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Identification of bombesin (Bn) receptor subtype-specific ligands: effect of N-methyl scanning, truncation, substitution and evaluation of putative reported selective ligands

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ABBREVIATIONS: Bn, bombesin**;** GRPR, gastrin-releasing peptide receptor; NMBR, neuromedin B receptor; BRS-3, bombesin receptor subtype 3; [^{3}H]IP, [^{3}H] labeled inositol phosphates; fBB4,frog bombesin receptor subtype 4; CNS, central nervous system; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; βAla, βAlanine; Nle,

norleucine; NMe, N-methyl; His(tBzl), histidine(tau benzl); , Bzl, benzyl; COOH, carboxyl terminal; CCK, cholecystokinin; Apa, 3-amino, propionic acid; FLIPR, fluorometric imaging plate reader; $[Ca^{2+}]_i$, intracellular calcium; Nip, piperidine-3 carboxylic acid; Ac, acetyl;

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ABSTRACT

Mammalian bombesin (Bn) receptors include the gastrin-releasing peptide receptor (GRPR), neuromedin B receptor (NMBR) and bombesin receptor subtype 3 (BRS-3). These receptors are involved in a variety of physiological/pathologic processes including thermoregulation, secretion, motility, chemotaxis and mitogenic effects on both normal and malignant cells. Tumors frequently overexpress these receptors and their presence is now used for imaging and receptor-mediated cytotoxicity. For these reasons there is an increased need to develop synthetic, selective receptor subtype specific ligands, especially agonists for these receptors. In this study we used a number of strategies to identify useful receptor subtype selective ligands including synthesizing new analogs (N-methyl-substituted constrained analogs, truncations, substitutions) in [DTyr^{6},βAla^{11},Phe^{13},Nle^{14}]Bn(6-14), which has high affinity for all Bn receptors and is metabolically stable, as well as completely pharmacologically characterized analogs recently reported to be selective for these receptors in $[Ca^{2+}]_i$ assays. Affinities and potencies of each analog were determined for each human Bn receptor subtype. N-methyl substitutions in position 14,12,11,10,9,8 did not result in selective analogs and except in position 11, which markedly decreased affinity/potency. N terminal truncations or position 12 substitutions did not increase selectivity as previously reported by others. Of the four shortened analogs of [DPhe^{6},βAla^{11},Phe^{13},Nle^{14}]Bn(6-14) reported to be potent selective BRS-3 ligands on $[Ca^{2+}]_i$ assays, only Ac-Phe,Trp,Ala,His(tBzl),Nip,Gly,Arg-NH_2 had moderate selectivity for hBRS-3, however it was less selective than previously reported Apa^{11} analogs, demonstrating these are still the most selective BRS-3 analogs available. However, both of these analogs should be useful templates to develop more selective BRS-3 ligands.

INTRODUCTION

There are four bombesin (Bn) receptor subtypes that have been cloned and identified. The first two, the neuromedin B receptor (NMBR) and gastrin-releasing peptide receptor (GRPR), designated BB1 and BB2, respectively, are widely distributed in human and mammalian tissues (Bunnett, 1994;Ohki-Hamazaki, et al., 2005). A third mammalian bombesin receptor subtype, BRS-3, is found in the CNS and gastrointestinal tract, however its endogenous ligand is presently unknown (Mantey, et al., 1997). The fourth Bn receptor subtype, BB4, has only been identified in frogs and at present, no mammalian equivalent of this receptor has been described (Nagalla, et al., 1995;Katsuno, et al., 1999). Bn receptors are widely distributed in the CNS, the gastrointestinal tract, lungs, reproductive organs, and are one of the most frequently over-expressed G protein-coupled receptors in many types of human cancers including those from the gastrointestinal tract, breast, lung and prostate (Jensen and Moody, 2006;Bunnett, 1994;Ohki-Hamazaki, et al., 2005;Reubi, et al., 2002).

Studies suggest that Bn receptors are involved in a wide variety of physiological and pathological processes including in the CNS (satiety, thermo-regulation, regulation of blood pressure), in regulation of metabolism (glucose metabolism and energy balances), in the regulation of normal growth and development, in the regulation of immunologic responses and in mediating various numerous gastrointestinal responses (motility, secretion) (Bunnett, 1994;Ohki-Hamazaki, et al., 2005). Bn peptides have potent growth effects on numerous human tumors and function as autocrine growth factors (Jensen and Moody, 2006). Furthermore, because so many human neoplasms over-express Bn receptor subtypes their presence is currently being extensively evaluated both to allow localization of these tumors by imaging methods as well as to deliver cytotoxic agents to these tumors (Moody, et al., 2004).

Because of their widespread roles in both normal and neoplastic tissues, the development of synthetic ligands, which are selective for one Bn receptor subtype and function as selective agonists or antagonists, is important. For imaging studies and the use of Bn receptor over- or ectopic- expression to deliver receptor mediated cytotoxic agents, the development of selective agonists, particularly, if metabolically stable, is important because they are rapidly internalized (Benya, et al., 1992;Mantey, et al., 1993). There are relatively few selective synthetic Bn analogs that are metabolically stable for the various Bn receptor subtypes. This has been a particular problem with the BRS-3 receptor because its natural ligand is unknown and only a few synthetic ligands have been described which interact with this receptor with moderate to high affinity (Mantey, et al., 1997;Pradhan, et al., 1998;Ryan, et al., 1998b;Weber, et al., 2002;Weber, et al., 2003;Boyle, et al., 2005). This is important because recent studies using BRS-3 deficient mice, produced by targeted disruption, develop hypertension, obesity and diabetes (Ohki-Hamazaki, et al., 1997), however little is known of the role of BRS-3 in these processes because of the lack of selective ligands.

In this study, in an attempt to identify selective ligands for Bn receptor subtypes, we used a number of different strategies. First, we have synthesized N-methyl substituted constrained analogs analogs of [DTyr^{6},βAla^{11},Phe^{13}, Nle^{14}]Bn(6-14), because this analog has been shown to be metabolically stable and to have high affinity and potency for activating all Bn receptor subtypes (Ryan, et al., 1998b;Ryan, et al., 1998a;Reubi, et al., 2002;Pradhan, et al., 1998;Mantey, et al., 1997;Moody, et al., 2004). This approach was used because it has successfully yielded highly selective peptide ligands with a number of other receptors (Rajeswaran, et al., 2001;Reissmann, et al., 1996;Pradhan, et al., 1998). The second approach involved synthesizing analogs of [DTyr^{6},βAla^{11},Phe^{13},Nle^{14}]Bn(6-14) that were N-terminally truncated or had selective substitutions because this was reported to result in increasing

selectivity for one type of Bn receptor in a recent study (Darker, et al., 2001). The final approach used was to synthesize various shortened [DTyr^{6},βAla^{11},Phe^{13},Nle^{14}]Bn(6-14) analogs recently reported to be selective for a Bn receptor subtype and fully characterize them pharmacologically by binding studies and assays assessing receptor activation at each of the three human Bn receptor subtypes. This latter strategy was included because these agents were not fully characterized at each receptor pharmacologically in these studies with only calcium or calcium FLIPR assay results available in most cases. Finally we compared the selectivity for the BRS-3 receptor of the most potent and selective shortened [DTyr^{6},βAla^{11},Phe^{13},Nle^{14}]Bn(6-14) analogs to two full-length analogs we recently described (Mantey, et al., 2001;Mantey, et al., 2004).

