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-Tyr⁶,β-Ala¹¹,Phe¹³,Nle¹⁴]-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.¹²⁵I-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 toxicity in an MTT assay for cells overexpressing any of the three BN receptor classes and functions as a prodrug for receptors specific to tumor components (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) 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-Tyr⁶,β-Ala¹¹,Phe¹³,Nle¹⁴]-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 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).
with 10% fetal bovine serum, 100 units/ml penicillin, and 100 mg/ml streptomycin, or NCI-H1299 (human NSCLC), and HEK 293 (human embryonic kidney) cells from ATCC (Manassas, VA); Dulbecco's modified Eagle's medium, sodium fumarate, 0.01% (w/v) soybean trypsin inhibitor, 1% amino acid mixture, 106), or NCI-H1299 cells were incubated with 50 μM 125I-labeled L2-BA3 (mixture of polymers) for 16 h at 4°C. Two sets of cells were incubated with 0.5 μl of 1.5 M diethylthio-}

Camptothecin-Bombesin Conjugates

**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 modified Eagle's medium, sodium fumarate, 0.01% (w/v) soybean trypsin inhibitor, 1% amino acid mixture, 106), or NCI-H1299 cells were incubated with 50 μM 125I-labeled L2-BA3 (mixture of polymers) for 16 h at 4°C. Two sets of cells were incubated with 0.5 μl of 1.5 M diethyldithiocarbamylamine (acid-stripped) or internalized (not acid-stripped). The internalization experiments were 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 MgCl2, 2.5 mM NaH2PO4, 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 HLGPR (0.3 × 106), nHBRS3 (0.03 × 106), and HBRS3 (0.3 × 106), or NCI-H1299 cells were incubated with 50 μM 125I-labeled ligand at 22°C for 60 min. Aliquots (100 μl) were removed and centrifuged through 300 μl of 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 125I-ligand added that is surface-bound in the exponential growth phase after incubation at 37°C in 5% CO2, 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-fluorenylmethoxycarbonyl) protection strategy (3). Sequences were assembled on Rink amide resin (Advanced ChemTech, Louisville, KY), and Trt group protection was employed for the Trp-His-Trp-Thr peptide. One set of the peptides was purified by reverse-phase HPLC (C18 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-L1BA1 had an internal mass of 1647.5 and was found to have a mass of 1647.1. CPT-L1BA2 (mixture of polymers) had an average mass of 3000; and CPT-L2BA3 had a calculated mass of 1709.9 and was found to have a mass of 1709.1.

**Preparation of 125I-Labeled Peptides**—

**Binding of 125I-Labeled Related-BN Peptides to Various Cells**—

**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 1.5-ml Microfuge tubes at 10,000 × g for 1 min using a Beckman Microfuge B. The pellets were washed twice with incubation buffer and counted for radioactivity in a liquid scintillation counter.

**RESULTS**

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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 or hNMBR [57] were incubated at 37 °C for 15 min with 0.4 nM ³²P-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 µM 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 buffer with protease inhibitors containing 1% Triton X-100 (pH 6), and centrifugation, cells were resuspended in 1 ml of standard binding medium (100/425/426) 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 [³²P]Inositol Phosphates ([³²P]IP)—Changes in total [³²P]IP were measured as described previously (14, 15). Briefly, hGRPR-, hNMBR-transfected Balb/c 3T3 cells were subcultured into 24-well plates (5 × 10⁴ cells/well) in regular propagation medium and then incubated for 24 h at 37 °C in a 5% CO₂ atmosphere. The cells were then incubated with 3 µCi/ml myo-[²-³²P]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% (w/v) hydrochloric acid in methanol. Total [³²P]IP was isolated by anion exchange chromatography as described previously (14, 15). Briefly, samples were loaded onto Dowex AGI-X8 anion exchange resin columns, washed with 5 ml of distilled water to remove free [³²P]inositol, and then washed with 2 ml of 50 mM disodium tetraborate, 60 mM sodium formate solution to remove [³²P]glycerophosphorylinositol. Two milliliter of 1 M ammonium formate, 100 mM formic acid solution were added to the columns to elute total [³²P]IP. Each eluate was mixed with scintillation mixture and measured for radioactivity in a scintillation counter.

Cytosolic Calcium [Ca²⁺]: Measurement—The ability of the CPT-BN conjugates to alter cytosolic [Ca²⁺], was investigated as described previously (17). NCI-H1299 cells and NCI-H345 cells were harvested (2.5 × 10⁶/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 × g for 10 min and resuspended at the same concentration in new SIT medium (RPMI 1640 containing 30 mM 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 in the various peptides are shown in parenthesis (18).

