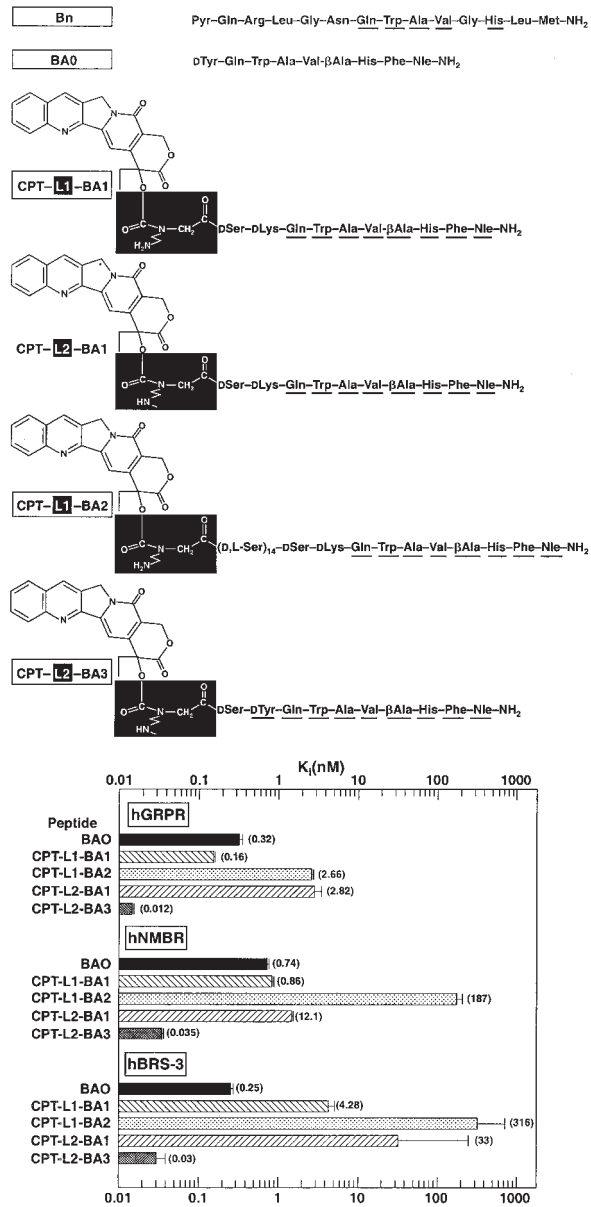

FIG. 1. **Structures of BN, various synthetic BN analogs, their CPT conjugates studied, and their affinities for human BN receptors.** *Top*, the synthetic BN analog, BA0, has been shown to be a universal high affinity ligand for all known mammalian BN receptors (5, 11, 12) and was used as the starting peptide to make other BN-related peptides (BA1, BA2, and BA3). These peptides were coupled through nucleophilic carbamate linking groups based on either *N*-aminoethyl-glycine (L1) or the more labile *N*-(*N*-Me-aminoethyl)-glycine (L2) (3) (shown in *black*) to camptothecin. Amino acid identities to BA0 in the various peptides are *underlined*. *Bottom*, Balb/c 3T3 cells stably transfected with hGRPR (0.3×10^6 cells/ml) (14), hNMBR (0.03×10^6 cells/ml) (14), or hBRS3 (0.3×10^6 cells/ml) were incubated for 60 min at 22 °C with 50 pM ^{125}I-[Tyr^4]BN, ^{125}I-[D-Tyr^0]NMB, or ^{125}I-BA0, respectively, with concentrations of unlabeled peptides from 0.01 nM to 1 μM. 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 (16). All values are means ± S.E. from at least three experiments, and in each experiment each value was determined in duplicate. Values in *parenthesis* are the K_i value in nM for the indicated BN analog.

TABLE I

The affinities and potencies of various synthetic BN analogs and their camptothecin conjugates for human BN receptors

Balb/c 3T3 cells stably expressing hGRPR (0.3×10^6 cells/ml), hNMBR (0.03×10^6 cells/ml), or hBRS3 (0.3×10^6 cells/ml) were incubated with 50 pM iodinated ligand, with or without increasing concentrations of unlabeled ligand for 60 min at 22 °C as described in the legend to Fig. 2. The affinities were calculated using the Cheng-Prusoff equation (16). To assess phospholipase C activation, the Balb/c 3T3 cells stably transfected with hGRPR, hNMBR, or hBRS3 were incubated with [^{3}H]inositol, and total [^{3}H]IP was determined as stated in the legend to Fig. 2. A dose-response curve was determined for each ligand with concentrations of 0.001 nm to 1 m. For each ligand, a concentration causing a half-maximal increase (EC_{50}) was calculated using KaleidaGraph. Each value is a mean ± 1 S.E. from at least three experiments.

BN receptor cells	Peptide	K_i for ^{125}I-ligands				EC_{50} for [^{3}H]IP
		^{125}I-BA0	^{125}I-L2-BA3	^{125}I-CPT-L2-BA3	^{125}I-[Tyr^4]BN	
		nM				*nM*
hGRP	BA0	0.32 ± 0.012	0.74 ± 0.05	0.44 ± 0.11	0.19 ± 0.14	2.01 ± 0.01
	L2-BA3	0.22 ± 0.013	0.14 ± 0.01	0.22 ± 0.02	0.18 ± 0.02	0.59 ± 0.03
	CPT-L2-BA3	0.012 ± 0.001	0.006 ± 0.001	0.028 ± 0.007	0.009 ± 0.001	0.78 ± 0.13
hNMBR	BA0	0.74 ± 0.05	1.74 ± 0.09	2.0 ± 0.05	2.2 ± 0.18	2.39 ± 0.21
	L2-BA3	0.14 ± 0.01	0.38 ± 0.01	0.50 ± 0.10	0.59 ± 0.04	0.91 ± 0.07
	CPT-L2-BA3	0.035 ± 0.003	0.09 ± 0.03	0.035 ± 0.01	0.21 ± 0.02	1.66 ± 0.13
hBRS3	BA0	0.25 ± 0.10	0.45 ± 0.04	0.47 ± 0.03	No binding	1.02 ± 0.01
	L2-BA3	0.42 ± 0.03	1.0 ± 0.1	1.2 ± 0.02	No binding	0.79 ± 0.03
	CPT-L2-BA3	0.031 ± 0.008	0.21 ± 0.05	0.25 ± 0.14	No binding	0.62 ± 0.10

Preparation of HA-GRPR—The cDNA of the mouse GRPR used was identical to that described previously (14, 19). A sequence encoding the HA epitope tag (YPYDVPDYA) was inserted between the first (Met) and second (Ala) amino acid residue of the GRPR using the ExSite PCR-based site-directed mutagenesis kit, following the manufacturer's instructions. Nucleotide sequence analysis of the entire coding region was performed using an automated DNA sequencer (ABI Prism 377 DNA sequencer; Applied Biosystems Inc., Foster City, CA).