MATERIALS AND METHODS

Materials – The following cells and materials were obtained from the sources indicated: BALB 3T3 (mouse fibroblast) cells were from American Type Culture Collection (ATCC), Rockville, MD; Dulbecco's minimum essential medium (DMEM), phosphate-buffered saline (PBS), Roswell Park Memorial Institute (RPMI-1640), trypsin-EDTA and fetal bovine serum (FBS), from Biofluids, Rockville, MD; G418 sulfate from Life Technologies, Inc., Grand Island, NY; Na^{125}I (2200 Ci/mmol) and myo-[2-^{3}H]Inositol (20 Ci/mmol) were from Amersham Pharmacia Biotech; formic acid, ammonium formate, disodium tetraborate, soybean trypsin inhibitor, bacitracin and AG 1-X8 resin from Bio-Rad, Richmond, CA; bombesin (Bn), gastrin-releasing peptide (GRP), neuromedin B (NMB) and [Tyr^{4}]Bn were from Bachem, Torrence CA; and bovine serum albumin (BSA) from ICN Pharmaceutical Inc., Aurora, OH.

Cell Culture - Balb 3T3 cells stably expressing human BRS-3 receptor (hBRS-3), human NMB receptor (hNMBR) or human GRP receptor (hGRPR) were made as described previously (Mantey, et al., 1997;Benya, et al., 1995;Ryan, et al., 1998a), were grown in Dulbecco's modified Eagle's cell medium

(DMEM) supplemented with 300 mg/liter of G418 sulfate. The cells were mycoplasma free and were used when they were in exponential growth phase after incubation at 37 ^{o}C in 5% CO_2, 95% air.

Strategies used and preparation of peptides – In this study we attempted to develop or identify selective ligands for human Bn receptors, primarily concentrating on the orphan receptor, human BRS-3. We have previously reported that the Bn analogue, [DPhe^{6}, βAla^{11},Phe^{13},Nle^{14}]Bn(6-14) (Peptide 2, Table 1), has high affinity for all known Bn receptors (Mantey, et al., 1997;Katsuno, et al., 1999;Ryan, et al., 1998a;Pradhan, et al., 1998;Mantey, et al., 2001). Recently analogs of [DPhe^{6}, βAla^{11},Phe^{13},Nle^{14}]Bn(6-14) have been described (Darker, et al., 2001;Weber, et al., 2003;Weber, et al., 2002;Mantey, et al., 2004;Mantey, et al., 2001) that are reported to have selectivity for hBRS-3 or the other Bn receptor subtypes. In three studies (Weber, et al., 2003;Weber, et al., 2002;Boyle, et al., 2005) this selectivity was based on assessment of changes in $[Ca^{2+}]_i$ using a FLIPR assay with no direct assessment of receptor affinities. In the present study we have used a number of strategies known to produce receptor subtype selective analogs with other peptides to synthesize possible new analogs selective for human Bn receptor subtypes. Also we have synthesized these recently described Bn receptor subtype selective as well as other related analogs to fully characterize their selectivity for the human Bn receptors and attempt to identify analogs with specificity for hBRS-3 or the other Bn receptors. The peptides were synthesized using standard solid-phase methods as described previously (Mantey, et al., 2001). Briefly, solid-phase syntheses of peptide amides were carried out using Boc chemistry on methylbenzhydrylamine resin (Advanced ChemTech, Louisville, KY) followed by HF-cleavage of free peptide amides. The crude peptides were purified by preparative high liquid chromatography (HPLC) on columns (2.5 x 50 cm) of Vydac C18 silica (10 μm), which was eluted, with linear gradients of acetonitrile in 0.1% (v/v) trifluoroacetic acid. Homogeneity of the peptides was assessed by analytical reverse-phase HPLC and the purity was usually 97% or higher. Amino acid analysis (only amino acids with primary amino acid groups were quantitated) gave the expected amino acid ratios. Peptide molecular masses were obtained

by matrix-assisted laser desorption mass spectrometry (Thermo Bioanalysis Corp., Hemel, Helmstead, UK) and all corresponded well with calculated values.

Preparation of ^{125}I-[DTyr^{6}, βAla^{11},Phe^{13},Nle^{14}]Bn(6-14)– This radioligand, with specific activity of 2200 Ci/mmol, was prepared as previously described (Mantey, et al., 1997;Ryan, et al., 1998b). Briefly, 0.8 μg of IOD-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). To this tube 20 μl of potassium phosphate solution (pH 7.4), 8 μg of peptide in 4 μl of water, 2 mCi (20 μl) of $Na^{125}I$ were added and incubated for 6 min at room temperature. The incubation was stopped with 300 μl of water. The radiolabeled peptide was separated using a 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 ^{o}C.

Binding of ^{125}I-Labeled BN-related Peptides to various cells– Binding was performed as described previously (Mantey, et al., 1993;Mantey, et al., 1997;Ryan, et al., 1998b). The standard binding buffer contained 24.5 mM HEPES (pH 7.4), 98 mM NaCl, 6 mM KCl, 5 mM $MgCl_2$, 2.5 mM NaH_2PO_4, 5 mM sodium pyruvate, 5 mM sodium fumarate, 0.01% (w/v) soybean trypsin inhibitor, 1% amino acid mixture, 0.2% (w/v) bovine serum albumin and 0.05% (w/v) bacitracin. BALB 3T3 cells stably expressing hGRPR (0.3×10^6), hNMBR (0.03×10^6), or hBRS-3 (0.3×10^6) were incubated with 50 pM ^{125}I-labeled ligand at 22 ^{o}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 x g for 1 min using a Beckman Micro-centrifuge B. The pellets were washed twice with buffer and counted for radioactivity in a gamma counter. The nonsaturable binding was the amount of radioactivity associated with cells in incubations containing 50 pM radioligand (2200 Ci/mmol) and 1 μM unlabeled ligand. Nonsaturable binding was <10% of total

binding in all the experiments. Receptor affinities were determined using a least-square curve-fitting program (LIGAND) and the Cheng-Prusoff equation.

Measurement of [^{3}H]IP - Changes in total [^{3}H]inositol phosphates ([^{3}H]IP) was measured as described previously (Benya, et al., 1994;Ryan, et al., 1998a;Benya, et al., 1995;Benya, et al., 1992). Briefly, hBRS-3-, hGRPR- or hNMBR- transfected Balb 3T3 cells were subcultured into 24-well plates (5 X 10^4 cells/well) in regular propagation media and then incubated for 24 hr at 37^{o}C in a 5% CO_2 atmosphere. The cells were then incubated with 3 Ci/ml of myo-[2-^{3}H] inositol in growth media supplemented with 2% FBS for an additional 24 hr. Before assay, the 24-well plates were washed by incubating for 30 min at 37^{o}C with 1 ml/well of PBS (pH 7.0) containing 20 mM lithium chloride. The wash buffer was aspirated and replaced with 500 µl of IP assay buffer containing 135 mM sodium chloride, 20 mM HEPES (pH 7.4), 2 mM calcium chloride, 1.2 mM magnesium sulfate, 1 mM EGTA, 20 mM lithium chloride, 11.1 mM glucose, 0.05% BSA (w/v) and incubated with or without any of the peptides studied. After 60 min of incubation at 37^{0}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 (Benya, et al., 1994;Ryan, et al., 1998a;Benya, et al., 1992). 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, 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 cocktail and measured for radioactivity in a scintillation counter.