Proliferation Assays—Growth studies were performed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. Growth studies were conducted using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) assay. Cells were plated in 24-well plates (5 × 10⁴ cells/well) in regular propagation medium and cultured for 3 days. The basal 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 (FMIC Corp., Rockford, ME). CPT-BN conjugates, and 5 × 10⁵ 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 scanned for colony formation. The number of colonies larger than 50 µm in diameter were counted using an Omnicon image analysis system.
The affinities and potencies of various synthetic BN analogs and their camptothecin conjugates for human BN receptors

**Table I**

<table>
<thead>
<tr>
<th>BN receptor cells</th>
<th>Peptide</th>
<th>125I-Ba0</th>
<th>125I-L2-BA3</th>
<th>125I-CPT-L2-BA3</th>
<th>125I-TyrBN</th>
<th>EC50 for 125I-Ba0</th>
</tr>
</thead>
<tbody>
<tr>
<td>hGRPR</td>
<td>BA0</td>
<td>0.32 ± 0.012</td>
<td>0.74 ± 0.05</td>
<td>0.44 ± 0.11</td>
<td>0.19 ± 0.14</td>
<td>2.01 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>L2-BA3</td>
<td>0.22 ± 0.013</td>
<td>0.14 ± 0.01</td>
<td>0.23 ± 0.02</td>
<td>0.18 ± 0.02</td>
<td>0.59 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>CPT-L2-BA3</td>
<td>0.012 ± 0.001</td>
<td>0.006 ± 0.01</td>
<td>0.029 ± 0.007</td>
<td>0.009 ± 0.007</td>
<td>0.78 ± 0.13</td>
</tr>
<tr>
<td>hNMBR</td>
<td>BA0</td>
<td>0.74 ± 0.05</td>
<td>1.74 ± 0.09</td>
<td>2.0 ± 0.05</td>
<td>2.2 ± 0.18</td>
<td>2.39 ± 0.21</td>
</tr>
<tr>
<td></td>
<td>L2-BA3</td>
<td>0.14 ± 0.01</td>
<td>0.38 ± 0.01</td>
<td>0.50 ± 0.10</td>
<td>0.59 ± 0.04</td>
<td>0.91 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>CPT-L2-BA3</td>
<td>0.035 ± 0.003</td>
<td>0.09 ± 0.03</td>
<td>0.033 ± 0.01</td>
<td>0.21 ± 0.02</td>
<td>1.66 ± 0.13</td>
</tr>
<tr>
<td>hBRS3</td>
<td>BA0</td>
<td>0.25 ± 0.10</td>
<td>0.45 ± 0.04</td>
<td>0.47 ± 0.03</td>
<td>1.2 ± 0.02</td>
<td>1.02 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>L2-BA3</td>
<td>0.42 ± 0.02</td>
<td>1.0 ± 0.1</td>
<td>1.0 ± 0.2</td>
<td>0.79 ± 0.03</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CPT-L2-BA3</td>
<td>0.031 ± 0.008</td>
<td>0.21 ± 0.05</td>
<td>0.25 ± 0.14</td>
<td>No binding</td>
<td>0.62 ± 0.10</td>
</tr>
</tbody>
</table>

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 sequencing apparatus (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 × 10⁶ 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% for 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 secondary antibody. Nuclei were visualized after 4’,6-diamidino-2-phenylindole counterstaining. Coverslips were mounted using Vectashield 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 SH 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 the CPT (Fig. 1, top) and the NßH of human BN receptor, BA0, BA0, L2-BA3, and CPT-L2-BA3 had a 30-fold higher affinity than BA0 for human GRPR (Fig. 1, top). The affinities and potencies of various synthetic BN analogs and their camptothecin conjugates for human BN receptors