Fluorescent Microscopy of GRPR-bound CPT-L2-BA3—HEK 293 cells were seeded on 6-well plates at a density of 0.5×10^6 cells/well. On the following day, cells were transfected using 3 μl of Fugene 6 reagent and 1 μg of HA-GRPR-pcDNA3 following the manufacturer's protocol. One day after transfection, the cells were trypsinized and plated on polylysine-coated glass coverslips in 12 wells at a density of 100,000 cells/well. Two days later, cells were washed with phosphate-buffered saline and treated with or without the CPT-L2-BA3 (3 nM) in Dulbecco's modified Eagle's medium for various incubation times at 37 °C. The cells were washed three times in buffer to remove the CPT-L2-BA3. The cells containing bound CPT-L2-BA3 were fixed (paraformaldehyde 4%, 10 min, 22 °C), permeabilized (0.5% Triton X-100, 5 min, 22 °C), and blocked (2% bovine serum albumin, 2% donkey serum in phosphate-buffered saline, 30 min, 22 °C). Cells were stained with a polyclonal rabbit anti-bombesin antibody (1:500 dilution) as first antibody and a fluorescein isothiocyanate-labeled donkey anti-rabbit antibody antibody (1:200 dilution) as secondary antibody. The HA-GRPR was stained using a mouse anti-HA antibody (1:100 dilution) as first antibody and a TRITC-labeled donkey anti-mouse antibody (1:200 dilution) as the secondary antibody. Nuclei were visualized after 4′,6-diamidino-2-phenylindole counterstaining. Coverslips were mounted using Vectorshield and fixed on glass slides with nail polish. Imaging was done using a Nikon fluorescent microscope.

RESULTS

CPT-L2-BA3 Binds with High Affinity to hGRPR, hNMBR, and hBRS3 and Has Biological Activity—In developing an agonist that could be coupled to cytotoxic compounds and still bind with high affinity to human BN receptors, we started with the standard BN analog, BA0 (Fig. 1, *top*). Previous studies by us (11, 12) and others (5) have demonstrated that this synthetic BN ligand is a universal high affinity agonist for all human BN receptor subtypes as well as the amphibian BN receptor, BB4. Additionally, a new carbamate linker system was utilized, which consisted of a series of BINAR groups, which allows more facile intracellular cleavage of free cytotoxic agents containing reactive OH groups (3). This BINAR linker system, when coupled to CPT or combretastatin, allows adjustable rate release of the free cytotoxic agent (3). In the present study, we coupled analogs of the BN receptor universal agonist, BA0, to the two BINAR linkers, which were found previously to have stabilities within a useful range and display the greatest cytotoxicity with camptothecin when coupled to somatostatin analogs (3) (Fig. 1, *top*). These consisted of an ethylenediamine linker (L1) and *N*-methylenediamine linker (L2) (Fig. 1, *top*). Each of these BINAR linkers was coupled to the tertiary hydroxyl group in ring position 20 of CPT (Fig. 1, *top*) and the NH_2 termini of analogs of the BN receptor universal agonist BA0 in which various polar sequences were added in its NH_2 terminus (Fig. 1, *top*). BA0 bound with high affinity to 3T3 cells stably transfected hGRP, hNMBR, and hBRS3 with K_i values of 0.32, 0.74, and 0.25 nM, respectively (Fig. 1, *bottom*; Table I). Each of the CTP-BN-conjugates was then examined for the ability to interact with the three human BN receptor subtypes (*i.e.* hGRPR, hNMBR, and hBRS3) (Fig. 1, *bottom*). For each of these three human BN receptor subtypes, only CTP-L2-BA3 retained the high affinity seen with the BA0 analog for all three human BN receptor subtypes (Fig. 1, *bottom*; Table I). In fact, CPT-L2-BA3 had a 30-fold higher affinity than BA0 for human GRPR (K_i of 0.012 ± 0.002 *versus* 0.32 ± 0.02) (Fig. 1, *bottom*; Table I), a 21 times higher affinity for the human NMBR (K_i of 0.035 ± 0.003 *versus* 0.74 ± 0.05) (Fig. 2, Table I), and an 8-fold higher affinity than BA0 for the human BRS3-containing cells (K_i of 0.03 ± 0.01 *versus* 0.25 ± 0.01) (Fig. 1, *bottom*; Table I). In contrast, CPT-L1-BA2 and CPT-L2-BA1 had 8-fold lower affinities than BA0 for hGRPR; 252- and 16-fold lower, respectively, for the hNMBR; and 17- and 1260-fold lower, respectively, for hBRS-3. The CPT-L1-BA1 analog retained equal high affinity to BA0 for the hGRPR and hNMBR but showed a 17-fold lower affinity than BA0 for hBRS-3 (Fig. 1, *bottom*; Table I). Because of its high affinity for all three BN receptor subtypes, we selected CPT-L2-BA3 as the CPT-BN conjugate for full characterization in this study.

In comparing the effect on receptor affinity of the addition of CPT with the effect of the addition of the L2 linker alone to the lead compound, BA0, two different types of pharmacological studies were performed (Table I). First, the ability of unlabeled BA0, the L2-BA3 analog, and CPT-L2-BA3 to inhibit binding to Balb/c 3T3 cells containing hGRPR, hNMBR, and hBRS-3 was determined (Table I). For all three BN receptor subtypes, CPT-L2-BA3 was the most potent in inhibiting binding (Table I). Second, we examined the pharmacology of the radiolabeled analogs of each of these BN-related peptides, because with some peptides, insertions of a radiolabel can alter the pharmacology from the unlabeled compound (20) (Table I). Each of the three peptides (*i.e.* BA0, L2-BA3, and CPT-L2-BA3) was iodinated on the tyrosine residue (Fig. 1), and their affinity for the hGRPR was determined (Fig. 2, Table I) as well as for both hNMBR and hBRS3 (Table I). Each of the radiolabeled compounds demonstrated specific binding to the hGRPR-containing cells, and the specific binding of ^{125}I-BA0, ^{125}I-L2-BA3, and