Measurement [Ca^{2+}]$_i$ using ^{45}Ca efflux and Fura-2. - Changes in [Ca^{2+}]$_i$ using ^{45}Ca efflux and Fura-2 were measured as described previously (Benya, et al., 1992). In a previous study both methods demonstrate similar dose-response curves for Bn-related peptides (Benya, et al., 1992). Briefly, for [Ca^{2+}]$_i$ measurements using Fura-2, hBRS-3-, hGRPR- or hNMBR- transfected Balb 3T3 cells were

resuspended in binding buffer without bacitracin containing $4x10^6$ cells and 2 μM fura-2 for 45 min at 37°C. Fura-2 loaded cells were washed three times in binding buffer and 2 ml samples were placed in quartz cuvettes in a Delta PTI Scan I spectrophotometer (PTI Instruments, Gaithersburg, MD). $[Ca^{2+}]_i$ was measured as described previously (Benya, et al., 1992) after detecting fluorescence at 500 nm following excitation at 340 nm and 380 nm. For measuring changes of $[Ca^{2+}]_i$ using ^{45}Ca efflux, hBRS-3-, hGRPR- or hNMBR- transfected Balb 3T3 cells were subcultured into 24-well plates ($5 X 10^4$ cells/well) in regular propagation media. After aspirating the media 1 ml of phosphate-free binding buffer containing 5 μCi/ml of ^{45}Ca was added to each well and the cells incubated for 90 min at 37°C in a 5% CO_2 atmosphere. Immediately before the assay the cells were rapidly washed once in phosphate-free buffer and then incubated at 22°C in buffer containg the appropriated concentration of peptide. After 5 min the supernatant was removed and discarded; the cells were lysed in 1% HCl in methanol and the cell-associated radioactivity was determined in a liquid scintillation counter.

RESULTS

Because with a number of other receptors insertion of N-methyl groups in ligands results in subtype selective analogues (Lin, et al., 1990;Pradhan, et al., 1998;Reissmann, et al., 1996), a similar strategy was applied to the Bn analog, [DPhe^6, βAla^{11},Phe^{13},Nle^{14}]Bn(6-14) (Peptide 1, Table 1), which has high affinity for all human Bn receptor subtypes (Mantey, et al., 1997;Ryan, et al., 1998a;Pradhan, et al., 1998;Mantey, et al., 2001;Mantey, et al., 2004) (Fig.1 and Table 2). N-methyl groups were added to the Nle^{14}, His^{12}, βAla^{11}, Val^{10} and Ala^9 (Peptides 3-7, Table 1) of the non-selective peptide, [DTyr^6, βAla^{11},Phe^{13},Nle^{14}]Bn(6-14) or to Trp^8 on the truncated analog [βAla^{11},Phe^{13},Nle^{14}]Bn(8-14) (Peptide 8, Table 1). The ability of the N-methyl peptides to

inhibit binding of ^{125}I-[DTyr^{6}, βAla^{11},Phe^{13},Nle^{14}]Bn(6-14) and stimulate the generation of [^{3}H]IP at the three human Bn receptor subtypes were investigated, because the activation of each of the three human Bn subtypes causes phospholipase C activation (Benya, et al., 1995;Ryan, et al., 1998b;Ryan, et al., 1998a). [DTyr^{6}, βAla^{11},Phe^{13},Nle^{14}]Bn(6-14) had greater potency for each of the three human Bn receptor subtypes than Bn and Bn had a much greater affinity for the hGRPR and hNMBR than the hBRS-3 (compare Bn and Peptide 1, Table 2). Except for position 11 (Peptide 5, Table 2), the insertion of N-methyl groups at positions 14, 12, 10 or 9 of [DTyr^{6}, βAla^{11},Phe^{13},Nle^{14}]Bn(6-14), resulted in marked (> 20-fold) decreases in affinity for each of the three human Bn receptors (Table 2, Fig. 1). In contrast, the insertion at the βAla^{11} position of an N-methyl group (Peptide 5, Table 2) resulted in only 2- to 16-fold decrease in affinity for the human Bn receptors. Neither the insertion of the N-methyl groups in various positions of [DTyr^{6}, βAla^{11},Phe^{13},Nle^{14}]Bn(6-14) nor in Trp^{8} of [DTyr^{8}, βAla^{11},Phe^{13},Nle^{14}]Bn(8-14), (Peptide 8, Table 2) resulted in an increase in selectivity for hBRS-3 over hGRPR or hNMBR for any of the N-methyl-substituted analogs. The addition of N-methyl to βAla^{11} (Peptide 5, Table 2) resulted in a small increase in selectivity for the hGRPR. Specifically, with this analog (Peptide 5, Table 2) the hGRPR had an 8-fold greater affinity over the hNMBR or hBRS-3 compared to [DTyr^{6}, βAla^{11},Phe^{13},Nle^{14}]Bn(6-14). Each of the N-methyl substituted peptides (Peptides 3-8, Table 2, Fig. 1) was an agonist stimulating an increase in [^{3}H]IP (Fig. 1, bottom panels). Their relative potencies for human Bn receptors were similar to the results for the affinities from binding studies and no analog showed greater selective potency for activating hBRS-3.

Recently analogs of [DPhe^{6}, βAla^{11},Phe^{13},Nle^{14}]Bn(6-14) (Darker, et al., 2001) have been reported to function as highly selective GRPR agonists in a [Ca^{2+}]$_{i}$ FLIPR assay. We synthesized three of the most potent analogs [Peptide 9 (compound 6 in (Darker, et al., 2001); Peptide 10 (Compound 11 in (Darker, et al., 2001) and Peptide 12 (Compound 13) in (Darker, et

al., 2001), Table 3] as well as 6 new related analogs, primarily with different position 12 substitutions (Peptides 12-17, Table 6) and assessed their abilities to interact with and activate each of the three human Bn receptors (Fig. 2 and 3, Table 3). In binding studies, we found (pGlu^{7},βAla^{11},Phe^{13},Nle^{14})Bn(7-14) [Peptide 9, Table 3] to have a 300- to 1600-fold reduced affinity for each of the three human Bn receptors compared to [DPhe^{6}, βAla^{11},Phe^{13},Nle^{14}]Bn(6-14) and found no hGRPR selectivity for any human Bn receptor subtype (Fig. 2, Table 3, Top). Furthermore, the Phe^{6} truncation made the peptide equipotent for activating and stimulating phospholipase C activity at the hGRPR and the hNMBR and 5-fold less potent for activating the hBRS-3 (Peptide 9, Fig.2, Table 3). In binding studies we found that Peptide 10 and 11 had a 100 to 8000-fold lower affinity for hGRPR than [DPhe^{6}, βAla^{11},Phe^{13},Nle^{14}]Bn(6-14) (Table 3, Fig. 2). Furthermore, neither peptide 10 nor 11 shows selectivity in our binding or [^{3}H]IP assays for the hGRPR (Fig. 2, Table 3). In the calcium FLIPR assay in other cells (Darker, et al., 2001), peptides 9 and 10 (Table 3) were reported to have to have a 170- and >5000-fold higher affinity for hGRPR than hNMBR and 1000- and 10000-fold for hGRPR over BRS-3. To be certain that our results did not differ from the previous study (Darker, et al., 2001) because of the different assays used in the two studies we assessed the ability of peptides 2,9,11 (Table 1) to cause mobilization of cellular calcium by assessing changes ^{45}Ca outflux (Figure 2) or cause increases in $[Ca^{2+}]_i$ using Fura-2. In both assays the EC50's for altering cellular calcium at the hGRPR and hNMBR for peptide 9 (0.5-1 nM), peptide 11 (1-3 nM) or peptide 2 (0.003 nM) did not differ which resembled our results with stimulating an increase in [^{3}H]IP (Table 3). Also similar to our results with [^{3}H]IP , their relative affinities for stimulating hBRS-3 were peptide 2 (EC50-0.3nM)>peptide 9, peptide 11 (Figure 2).