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$^{125}$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 CPT-L2-BA3 was evaluated using Balb/c 3T3 cells transfected with BN receptors and loaded with [H]$^3$IP (Fig. 2, bottom). Each of the three
ligands (BA0, L2-BA3, and CPT-L2-BA3) were agonists, stimulating increases in [3H]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 [3H]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 and hBRS3 cells the EC50 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. 3A, Table II). 125I-CPT-L2-BA3 was internalized more rapidly by hNMBR and demonstrated 1.3-fold greater maximal internalization than 125I-BA0. Similar internalization data were obtained using Balb/c 3T3 cells containing hNMBR (Fig. 3B, Table II). With hGRPR cells, 125I-BA0, 125I-L2-BA3, and 125I-CPT-L2-BA3 were rapidly internalized with 20–30% of the ligand internalized after 2.5 min (Fig. 3C). For 125I-CPT-L2-BA3, maximal internalization occurred at 15.3 ± 0.8 min with 42.7 ± 2.2% of the bound ligand internalized (Fig. 3C, Table II). 125I-CPT-L2-BA3 demonstrated 1.8- and 1.5-fold greater maximal internalization by hGRPR cells than 125I-BA0 or 125I-L2-BA3 (Fig. 3C, Table II). These results indicate that 125I-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. 4B). Two min after CPT-L2-BA3 (10 nM) was added, both the GRP receptors (Fig. 4E) and BN-like immunoreactivity (green) (Fig. 4D), were localized primarily to the plasma membrane and were frequently co-localized (yellow) (Fig. 4F). 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. 4H). Similarly, the BB-like immunoreactivity had internalized into intracellular patches (Fig. 4G); however, there was minimal co-localization with BN-like immunoreactivity (Fig. 4I). These results show that after 20 min, the HA-GRP receptor (Fig. 4I) 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 125I-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 125I-CPT-L2-BA3, 25% as 125I-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.
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

<table>
<thead>
<tr>
<th>Ligand</th>
<th>hGRPR T$_{\text{max}}$</th>
<th>Max internalized</th>
<th>hNMBR T$_{\text{max}}$</th>
<th>Max internalized</th>
<th>hBRs T$_{\text{max}}$</th>
<th>Max internalized</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{125}$I-BA0</td>
<td>7.5 ± 0.6</td>
<td>24.2 ± 2.1</td>
<td>30 ± 3</td>
<td>26 ± 3</td>
<td>7.5 ± 0.7</td>
<td>31 ± 3</td>
</tr>
<tr>
<td>$^{125}$I-L2-BA3</td>
<td>15.2 ± 1.2</td>
<td>28.9 ± 2.3</td>
<td>30 ± 3</td>
<td>34 ± 4</td>
<td>7.5 ± 1.1</td>
<td>40 ± 6</td>
</tr>
<tr>
<td>$^{125}$I-CPT-L2-BA3</td>
<td>15.3 ± 0.8*</td>
<td>42.7 ± 2.2</td>
<td>15 ± 1$^b$</td>
<td>34 ± 2$^b$</td>
<td>7.5 ± 1.2</td>
<td>42 ± 6</td>
</tr>
</tbody>
</table>

* $p < 0.001$ compared with $^{125}$I-BA0.

† $p < 0.05$ compared with $^{125}$I-BA0.

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 ($K_f = 0.8 \pm 0.2 \text{ nm}$) had a 5-fold greater affinity for hGRPR receptors on these cells than BA0 ($K_f = 4.3 \pm 0.4 \text{ nm}$). CPT-L2-BA3 (10 nM) stimulated a rapid increase in cytosolic Ca$^{2+}$ in Fura-2-loaded NCI-H1299 cells (Fig. 5). BW2258U89 (10 \mu M), 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$^{2+}$ of 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 GRP-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 NSCCL 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 nM) and were absent using 1000 nM CPT-L2-BA3 (1 \mu M). 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 $[^{3}H]$thymidine 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+}], in NCI-H1299 cells. Top, NCI-H1299 cells were loaded with 5 μM Fluo-2/AM as described under “Experimental Procedures,” and changes in cytosolic Ca^{2+} ([Ca^{2+}]_i) were determined in a Delta PTI Scan 1 spectrophotofluorometer 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 GRPR antagonist (10 μM), (3-phenylpropanoyl)-6-[His^{7},n-Ala^{11},Pro^{13},\phi_{13-14},Phe^{14}]-BN-(6-14) (BW 2558489) (17). CPT-L2-BA3 stimulates increases in [Ca^{2+}], and its effect at lower concentrations is inhibited by the GRPR antagonist but only 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 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},\beta-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 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-methylmethylenediamine (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
unknown. Previously, Gln\textsuperscript{121}, Phe\textsuperscript{185}, Ala\textsuperscript{198}, Pro\textsuperscript{199}, Arg\textsuperscript{288}, and Ala\textsuperscript{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 \textsuperscript{125I}-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 assays, CPT-L2-BA3 inhibited the growth of NCI-H11001/hGRPR cells. In contrast, a bombesin analog not coupled to CPT would suggest CPT-L2-BA3 and results of similar compounds in the literature demonstrate the ability of the bombesin receptor-containing cells.

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