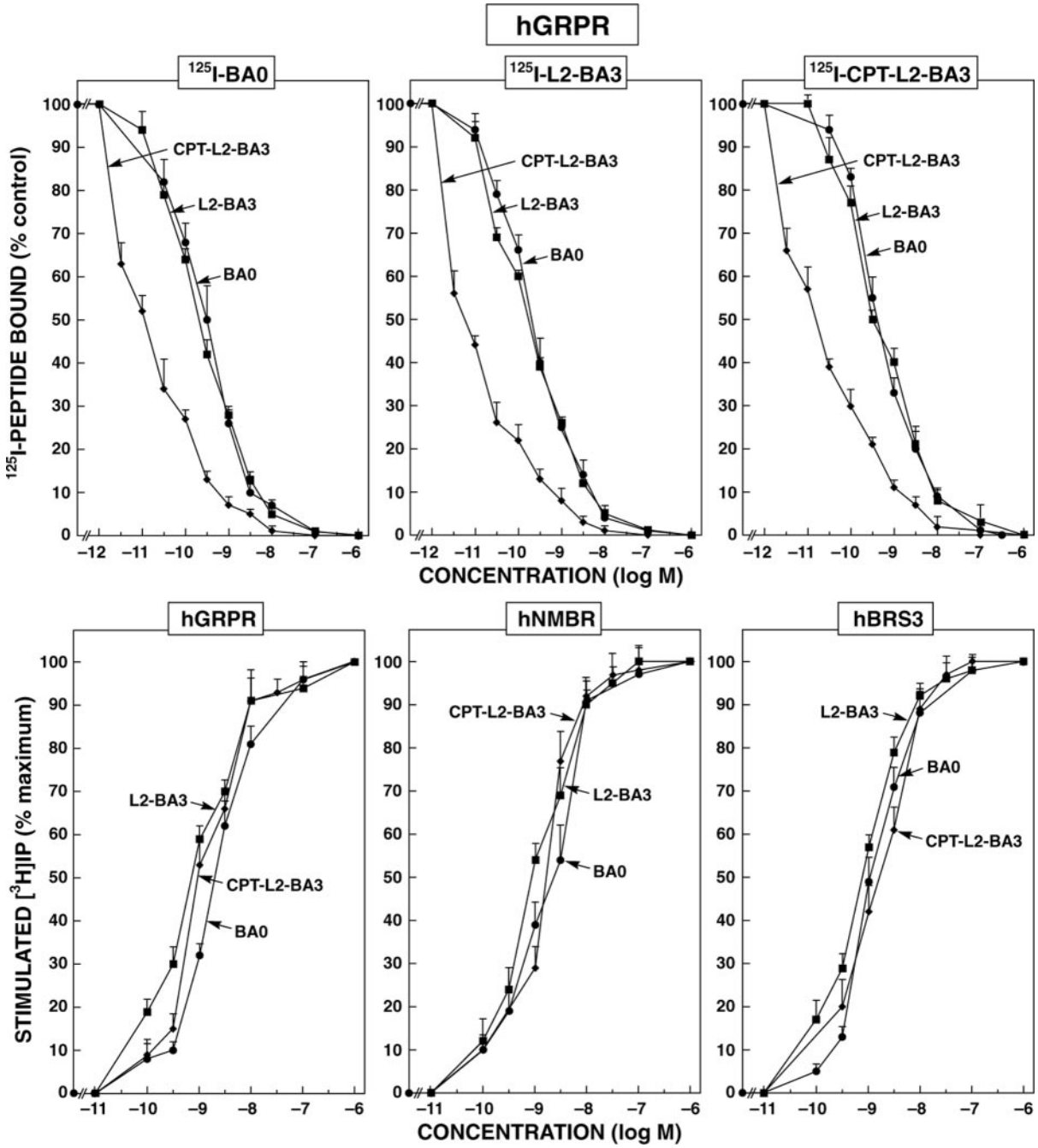

FIG. 2. **Comparison of the ability of BA0, L2-BA3 and CPT-L2-BA3 to inhibit binding of ^{125}I-BA0 (*top left panel*), ^{125}I-L2-BA3 (*top middle panel*), or ^{125}I-CPT-L2-BA3 (*top right panel*) to hBRPR cells or stimulate accumulation of [^{3}H]IP (*bottom panel*).** *Top panel*, Balb/c 3T3 cells stably transfected with hGRPR (0.3×10^6 cells/ml) were incubated for 60 min at 22 °C with 50 pM ^{125}I-BA0, ^{125}I-L2-BA3, or ^{125}I-CPT-L2-BA3 and the indicated concentrations of unlabeled peptides. Results are expressed as the percentage of saturable binding without unlabeled peptide. Results are means ± S.E. from at least three experiments, and in each experiment each data point was determined in duplicate. *Bottom panel*, hGRPR, hNMBR, and hBRS3 transfected Balb/c 3T3 cells in 24-well plates were labeled with myo-[2-^{3}H]inositol. The cells were incubated for 60 min at 37 °C with the indicated concentrations of the various peptides in IP assay buffer, and total [^{3}H]IP was determined as described under "Experimental Procedures." Results are expressed as the percentage of stimulation caused by a maximally effective concentration of agonist (*i.e.* 1 μM BA0). Results are means ± 1 S.E. of at least three experiments, and in each experiment, each value was determined in duplicate. Control and 1 μM BA0-stimulated values for hGRPR were 396 ± 50 and 6386 ± 818 cpm, respectively; for hNMBR cells, the values were 2566 ± 191 and 63076 ± 2636 cpm, respectively; and for hBRS3 cells, the values were 3076 ± 493 and 14,618 ± 432 cpm, respectively.

^{125}I-CPT-L2-BA3 were all inhibited in a similar manner, showing similar relative affinities of CPT-L2-BA3 > L2-BA3 ~ BA0 and similar absolute affinities to that seen with the native ligand, ^{125}I-[Tyr^4]BN (Table I). Similar results were seen with ^{125}I-BA0, ^{125}I-L2-BA3, and ^{125}I-CPT-L2-BA3 binding to hNMBR- and hBRS3-containing cells (Table I). With each of these human BN receptor subtypes, similar relative affinities of CPT-L2-BA3 > L2-BA3 ~ BA0 were obtained and similar absolute affinities (Table I). These pharmacologic studies demonstrate that each of these radioligands can bind to each of the human BN receptors and show similar relative affinities, with ^{125}I-CPT-L2-BA3 having the highest affinity for each human BN receptor subtype.