Because the substitution of alanine in the 12th position of [DPhe^{6}, βAla^{11},Phe^{13},Nle^{14}]Bn(6-14) was reported to result in hGRPR selective analogs with high

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affinity for hGRPR (Darker, et al., 2001), we synthesized 6 related peptides to explore the importance of substitution in this position for determining Bn receptor selectivity (Peptides 12-17, Table 3, Fig. 3). Four analogs in this group of peptides (Peptides 12, 13, 14, and 16, Table 3) had a substitution of Tyr^{12} or Phe^{12} instead of Ala^{12} in peptide 11 (Table 1) in place of the His^{12} in [DTyr^{6}, βAla^{11},Phe^{13},Nle^{14}]Bn(6-14). The substitution of a Tyr^{12} or Phe^{12} moiety (Peptides 12 and 14, Table 3) resulted in two peptides, that when compared to [DTyr^{6}, βAla^{11},Phe^{13},Nle^{14}]Bn(6-14) (Peptide 1, Table 1) had a 32- and 208-fold decrease, respectively, in affinity for the hGRPR and neither substitution increased its selectivity for hGRPR (Table 3, Fig. 3). In contrast to the effect on hGRPR, the substitution of Tyr^{12} resulted in an analog (Peptide 12, Table 3, Fig. 3) that showed some selectivity for hBRS-3 (10- and 60-fold selective for hBRS-3 over hGRPR and hNMBR, respectively) in binding studies. The hGRPR, hNMBR and hBRS-3 demonstrate different degrees of receptor spareness for stimulating changes in [3H]IP (i.3. peptide 2 demonstrates 3-, 14-, and 0-fold spareness,respectively)(Table 2). Because of the different receptor spareness that occurs with different human Bn receptors (Ryan, et al., 1998a;Ryan, et al., 1998b;Ryan, et al., 1999;Benya, et al., 1995), there was no selectivity in activating hBRS-3 over the other Bn receptors (Table 3, Fig. 3).

In a previous study (Mantey, et al., 1997) the presence of βAla^{11} was reported to be important for high affinity interaction with hBRS-3. To examine this point further, Peptide 14, with a βAla in position 11 and Peptide 13, without a βAla^{11} were compared. Both peptides had a marked decrease in affinity and potency for activating each human Bn receptor subtype, compared to [DTyr^{6}, βAla^{11},Phe^{13},Nle^{14}]Bn(6-14) (Peptide 1, Table 3). Each of the two analogs demonstrated similar decreases in affinity for each receptor subtype demonstrating that, for low affinity Bn analogs, the presence of the βAla^{11} did not have a greater effect on hBRS-3 affinity than the other human Bn receptors. Because N-terminal truncation of [DPhe^{6},

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βAla[11],Phe[13],Nle[14]]Bn(6-14) is reported to result in analogs that retain high affinity for the GRPR and are GRPR preferring (Darker, et al., 2001), we synthesized two N-terminal truncated analogs, [DPhe[7], βAla[11],Phe[13],Nle[14]]Bn(7-14) (Peptides 15, Table 3, Fig. 3) and [pGlu[8], βAla[11],Phe[13],Nle[14]]Bn(8-14) (Peptides 17, Table 3, Fig. 3). Each of these two analogs (Peptides 15, and 17, Table 3, Fig. 3) demonstrated >1000-fold decrease in affinity for each of the three human Bn receptors and had either no or minimal (< 3-fold) selectivity for the hGRPR. In general, the ability of each of the new analogs in this series (Peptides 9-17, Table 3, Figs. 2, 3) to activate the three human Bn receptors correlated with their binding affinities if differences in receptor spareness are considered, and no antagonists or partial agonists were found (Table 2, Figs. 2, 3).

Recently, three studies (Weber, et al., 2002;Weber, et al., 2003;Boyle, et al., 2005) which performed structure/activity studies on [DPhe[6], βAla[11],Phe[13],Nle[14]]Bn(6-14) using a calcium or FLIPR calcium assay have described a number of shortened analogs (Peptides 18, 19, 20 and 23, Table 4) with selectivity for hBRS-3. We synthesized these four peptides and compared their selectivity for the different human Bn receptors to two other [DTyr[6], βAla[11],Phe[13],Nle[14]]Bn(6-14) analogs, [DTyr[6], (R)Apa[11],Phe[13],Nle[14]]Bn(6-14) (Table 3, Peptide 21) and [DTyr[6], (R)Apa[11]-4Cl,Phe[13],Nle[14]]Bn(6-14) (Table 4, Peptide 22) (Mantey, et al., 2001;Mantey, et al., 2004) that we have previously reported were selective for the hBRS-3 (Table 3). As was seen in previous studies (Mantey, et al., 2001;Mantey, et al., 2004), [DTyr[6], (R)Apa[11],Phe[13],Nle[14]]Bn(6-14) (peptide 21, Table 4) and its chloro-substituted analog, [DTyr[6], (R)Apa[11],4ClPhe[13],Nle[14]]Bn(6-14) (Table 4, Peptide 22), when compared to the nonselective peptide, [DTyr[6], βAla[11],Phe[13],Nle[14]]Bn(6-14), had lower affinity for all the human Bn receptor subtypes but showed less of a decrease in affinity for hBRS-3, resulting in a 50-229-fold selectivity for the