The biological activity of BA0, L2-BA3, and CTP-L2-BA3 was evaluated using Balb/c 3T3 cells transfected with BN receptors and loaded with [^{3}H]IP (Fig. 2, *bottom*). Each of the three

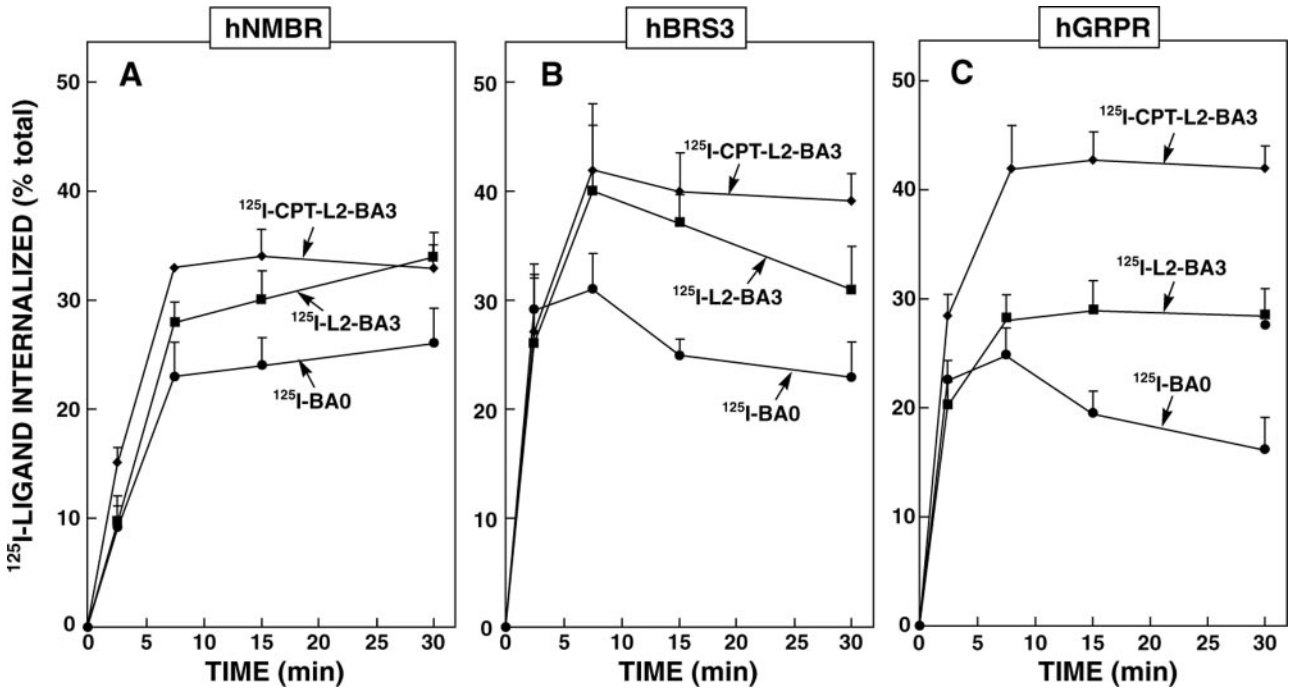

FIG. 3. **Ability of Balb/c 3T3 cells possessing various classes of mammalian BN receptors to internalize ^{125}I-BA0, ^{125}I-L2-BA3, or ^{125}I-CPT-L2-BA3.** hNMBR (0.03×10^6 cells/ml) (*A*), hBRS3 (0.3×10^6 cells/ml) (*B*), or hGRPR (0.3×10^6 cells/ml) (*C*) transfected Balb/c 3T3 cells were incubated with 50 pM ^{125}I-BA0, ^{125}I-L2-BA3, or ^{125}I-CPT-L2-BA3 for the indicated times. At each time point, the percentage of the indicated ^{125}I-ligand saturably bound that was internalized was determined using acid-stripping as outlined under "Experimental Procedures." Results are expressed as the percentage of the saturable ligand bound at each time point expressed as the percentage of total counts added to medium that was not removed by acid stripping (*i.e.* internalized). Results are means ± 1 S.E. of at least three experiments, and each point was determined in duplicate.

ligands (BA0, L2-BA3, and CTP-L2-BA3) were agonists, stimulating increases in [^{3}H]IP in a dose-dependent manner (Fig. 2, *bottom panel*). Each BN analog had similar efficacy, stimulating a 16-, 25-, and 5-fold increase in [^{3}H]IP at the hGRPR, hNMBR, and hBRS3, respectively. Each BN analog caused half-maximal stimulation in the nanomolar range for each of the BN receptor subtypes (Fig. 2, *bottom panel*). Specifically with the hGRPR, half-maximal stimulation of CPT-L2-BA3, L2-BA3, and BA0 occurred at 0.8–2 nM, and with the hNMBR cells and hBRS-3 cells the EC_{50} values ranged from 0.8 to 2.4 nM (Fig. 2, *bottom*; Table I). These results demonstrate that CPT-L2-BA3 had similar potency and efficacy for activating each of the three human BN receptor subtypes to either the parent compound BA0 or the BN linker analog L2-BA3.

CPT-L2-BA3 Is Internalized—The ability of each of the three radiolabeled BN analogs to be internalized by each of the three human BN receptor subtypes was examined (Fig. 3). With hNMBR cells, each of the three radioligands were rapidly internalized (Fig. 3*A*, Table II). ^{125}I-CPT-L2-BA3 was internalized more rapidly by hNMBR and demonstrated 1.3-fold greater maximal internalization than ^{125}I-BA0. Similar internalization data were obtained using Balb/c 3T3 cells containing hBRS3 (Fig. 3*B*, Table II). With hGRPR cells, ^{125}I-BA0, ^{125}I-L2-BA3, and ^{125}I-CPT-L2-BA3 were rapidly internalized with 20–30% of the ligand internalized after 2.5 min (Fig. 3*C*). For ^{125}I-CPT-L2-BA3, maximal internalization occurred at 15.3 ± 0.8 min with 42.7 ± 2.2% of the bound ligand internalized (Fig. 3*C*, Table II). ^{125}I-CPT-L2-BA3 demonstrated 1.8- and 1.5-fold greater maximal internalization by hGRPR cells than ^{125}I-BA0 or ^{125}I-L2-BA3 (Fig. 3*C*, Table II). These results indicate that ^{125}I-CPT-L2-BA3 is readily internalized by cells containing BN receptors at 37 °C.

To provide direct evidence for the internalization of CPT-L2-BA3 by GRP receptors, immunofluorescence microscopy techniques were used (Fig. 4). Using HEK cells transfected with an epitope-tagged GRPR (HA-GRPR), the receptor was localized (*red*) to the plasma membrane with no agonist present (Fig. 4*B*). Two min after CPT-L2-BA3 (10 nM) was added, both the GRP receptors (Fig. 4*E*) and BN-like immunoreactivity (*green*) (Fig. 4*D*), were localized primarily to the plasma membrane and were frequently co-localized (*yellow*) (Fig. 4*F*). Some of the GRP receptors (*red*), however, had already internalized after 2 min (Fig. 4, compare *B* and *E*). After a 20-min incubation with 10 nM CPT-L2-BA3, most of the GRP receptors were internalized (Fig. 4*H*). Similarly, the BB-like immunoreactivity had internalized into intracellular patches (Fig. 4*G*); however, there was minimal co-localization with BN-like immunoreactivity (Fig. 4*I*). These results show that after 20 min, the HA-GRP receptor (Fig. 4*I*) and CPT-BN conjugate have largely dissociated. These results demonstrate directly that the BN conjugate CPT-L2-BA3 is internalized by a GRPR-mediated process and accumulates rapidly in intracellular organelles after dissociating from the internalized GRPR.

To characterize the nature of the internalized radioactivity, Balb/c 3T3 cells not containing hGRPR and Balb/c 3T3 cells stably transfected with hGRPR were incubated with 0.4 nM ^{125}I-CPT-L2-BA3 for 15 min, and the surface-bound ligand was removed by acid stripping (data not shown). The internalized radioactivity was solubilized and sonicated and characterized by analyzing HPLC fractions. With the hGRP-containing Balb/c 3T3 cells, 40% of the internalized radioactivity eluted in the same fraction as intact ^{125}I-CPT-L2-BA3, 25% as ^{125}I-L2-BA3, and 35% as two uncharacterized metabolites (data not shown). In contrast, with the Balb/c 3T3 cells not containing hGRPRs, no internalization or metabolism of the ligand occurred. These results demonstrate that intact CPT-L2-BA3 is internalized and metabolized by releasing CPT and generating L2-BA3 as well as other metabolites. Furthermore, the presence of hGRPR is required for internalization and metabolism of CPT-L2-BA3.

TABLE II

Comparison of the kinetics and the maximum internalization of ^{125}I-BA0, ^{125}I-L2-BA3, or ^{125}I-CPT-L2-BA3 by human BN receptors

hGRPR-, hNMBR-, and hBRS3-transfected Balb/c 3T3 cells were incubated with the indicated ligands, and the rate and magnitude of internalization were determined. Results show the percentage of maximal saturable ligand binding at each time point that was not acid-stripped (*i.e.* maximum percentage internalized). T_{max} refers to the time in minutes for maximal internalization to occur. Results are from the data shown in Fig. 3 and are the mean ± S.E. of at least six experiments. Each point was determined in duplicate.

Ligand	hGRPR		hNMBR		hBRS	
	T_{max}	Maximum internalized	T_{max}	Maximum internalized	T_{max}	Maximum internalized
	min	%	*min*	%	*min*	%
^{125}I-BA0	7.5 ± 0.6	24.2 ± 2.1	30 ± 3	26 ± 3	7.5 ± 0.7	31 ± 3
^{125}I-L2-BA3	15.2 ± 1.2	28.9 ± 2.3	30 ± 3	34 ± 4	7.5 ± 1.1	40 ± 6
^{125}I-CPT-L2-BA3	15.3 ± 0.8[a]	42.7 ± 2.2[a]	15 ± 1[b]	34 ± 2[b]	7.5 ± 1.2	42 ± 6

[a] $p < 0.001$ compared with ^{125}I-BA0.
[b] $p < 0.05$ compared with ^{125}I-BA0.

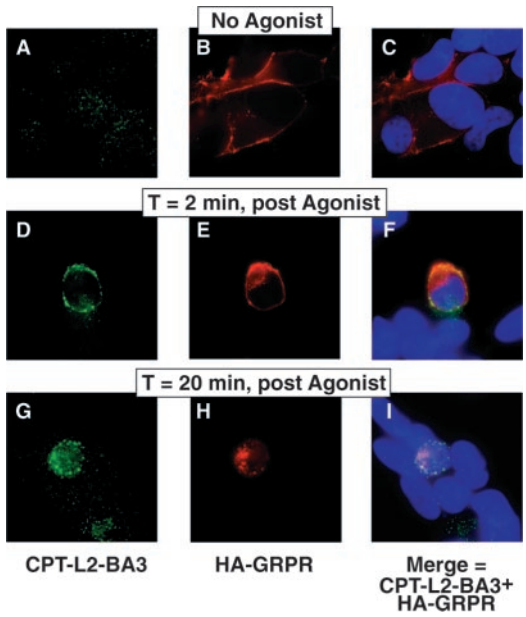

FIG. 4. **Internalization of the camptothecin-bombesin conjugate in HEK 293 cells transiently expressing HA-tagged GRP receptors.** HEK 293 cells (0.5×10^6 cells/6-well plate) were transfected with 1 μg of HA-GRPR-pcDNA3 and split to polylysine-coated 12-well plates the following day. Three days after transfection, cells were treated with (*D–I*) or without (*A–C*) CPT-L2-BA3 (3 nM) for the indicated periods of time. After removal of free CPT-L2-BA3, cells containing bound CPT-L2-BA3 were fixed, permeabilized, and stained with a polyclonal bombesin antibody (*left*) (*A*, *D*, *G*) or an HA antibody against the receptor (*middle*) (*B*, *E*, *H*), as described under "Experimental Procedures." *Merged images* (*C*, *F*, *I*) of the pictures are shown to reveal colocalization of the BN analog and the receptor (*right*). Shown are representative pictures of three independent experiments.

CPT-L2-BA3 Causes Elevation of Cytosolic Ca^{2+} in NCI-H1299 Cells—^{125}I-CPT-L2-BA3 bound with high affinity to NCI-H1299 cells and was internalized (data not shown). CPT-L2-BA3 (K_i = 0.8 ± 0.2 nM) had a 5-fold greater affinity for hGRP receptors on these cells than BA0 (K_i = 4.3 ± 0.4 nM). CPT-L2-BA3 (10 nM) stimulated a rapid increase in cytosolic Ca^{2+} in Fura-2-loaded NCI-H1299 cells (Fig. 5). BW2258U89 (10 μM), a specific GRP receptor antagonist (17), had no effect on cytosolic Ca^{2+} but completely inhibited the increase in cytosolic Ca^{2+} caused by 1 or 10 nM CPT-L2-BA3 (Fig. 5). The subsequent addition of 1000 nM CPT-L2-BA3, however, overcame the inhibition caused by the GRP receptor antagonist (Fig. 5). Similar results were obtained using NCI-H345 (SCLC) or hGRPR Balb/c 3T3 cells (data not shown). Because this antagonist binds with high affinity to hGRPR but not hNMBR or hBRS3 (11, 17), this result indicates that CPT-L2-BA3 is stimulating an increase in cytosolic calcium by activation of hGRPR on NCI-H1299 cells. CPT-L2-BA3 (0.1 nM) had no effect on cytosolic Ca^{2+}, but the subsequent addition of 10 nM GRP to NCI-H1299 cells increased cytosolic calcium (Fig. 5). CPT-L2-BA3 (1 nM) caused an increase in cytosolic Ca^{2+} ~20% of that seen with ionomycin (Fig. 5). A maximally effective concentration of CPT-L2-BA3 (*i.e.* 10 nM) caused a marked increase in cytosolic Ca^{2+}, and the subsequent addition of GRP (10 nM) had no effect (Fig. 5).