hBRS-3 over the other human Bn receptors. In our assays we found two of the recently described hBRS-3 selective peptides shortened analogs of [DPhe^{6}, βAla^{11},Phe^{13},Nle^{14}]Bn(6-14) (Peptides 18 and 20, Table 4, Fig. 4) had very low affinities for each of the human Bn receptors (> 5 μM), whether analyzed by binding studies or by their abilities to activate phospholipase C and increased [^{3}H]IP generation. We found the reported hBRS-3 selective short peptide (Weber, et al., 2003) Peptide 19 had both very low affinity for hBRS-3 and the other two human Bn receptors (i. e. > 10 μM), and very low potency (> 5 μM) for activating any of the three human Bn receptors and stimulating phospholipase C (Table 4, Fig. 4). In a third study using a FLIPR calcium assay (Boyle, et al., 2005) Ac-Phe-Trp-Ala-His(τBzl)-Nip-Gly-Arg-NH_2, (Peptide 23, Table 4) was reported to have equal high affinity to [DPhe^{6}, βAla^{11},Phe^{13},Nle^{14}]Bn(6-14) for hBRS-3 and to have greater than 1800-fold selectivity for hBRS-3 over hGRPR or hNMBR. In our binding study Peptide 23 had 185-fold lower affinity than [DPhe^{6}, βAla^{11},Phe^{13},Nle^{14}]Bn(6-14) for hBRS-3 (Table 4, Fig. 4) . However, Peptide 23 had some selectivity for hBRS-3 because it had a 14-fold higher affinity for hBRS-3 than hNMBR and >20-fold higher affinity for hBRS-3 than hGRPR (Table 4, Fig. 4). It's potency for activating hBRS-3 was 30-fold greater than for activating hNMBR and 74-fold greater than for activating hGRPR. In comparison to the previously described most selective hBRS-3 agonists (Mantey, et al., 2001;Mantey, et al., 2004), (Table 4, analog 21) and [DTyr^{6}, (R)Apa^{11}-4Cl,Phe^{13},Nle^{14}]Bn(6-14) (Table 4, analog 22) Peptide 23 was also an agonist at each of the human Bn receptors stimulating activation of phospholipase C in each (Fig. 4, Table 4). In terms of relative selectivity for activating each human Bn receptor subtype, Peptide 22 had the greatest selectivity for hBRS-3 over hGRPR (i.e. 98-fold) followed by Peptide 23 (i.e. 74-fold) and Peptide 21 (9-fold) (Table 4, Fig. 4). For selectivity of hBRS-3 over hNMBR, Peptide 23 had greatest selectivity (30-fold) compared to 20-fold for Peptide 22 and 6-fold for Peptide 21 (Fig. 4, Table 4).

DISCUSSION

In this study we synthesized a series of bombesin (Bn)-related analogs using different strategies to attempt to identify selective ligands for Bn receptor subtypes(GRPR, NMBR BRS-3) as well as fully characterized ligands recently reported to have selectivity for hGRPR or hBRS-3. First we synthesized conformationally constrained analogs by inserting N-methyl substitutions into the Bn COOH terminus, the biologically active portion(Jensen and Coy, 1991;Lin, et al., 1996). Second, we synthesized analogs with either N-terminal truncations or amino acid substitutions in the position equivalent to His^{12} of Bn. The former strategy was used because previous studies concluded the COOH terminus of GRP/Bn exists in a folded conformation with an antiparallel β-pleated sheet structure and a β-bend centered on $glycine^{11}$ (Coy, et al., 1988;Erne and Schwyzer, 1987;Kull, Jr., et al., 1992). It was proposed that this structure is maintained by hydrogen bonding between the Trp^{8}carbonyl(C=O) and Val^{10} NH, the Val^{10}C=O and NH of Leu^{13} as well as NH of Val^{10} and Leu^{13}C=O (Coy, et al., 1988). N-methyl subsitutions should introduce conformational restriction, disrupt hydrogen bonding and thus have pronounced effects on the conformation of the COOH terminus (Lin, et al., 1996;Lin, et al., 1995). Substitution of N-methyl groups to produce conformationally restricted analogs has been widely used with analogs of somatostatin (Rajeswaran, et al., 2001), bradykinin (Reissmann, et al., 1996), CCK (Pradhan, et al., 1998), endothelin (Cody, et al., 1997), tachykinins (Wormser, et al., 1986), GIP (Hinke, et al., 2003), enkephalins (Penkler, et al., 1993), angiotensin (Khosla, et al., 1976), insulin (Ogawa, et al., 1987) and galanin (Rivera, et al., 1994). N-methyl substitution can result in analogs with enhanced selectivity, potency or enhanced stability (Wormser, et al., 1986;Schmidt, et al., 1995;Pradhan, et al., 1998;Lin, et al., 1990;Cody, et al., 1997). Previously the results of N-methyl substitutions in the COOH terminus of Bn(7-14) was reported (Horwell,

et al., 1996). However, Bn(7-14) only has high affinity equal to the native ligand, GRP, for the GRPR whereas it has a 20-fold lower affinity for the NMBR than NMB and has a very low affinity, similar to Bn, for the hBRS-3 receptor (Horwell, et al., 1996;Lin, et al., 1996). In the present study, to investigate the effect of N-methyl substitutions on affinity/selectivity for the three human Bn receptors we made the substitutions in analogs of [DPhe^{6}, βAla^{11},Phe^{13},Nle^{14}]Bn(6-14) which has high affinity for all human Bn receptors, as well as the FBB4, the GRPR, and the NMBR from a number of species (Mantey, et al., 1997;Pradhan, et al., 1998;Ryan, et al., 1998a;Ryan, et al., 1998b). For the hGRPR, N-methyl substitution in Ala^{9} or Val^{10} had the greatest effect in decreasing affinity(>1500-fold), whereas insertion into His^{12} or Nle^{14} caused a 100-220-fold decrease in affinity and insertion into βAla^{11} had almost no effect (i. e. 2-fold decrease). These results have both similarities and differences from the previously reported N-methyl scan of Bn(7-14) (Horwell, et al., 1996). Our results are similar in that substitution on Ala^{9}, Val^{10}, Nle^{14} or Met^{14} caused a marked decrease in hGRPR affinity, and substitutions on Trp^{8} resulted in a <100-fold affinity decrease. However, our results differ in that we found no effect from insertion of N-methyl into βAla^{11} and a 112-fold decrease in affinity with His ^{12}, whereas in the previous study (Horwell, et al., 1996), the reverse was found, with no change in affinity for insertion of N-methyl on His^{12}, but a 50-fold decrease for Gly^{11}. A number of our results are consistent with the previously proposed β-pleated sheet model which envisages a β bend at Gly^{11} for the Bn COOH terminus upon binding with the GRPR. The insertion of N-methyl into the 11th position should lead to the stabilization of this conformation which is consistent with our finding that this analog retains high affinity. In this model the insertion of N-methyl in Val^{10} would be expected to disrupt hydrogen bonding and markedly decrease affinity, as was found. Unfortunately, the pharmacology of N-methyl substituted analogs for hBRS-3 and hNMBR generally mirrored the changes in affinity with each substitution seen with the hGRPR.

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These results would support the conclusion that the active conformation of [DTyr^{6}, βAla^{11},Phe^{13},Nle^{14}]Bn(6-14) for interacting with each of the three human Bn receptors is generally similar and thus this approach was unsuccessful at yielding potent subtype selective ligands.

The second strategy used to identify possible selective Bn receptor subtypes ligands was to synthesize N-terminal truncated and/or His^{12} substituted analogs of [DTyr^{6}, βAla^{11},Phe^{13},Nle^{14}]Bn(6-14), because a recent study (Darker, et al., 2001) reported this approach yields potent GRPR-selective agonists. Six such analogs were synthesized. Three had His^{12} replaced by Tyr^{12}, Phe^{12}, and Ala^{12} and another three were made with various N-terminal truncations of [DTyr^{6}, βAla^{11} ,Phe^{13},Nle^{14}]Bn(6-14). This strategy did not yield any GRPR selective agonists, although two analogs [DTyr^{6}, βAla^{11}, Tyr^{12},Phe^{13},Nle^{14}]Bn(6-14)(Peptide 12, Table 2) and [DPhe^{6}, βAla^{11}, Phe^{12},Phe^{13},Nle^{14}]Bn(6-14)(Peptide 16, Table 2) had moderate selectivity for hBRS-3 over hGRPR (11-25-fold) and hNMBR (59-2361-fold). These latter analogs may prove useful as templates in discovering more selective hBRS-3 ligands, but this approach does not appear useful for making GRPR selective ligands.