CPT-L2-BA3 Inhibits Cellular Proliferation—The ability of CPT-L2-BA3 to inhibit cellular proliferation was investigated *in vitro*. Using the MTT assay, CPT-L2-BA3 caused a dose-dependent inhibition of proliferation by NCI-H1299 cells and hGRPR-transfected Balb/c 3T3 cells with concentrations from 100 to 3000 nM (Fig. 6). CPT-L2-BA3 had a half-maximal (IC_{50}) inhibitory growth effect in NCI-H1299 cells of 190 ± 20 nM, whereas the IC_{50} was 500 ± 40 nM and 2450 nM for GRPR-transfected Balb/c 3T3 and control Balb/c 3T3 cells not containing human BN receptors, respectively (Fig. 6). Also, the IC_{50} was 800 and 600 nM for Balb/c 3T3 cells transfected with hNMBR or hBRS3 (data not shown). The results indicate that CPT-L2-BA3 was more potent at inhibiting the proliferation of cells containing BN receptors than cells lacking BN receptors.

Using a clonogenic assay, CPT-L2-BA3 also inhibited cellular proliferation of NCI-H1299 NSCLC cells in a concentration-dependent manner (Fig. 6). Large robust NCI-H1299 colonies formed in the absence of CPT-BN conjugates (control). The colonies decreased in number and size using 100 or 300 nM CPT-L2-BA3 (0.1 or 0.3 μM) and were absent using 1000 nM CPT-L2-BA3 (1 μM). For the NCI-H1299 cells, the IC_{50} for CPT-L2-BA3 to inhibit proliferation was 170 ± 20 nM. In contrast, 1000 nM BA0 did not alter proliferation (data not shown). CPT-L2-BA3 (1000 nM) decreased the viability of NCI-H1299 cells based on trypan blue exclusion and decreased [^{3}H]leucine incorporation into proteins as well as [^{3}H]thymidine incorporation into DNA (data not shown). These results indicate that CPT-L2-BA3 was cytotoxic for NCI-H1299 cells.

DISCUSSION

Recent studies, primarily using radiolabeled somatostatin analogs (6, 7), demonstrate that the frequent ectopic expression or overexpression of peptide hormone receptors by tumors (4, 7) can be utilized to administer tumor-selective cytotoxicity (6, 7). A similar approach is now being investigated using peptide receptor ligands coupled to chemotherapeutic agents for a number of peptide hormone receptors frequently present on common malignant tumors (*i.e.* prostate, breast, central nervous system tumors, lung cancer, various gastro-intestinal malignancies, and ovarian cancer) (4, 8, 21, 22). A cytotoxic agent using bombesin receptors as a molecular target would be useful for a number of reasons. First, BN receptor expression and/or overexpression occurs on almost all prostate cancers (5, 23), 33–72% of breast cancers (23, 24), 44–100% of small cell or non-small cell lung cancers (23, 25), 57% of gastric cancers (26), 100% of some types of malignant gliomas (24), 100% of ovarian cancer (27), 100% of squamous cell carcinoids of the head and

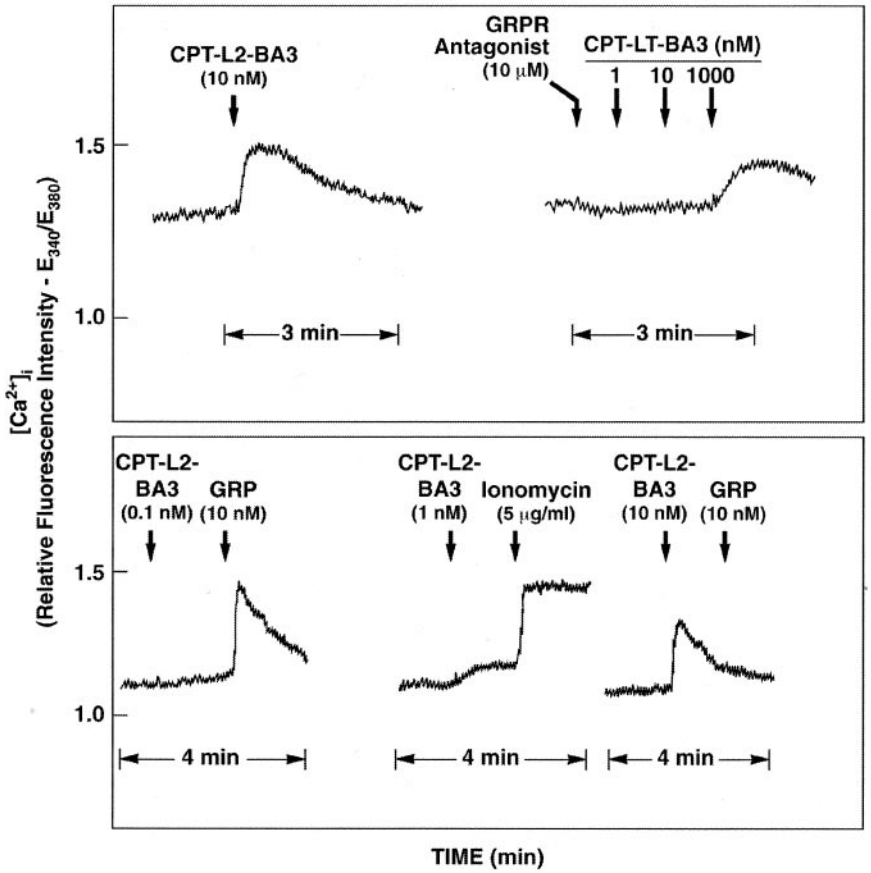

FIG. 5. **Effect of CPT-L2-BA3 alone or in combination with a GRPR receptor antagonist on $[Ca^{2+}]_i$ in NCI-H1299 cells.** *Top*, NCI-H1299 cells were loaded with 5 μM Fura-2/AM as described under "Experimental Procedures," and changes in cytosolic Ca^{2+} ($[Ca^{2+}]_i$) were determined in a Delta PTI Scan 1 spectrofluorometer using a cell density of 2.5×10^6 cells/ml. In the *top panel* are shown results with different concentrations of CPT-L2-BA3 in the presence or absence of an hGRPR antagonist (10 μM), (3-phenylpropanoyl-6)-[His^7,D-Ala^{11},D-Pro^{13},ψ13–14,Phe^{14}]-BN-(6–14) (BW 2258489) (17). CPT-L2-BA3 stimulates increases in $[Ca^{2+}]_i$, and its effect at lower concentrations is inhibited by the GRPR antagonist but overcome at higher concentrations (*i.e.* 1000 nM) of CPT-L2-BA3. These experiments are representative of three others. *Bottom*, the effects of increasing concentrations of CPT-L2-BA3, 10 nM GRP, or ionomycin are shown. These experiments are representative of three others.