The final strategy used to identify useful selective ligands was to fully characterize pharmacologically compounds that have been recently described as having selectivity for a Bn receptor subtype. This pharmacological characterization was performed because in the studies describing these selective compounds (Darker, et al., 2001;Weber, et al., 2002;Weber, et al., 2003;Boyle, et al., 2005) receptor affinity for each human receptor subtype was not determined from binding studies and also characterization of receptor potency by receptor subtype activation was often incomplete. This occurred because potencies were derived from calcium FLIPR assays and detailed dose-response curves as well as determining whether agonists were full or partial agonists were usually lacking or unclear. In the present study we performed complete dose-

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response curves with binding studies using a universal ligand, ^{125}I-[DTyr^{6},βAla^{11},Phe^{13},Nle^{14}]Bn(6-14), that had high affinity for all human Bn receptor subtypes (Pradhan, et al., 1998;Mantey, et al., 1997;Ryan, et al., 1998b) as well as determine their potencies to stimulating phospholipase C and for three selected peptides determined their abilities to alter cellular cytosolic calcium.

One study (Darker, et al., 2001) reported three truncated and His^{12} substituted analogs of [DPhe^{6},βAla^{11},Phe^{13},Nle^{14}]Bn(6-14) had hGRPR selectivity in the calcium FLIPR assay. [pGlu^{7},βAla^{11},Phe^{13},Nle^{14}]Bn(7-14)(Peptide 9, Table 2), [DPhe^{6},Ala^{8},βAla^{11},Phe^{13},Nle^{14}]Bn(6-14), (Peptide 10,Table 2) and [DPhe^{6},βAla^{11},Ala^{12},Phe^{13},Nle^{14}]Bn(6-14)(Peptide 11, Table 2) were reported to have affinities of 0.01-2 nM for the hGRPR and to have a 5570-, 4- and 170-fold greater affinity for hGRPR than hNMBR, respectively, and a 160-100,000-fold greater affinity for hGRPR than hBRS-3 (Darker, et al., 2001). In our binding studies each of these three analogs had relatively low affinity for hGRPR (63-5000 nM) and none was hGRPR-preferring. Each functioned as an agonist at each of the Bn receptor subtype, however, they had either no hGRPR selectivity(Peptides 9, 11, Table 2) or less than a 1-fold selectivity(Peptide 10). This difference from the previous study (Darker, et al., 2001) was not due to differences in the assays used, because when we assessed these peptides' abilities to cause changes in cellular calcium in our cells, no Bn receptor subtype selectivity was seen. These results suggest that some difference in the Bn receptor transfected cells used in these two studies (receptor number, G proteins, coupling, etc) likely is the reason for these differences. In previous studies (Benya, et al., 1995;Benya, et al., 1994;Benya, et al., 1992;Ryan, et al., 1998b;Ryan, et al., 1998a) we have demonstrated that Bn receptors stably expressed in BALB 3T3 cells, as used in the present study, behaved with similar pharmacology and cell activation to wild type nontransfected Bn receptors.

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The second group of possible selective Bn receptor ligands investigated was recently reported shortened, selective hBRS-3 ligands (Weber, et al., 2003;Weber, et al., 2002;Boyle, et al., 2005). Each of these ligands had been identified from structure-function studies on [DPhe^{6},βAla^{11},Phe^{13},Nle^{14}]Bn(6-14) which we and others have previously shown has a high affinity for hBRS-3, but is not selective for hBRS-3, because it also has high affinity for hGRPR and hNMBR (Mantey, et al., 1997;Mantey, et al., 2001;Pradhan, et al., 1998;Reubi, et al., 2002;Ryan, et al., 1998b). In the present study, four of these shortened [DTyr^{6},βAla^{11},Phe^{13},Nle^{14}]Bn(6-14) analogs, were synthesized: [H-DPhe-Gln-DTrp-NH$(CH_2)_2C_6H_5$(Peptide 18, Table 3 (Weber, et al., 2002); 3-phenyl-propionyl-Ala-DTrp-NH$(CH_2)_2C_6H_5$(Peptide 19, Table 3) (Weber, et al., 2003), HDPhe-Gl-DTrp-Phe-NH_2(Peptide # 20) (Weber, et al., 2002) and Ac-Phe-Trp-Ala-His(τBzl)-Nip-Gly-Arg-NH_2(Peptide 23, Table 3) (Boyle, et al., 2005) and fully characterize pharmacologically at each Bn receptor subtype. In the original studies (Weber, et al., 2002;Weber, et al., 2003;Boyle, et al., 2005) of these shortened analogs, the reported hBRS-3 selectivity was based primarily on results from calcium or calcium FLIPR studies. In these studies the above shortened analogs, were reported to have selectivity for hBRS-3 over hGRPR or hNMBR of 1941-fold(Peptide 19) (Weber, et al., 2003), 151-fold(Peptide 23) (Boyle, et al., 2005), >50-fold(Peptide 18) (Weber, et al., 2002), >3-fold (Peptide 20) (Weber, et al., 2002). Furthermore, Peptide 23 (Boyle, et al., 2005), and Peptide 19 (Weber, et al., 2003) were reported to retain similar high affinity (i.e. nanomolar range) to [DTyr^{6},βAla^{11},Phe^{13},Nle^{14}]Bn(6-14) for the hBRS-3. In the present study we found that three of these shortened [DTyr^{6},βAla^{11},Phe^{13},Nle^{14}]Bn(6-14) analogs, Peptide 18 (Weber, et al., 2002), Peptide 19 (Weber, et al., 2003) and Peptide 20 (Weber, et al., 2002) not only had very low affinities (i.e. $\geq$ 5 μM for hBRS-3), but also for hGRPR and hNMBR and therefore were not only not selective for hBRS-3, but had too low affinities to be useful. In contrast, Peptide 23 was

found to have moderate affinity for hBRS-3 (i.e. 259 nM) and also have 15-fold higher selectivity for hBRS-3 than hNMBR and 19-fold higher for hBRS-3 than for hGRPR. Furthermore, its potency for stimulating the hBRS-3 was >30-fold higher than for hNMBR or hGRPR. We have previously described two conformationally restrained analogs of [DTyr^{6},βAla^{11},Phe^{13},Nle^{14}]Bn(6-14) in which βAla^{11} was replaced by either aminopropionic acid (Peptide 21) (Mantey, et al., 2001) or 4-chloro-aminopropionic acid(Peptide 22) (Mantey, et al., 2004) which are hBRS-3 selective. We compared the affinities and selectivities of these two full-length analogs (Peptide 21,22) to that of the recently described shortened analog (Peptide 23), which also demonstrated hBRS-3 selectivity in our assays. The full length analogs (Peptides 21 and 22) had a 123- and 32-fold greater affinities for hBRS-3 than the shortened analog, Peptide 23, and they were 4- and 20-fold, respectively, more selective than Peptide 23 for the hBRS-3. Therefore, the full-length analogs with Apa^{11} substitution (Peptides 21 and 22) remain the most selective agonists for hBRS-3.

In conclusion, although this study does not identify more selective Bn receptor ligands, it provides important information and leads for future studies. It demonstrates selective incorporation of N-methyl moieties in Bn analogs does not yield selective analogs and that a number of truncated and position 12 substituted Bn analogs recently reported to be selective ligands or a series of new positions 12 substituted Bn analogs, were not high affinity selective Bn receptor ligands. However, the study demonstrates that the shortened analog, Peptide 23 represents an important lead compound with a novel structure that could be used as a template to develop more selective hBRS-3 ligand in the future.

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Footnotes

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FIGURE LEGENDS

Fig. 1. The ability of Bn and [DTyr^6,βAla^{11},Phe^{13},Nle^{14}]Bn(6-14) (Peptide # 1) with N-Methyl substituted analogs to inhibit binding and stimulate increase in [^{3}H]IP formation at the hGRPR, hNMBR and hBRS-3. In binding (top panel) Balb 3T3 cells stably transfected with hGRPR (0.3 X 10^6 cell/ml), hNMBR (0.03 X 10^6 cells/ml) or hBRS-3 (0.5 X 10^6 cells/ml) cells were incubated for 60 min at 22^{0}C with 50 pM I^{125}- [DTyr^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn(6-14), with or without the indicated concentrations of the various peptides added. Results are expressed as the percentage of saturable binding without unlabeled peptide added (percent control). Bottom panel, BALB 3T3 cells transfected with hGRPR, hNMBR or hBRS-3were subcultured and preincubated for 24 h at 37^{0}C with 3mCi/ml myo-[2-^{3}H]inositol. The cells were then incubated with the ligands at the concentrations indicated for 60 min at 37^{0}C. Values expressed are a percentage of total [^{3}H]IP release stimulated by 1 μM [DTyr^6,βAla^{11},Phe^{13},Nle^{14}]Bn(6-14). Control and 1 μM [DTyr^6, βAla^{11},Phe^{13},Nle^{14}]Bn(6-14)-stimulated values for hGRPR were 1510 ± 98 and 8200 ± 210 dpm, respectively; for hNMBR 1950 ± 82 and 22820 ± 540 dpm, respectively; and for hBRS-3 2510 ± 120 and 12600 ±2305 dpm, respectively. Results are the mean ± SEM from at least three experiments, and in each experiment the data points were determined in duplicate. Numbers refer to the peptide number in Table 1. Abbreviations: See Table 1 legend.

Fig. 2. The ability to interact with human Bn receptors of various analogs of [DPhe^6,βAla^{11},Phe^{13},Nle^{14}]Bn(6-14) reported to have selectivity for hGRPR, hNMBR and hBRS-3. The experimental conditions were similar to those outlined in the legend to Fig. 1 or as described in METHODS. The results in binding experiments (top panels) are expressed as the percentage of saturable binding without unlabeled peptide added (percent control). The results

for [^{3}H]IP (middle panels) and ^{45}Ca efflux (bottom panels) stimulation are expressed as percentages of the response stimulated by a maximally effective concentration of [DPhe^{6},βAla^{11},Phe^{13},Nle^{14}]Bn(6-14). Results are the mean ± SEM from at least three experiments, and in each experiment the data points were determined in duplicate. Numbers refer to the peptide number in Table 2.

Fig. 3. The affinities and potencies of various position 12 and truncated analogs of [DPhe^{6},βAla^{11},Phe^{13},Nle^{14}]Bn(6-14) for human GRP, NMB and BRS-3 receptors. The experimental conditions were similar to those outlined in the Fig. 1 legend and the results are means ± SEM from at least three experiments with each data point determined in duplicate. Numbers refer to peptide numbers in Table 2. Abbreviations: see Fig. 1 and legend of Table 1.

Fig. 4. Comparison of the ability of various short analogs and conformationally restricted analogues of [DPhe^{6},βAla^{11},Phe^{13},Nle^{14}]Bn(6-14) reported to have hBRS-3 selectivity to interact with human Bn receptors. The experimental conditions were similar to those outlined in the Fig. 1 legend and the results are means ± SEM from at least three experiments with each data point determined in duplicate. Numbers refer to peptide numbers in Table 3. Abbreviations: see Fig. 1 and legend of Table 1.

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Table 1. Peptide number and structure of peptides studied.

Peptide Number	Peptide Structure
Bn	pGlu,Gln,Arg,Leu,Gly,Asn,Gln,Trp,Ala,Val,Gly,His,Leu,Met-NH_2
1	[DTyr^{6}, βAla^{11},Phe^{13},Nle^{14}]Bn(6-14)
2	[DPhe^{6}, βAla^{11},Phe^{13},Nle^{14}]Bn(6-14)
3	[DTyr^{6},βAla^{11},Phe^{13},NMe-Nle^{14}]Bn(6-14)
4	[DTyr^{6},βAla^{11},Phe^{13},NMe-His^{12},Nle^{14}]Bn(6-14)
5	[DTyr^{6},NMe-βAla^{11},Phe^{13},Nle^{14}]Bn(6-14)
6	[DTyr^{6},NMe-Val^{10},βAla^{11},Phe^{13},Nle^{14}]Bn(6-14)
7	[DTyr^{6},NMe-Ala^{9}βAla^{11},Phe^{13},Nle^{14}]Bn(6-14)
8	NMeTrp^{8},βAla^{11},Phe^{13},Nle^{14}]Bn(8-14)
9	[pGlu^{7},βAla^{11},Phe^{13},Nle^{14}]Bn(7-14), [# 6 in (Darker, et al., 2001)]
10	[DPhe^{6},Ala^{8},βAla^{11},Phe^{13},Nle^{14}]Bn(6-14), [#11 in (Darker, et al., 2001)]
11	[DPhe^{6},βAla^{11},Ala^{12},Phe^{13},Nle^{14}]Bn(6-14) [#13 in (Darker, et al., 2001)]
12	[DTyr^{6},βAla^{11},Tyr^{12}Phe^{13},Nle^{14}]Bn(6-14)
13	[DTyr^{6},Phe^{12},Phe^{13},Nle^{14}]Bn(6-14)
14	[DTyr^{6},βAla^{11},Phe^{12}Phe^{13},Nle^{14}]Bn(6-14)
15	[DPhe^{7},βAla^{11},Phe^{13},Nle^{14}]Bn(7-14)
16	[DPhe^{6},βAla^{11},Phe^{12}, Phe^{13},Nle^{14}]Bn(6-14)
17	[pGlu^{8},βAla^{11},Phe^{13},Nle^{14}]Bn(8-14)
18	H-DPhe,Gln,DTrp,NH$(CH_2)_2C_6H_5$ [[#68 in (Weber, et al., 2002)]
19	3-phenyl-propionyl-Ala,DTrp,NH$(CH_2)_2C_6H_5$ [# 17d in (Weber, et al., 2003)]
20	H-DPhe,Gln,DTrp,Phe-NH_2 [# 54 in (Weber, et al., 2002)]
21	[DTyr^{6},(R)-Apa^{11},Phe^{13},Nle^{14}]Bn(6-14) [# 14 in (Mantey, et al., 2001)]
22	[DTyr^{6},(R)-Apa^{11}-4Cl,Phe^{13},Nle^{14}]Bn(6-14) [# 7 in (Mantey, et al., 2004)]
23	Ac-Phe,Trp,Ala,His(tBzl),Nip,Gly,Arg-NH_2 [# 34 in (Boyle, et al., 2005)]

Abbreviations: βAla, β-alanine; Nle, norleucine; NMe, N-Methyl; Bzl, Benzyl

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Table 2. The ability of human Bn receptors to interact with and be activated by bombesin, [DTyr^6, βAla^{11}, Phe^{13},Nle^{14}]Bn(6-14 and various analogues with N-methyl-substitutions.