neck (28), and 40–80% of colon cancer (23, 29). Second, peptides, which function as agonists but not antagonists for each of the three BN receptor subclasses, are rapidly internalized (15, 30–32). Third, recent studies using radiolabeled synthetic analogs of BN-related peptides demonstrate that these can be used to target tumors/tissues containing BN receptors, and these analogs are internalized in high amounts by these tumors (9).

To develop a conjugated bombesin analog that would be selectively cytotoxic to tumor cells overexpressing mammalian BN receptors, two important obstacles needed to be overcome. First, the BN cytotoxic conjugate should bind with high affinity to hGRPR, hNMBR, and hBRS3. This is important because different tumors may express different classes of BN receptors in different amounts (5, 7). This requirement was met by using the novel bombesin analog, [D-Tyr^6,β-Ala^{11},Phe^{13},Nle^{14}]BN-(6–14), which binds with high affinity for each of the three classes of mammalian bombesin receptors (11, 12). Second, a coupling system needs to be used that can be cleaved by intracellular enzymes to selectively release the cytotoxic agent within the tumor cell (1). Various carbamate linkers were utilized (3), which contained BINAR groups that allow coupling to chemotherapeutic agents that contain a reactive hydroxy group. This BINAR linkage system, when used to couple somatostatin analogs to combretastatin or CPT, was found to retain high affinity binding for somatostatin receptors, be stable in plasma, and have tumor cytotoxicity at doses below the maximum tolerated equivalent dose of cytotoxic agent alone (3). In a previous study (3), different BINAR groups had different effects on receptor affinity or peptide stability. Therefore, we utilized two different BINAR carbamate linkers, an ethylenediamine-containing linker (L1) and an *N*-methylethylenediamine (L2) CPT conjugate, in combination with different BN analogs. Both BINAR linkers and the amino acids at the N-terminal of BA0 had a profound effect on binding affinity to BN receptors. Only one combination of BINAR linker and peptide conjugate coupled to camptothecin (CPT-L2-BA3) retained high affinity for all three BN receptors, and this analog was fully characterized in the present study.

A number of our results support the conclusion that the BINAR bombesin analog, L2-BA3, has the necessary characteristics to be an effective compound for site-specific delivery of chemotherapeutic agents to cells possessing mammalian bombesin receptors. These characteristics include the following. The bombesin analog, L2-BA3, functions as a high affinity agonist at each class of mammalian bombesin receptor and is rapidly internalized by each receptor class, and the subsequent conjugation of camptothecin has no detrimental affect on any of these properties. This bombesin analog had equal high affinity to the original universal ligand, BA0 (Fig. 1) (11, 12), for each of the three classes of mammalian bombesin receptors. Furthermore, activation of each of the mammalian bombesin receptors results in phospholipase C stimulation (15, 19, 33, 34), and when this was assessed, this analog had an equal or greater potency for activating each receptor and stimulating generation of phosphoinositides as well as being fully efficacious to the original universal ligand, BA0.

Using iodinated analogs of the bombesin analog, L2-BA3, and the universal ligand, BA0, we were able to show that ^{125}I-L2-BA3 was internalized by cells possessing each of the three mammalian bombesin receptors, and the internalization rates and magnitude of internalization were similar for the two bombesin analogs. Not only did the addition of camptothecin to the linker bombesin analog, L2-BA3, not decrease the receptor affinity, its addition in fact resulted in a 30-fold increase in affinity for GRP receptor, a 20-fold increase for the NMB receptor, and an 8-fold increase in affinity for the BRS-3 receptor. At present, the molecular basis for this increased affinity is

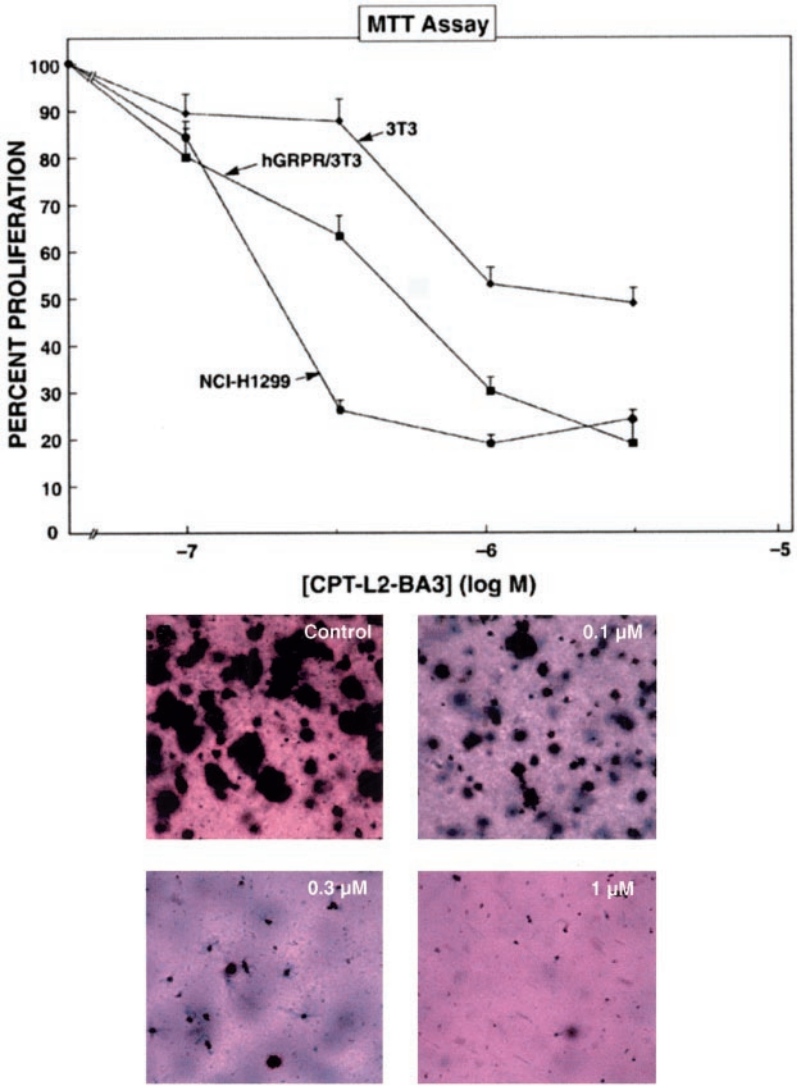

FIG. 6. **Ability of CPT-L2-BA3 to inhibit proliferation of various BN receptor-containing and non-BN receptor-containing cells.** *Top*, in the MTT assay, each of the cells (10^4/well) was incubated with the indicated concentration of CPT-L2-BA3 for 3 days, at which time MTT (1 mg/ml) was added. After this, 150 μl was added, and after 16 h the optical density was determined at 570 nm. Results are expressed as the percentage of proliferation seen with untreated cells. The mean value ± S.E. of eight determinations is indicated. This experiment is representative of four others. *Bottom*, in the clonogenic assay, colony number was determined 2 weeks after seeding with 5×10^4 NCI-H1299 cells. The colonies were determined in the absence of CPT-L2-BA3 (*i.e.* control) or presence of the indicated concentration of CPT-L2-BA3. This experiment is representative of three others.

unknown. Previously, Gln^{121}, Phe^{185}, Ala^{198}, Pro^{199}, Arg^{288}, and Ala^{308} of the GRPR were found to be important for high affinity binding by GRP (35, 36), and possibly CPT-L2-BA3 interacts with higher affinity with these amino acids or others due to the increased hydrophobicity caused by the addition of CPT to the L2-BA3. Furthermore, the camptothecin-coupled L2-BA3 had equal potency and efficacy to the uncoupled L2-BA3 for stimulating phospholipase C and, therefore, functioned as a full agonist. The coupling of camptothecin to the bombesin analog, L2-BA3, not only did not interfere with its internalization by the three mammalian bombesin receptors; its addition, in fact, significantly increased the amount internalized by 76% for the GRP receptor and 31% for the NMB receptor.

Previous studies demonstrate that GRPR agonists are rapidly internalized and also demonstrate that the BN agonist-GRPR complex is initially colocalized in endosomes and then moves to vesicles in the perinuclear space, where it is degraded (19, 32, 37, 38). Our results using fluorescent probes are consistent with the conclusion that CPT-L2-BA3 is being processed in a similar manner by the GRP receptor-bearing cells. Specifically, we found that the CPT-L2-BA3 colocalized on the cell surface with the GRPR was subsequently internalized in vesicles largely separate from the GRPR. These results indicate that CPT-L2-BA3 is internalized as a result of hGRPR-mediated endocytosis. After internalization, GRP receptor ligands are degraded intracellularly by lysosome-dependent and -independent mechanisms (39, 40). This could result in CPT-L2 being released into the cytosol and being metabolized by cytochrome P450 enzymes to release intracellular CPT. Our HPLC results of internalized radioactivity by hGRPR-containing Balb/c 3T3 cells after incubation with ^{125}I-CTP-L2-BA3 and removal of surface-bound ligand support this proposal. These results demonstrate that intact CPT-L2-BA3 was internalized, and it was metabolized to L2-BA3 by releasing free CPT. This internalization was dependent on the presence of GRPR on the Balb/c 3T3 cells and thus provides evidence that CPT-L2-BA3 was functioning as a prodrug by delivering the cytotoxic conjugate to GRPR-containing cells, where the cytotoxic agent could be released by intracellular metabolism. Unfortunately, we were unable to directly label camptothecin in our analogs without loss of activity, and, therefore, we could not directly study the kinetics of the intracellular generation of free camptothecin. Carbamate linkages are known to be metabolized by cytochrome P450, which is abundant in cancer cells (41). Cytochrome P450 is thought to metabolize CPT-somatostatin analogs, leading to cytotoxicity (3). Furthermore, P450 is known to be present in numerous human tumors including colon, breast, lung, liver, kidney, and prostate as well as to be up-regulated by human tumors (42).

A number of our results and those in the literature support the conclusion that the camptothecin-BINAR-bombesin analog, CPT-L2-BA3, will be a useful prototype agent for investigating

the ability of site-specific delivery of this chemotherapeutic agent to cause cytotoxicity in tumors overexpressing mammalian bombesin receptors. First, CPT-L2-BA3 bound with high affinity and was rapidly internalized by native hGRPR on NCI-H1299 cells. Second, the intact CPT-L2-BA3 was internalized and metabolized intracellularly, releasing free CPT. Third, CPT-L2-BA3 was biologically active in NCI-H1299 cells in that it elevated cytosolic Ca^{2+}, and its action was blocked by specific hGRPR antagonists. Fourth, using both the MTT and clonogenic growth assay, CPT-L2-BA3 inhibited the growth of NCI-H1299 cells. In contrast, a bombesin analog not coupled to CPT had no effect on growth. Fifth, in Balb/c 3T3 cells that were transfected with any one of the three classes of mammalian bombesin receptors, the CPT-L2-BA3 analog caused greater cytotoxicity than in the same cells not possessing bombesin receptors, demonstrating the ability of the bombesin receptor to cause enhanced cytotoxicity. Sixth, a number of properties of CPT-L2-BA3 and results of similar compounds in the literature would suggest *in vivo* that it should have an acceptable safety profile. CPT-L2-BA3 is more hydrophilic than native camptothecin, allowing greater aqueous solubility, and thus, greater concentrations should be available to act on targeted tissues (*i.e.* bombesin receptor-containing cells). Its selectivity for tissues overexpressing bombesin receptors should help target the analog, allowing greater cytoxicity with lower dosing. This proposal is supported by such results with doxorubicin analogs coupled to peptide delivery systems (8, 21).

In conclusion, our results show that CPT-L2-BA3 binds with high affinity to all mammalian BN receptor subtypes, functions as a fully potent and efficacious receptor agonist at each receptor class, is internalized, is metabolized intracellularly to L2-BA3-releasing camptothecin, and is cytotoxic for both tumor cells and cells transfected with human BN receptors. Its availability should be useful to explore the effectiveness of tumor-specific cytotoxicity delivery using a receptor-mediated mechanism.

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Development of High Affinity Camptothecin-Bombesin Conjugates That Have Targeted Cytotoxicity for Bombesin Receptor-containing Tumor Cells

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