	Bn-Receptor Transfected BALB 3T3 cells					
	K_i (nM)			Ec_{50} (nM)		
Peptide Number	hGRPR	hNMBR	hBRS-3	hGRPR	hNMBR	hBRS-3
Bn	0.36 ± 0.07	22 ± 1	>5000	0.45 ± 0.06	2.5 ± 0.1	>10000
1	0.53 ± 0.02	7.4 ± 0.2	0.54 ± 0.03	0.33 ± 0.01	0.18 ± 0.01	1.1 ± 0.1
2	0.63 ± 0.04	4.9 ± 0.6	1.4 ± 0.1	0.27 ± 0.01	0.50 ± 0.04	4.6 ± 0.3
3	132 ± 3	151 ± 5	79 ± 4	0.69 ± 0.01	0.35 ± 0.02	17 ± 1
4	56 ± 2	630 ± 20	204 ± 4	1.9 ± 0.1	6.2 ± 0.2	44.7 ± 1.6
5	1.0 ± 0.1	34.7 ± 1.2	8.1 ± 0.3	0.50 ± 0.02	0.61 ± 0.04	15.1 ± 0.9
6	832 ± 24	3630 ± 80	890 ± 41	112 ± 3	194 ± 12	760 ± 40
7	4360 ± 100	> 5000	>5000	255 ± 13	282 ± 15	> 5000
8	76 ± 11	1700 ± 170	115 ± 24	56 ± 5	66 ± 6	295 ± 31

Structures of peptides are listed in Table 1. Balb3T3 cells stably transfected with hGRPR (0.3 X 10^6 cells/ml), hNMBR (0.05 X 10^6 cells/ml) or hBRS-3 (0.5 X 10^6 cells/ml) were incubated with 50 pM iodinated [DTyr^6, βAla^{11},Phe^{13},Nle^{14}]Bn(6-14), with or without increasing concentrations of unlabeled ligand for 60 min at 22^{0}C as described in legend to Fig.1. The affinities of [DTyr^6,βAla^{11},Phe^{13},Nle^{14}]Bn(6-14)and Bn were calculated by a least-squares curve-fitting program (LIGAND). The remaining affinities were calculated using KaleidaGraph and the Cheng-Prusoff equation. All values are means ± SEM from at least three experiments. Balb 3T3 cells stably transfected with hBRS-3, hGRP-R or hNMB-R were incubated with [^{3}H]inositol and total [^{3}H]IP determined as described in Methods. For each peptide a dose-response curve was performed with concentrations from 0.01 nM to 1 μM. Results are expressed as the concentration causing one- half the maximal increase, EC_{50} seen with 1 uM peptide. Results were calculated from the dose-response curves shown in Fig. 1 for each peptide using KaleidaGraph. Each value is a mean ± SEM from at least three experiments. For hBRS-3/BALB 3T3 cells, the control and 1 μM DTyr^6,βAla^{11},Phe^{13},Nle^{14}]Bn(6-14) values were 10624 ± 571 dpm and 37660 ± 4106 dpm, respectively. For hGRP-R/BALB 3T3 cells, the control and 1 μM [DTyr^6,βAla^{11},Phe^{13},Nle^{14}]Bn(6-14) values were 9231 ± 2260 dpm and 43060 ± 9137 dpm, respectively. With hNMB-R/BALB 3T3 cells the control and 1 μM [DTyr^6,βAla^{11},Phe^{13},Nle^{14}]Bn(6-14) values were 2020 ± 166 dpm and 40667 ± 2371 dpm, respectively.

Abbreviations: βAla, β-alanine; Nle, norleucine; NMe, N-Methyl; Bzl, Benzyl

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Table 3**:** The affinities and potencies of human GRP, NMB and BRS-3 receptors for bombesin, [D-Tyr^{6}, βAla^{11},Phe^{13},Nle^{14}]Bn(6-14) and various synthetic peptides reported to be selective for BRS-3.

	Bn–Receptor transfected BALB 3T3 Cells					
	K_i (nM)			EC_{50} (nM)		
Peptide Number	hGRPR	hNMBR	hBRS-3	hGRPR	hNMBR	hBRS-3
Bn	0.36 ± 0.07	22.0 ± 0.9	>5000	0.45 ± 0.06	2.5 ±0.1	>5000
1	0.53 ± 0.02	4.9 ± 0.2	0.54 ± 0.03	0.33 ± 0.01	0.18 ± 0.01	1.1 ± 0.1
2	0.63 ± 0.04	7.4 ± 0.6	1.4 ± 0.1	0.27 ± 0.01	0.50 ± 0.04	4.6 ± 0.4
9	616 ± 45	1380 ± 100	890 ± 30	8.5 ± 0.5	7.8 ± 0.4	457 ± 14
10	63 ± 8	>5000	22.0 ± 1.9	28.2 ± 1.2	56 ± 2	186 ± 6
11	>5000	>5000	> 5000	977 ± 21	1020 ± 20	>5000
12	17.4 ± 0.5	95 ± 5	1.6 ± 0.1	9.0 ± 0.8	25 ± 3	11.7 ± 0.5
13	162 ± 6	1020 ± 60	316 ± 7	269 ± 12	58.9 ± 1.4	617 ± 17
14	110 ± 4	1300 ± 70	186 ± 7	270 ± 13	21.9 ± 0.9	6.4 ± 0.5
15	645 ± 39	>5000	1740 ± 40	12.0 ± 0.5	126 ± 3	880 ± 54
16	49.0 ± 1.5	>5000	1.9 ± 0.1	2.4 ± 0.4	5.4 ± 0.1	17.1 ± 1.9
17	>5000	>5000	>5000	>5000	>5000	>5000

The binding affinity (K_i) and the Ec_{50} for each peptide were determined as described in the legend for Table 1. . Structures of peptides are listed in Table 1. Balb 3T3 cells stably expressing hGRPR, hNMBR and hBRS-3 were incubated with 50 pM ^{125}I-ligand, with or without increasing concentrations of unlabeled ligand for 60 min at 22 ^{0}C as described in Fig. 1 Legend. The affinities were calculated using the Cheng-Prusoff equation. To assess phospholipase C activation, the cells were incubated with [^{3}H]inositol and total [^{3}H]IP was determined as stated in the methods. A dose-response curve was determined for each ligand with concentrations of 0.01 nM to 0.1 uM. For each ligand a concentration causing a half-maximal increase EC_{50} was calculated using KaleidaGraph. Each value is a mean ± 1 S.E.M from at least three experiments.

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Table 4. The affinities and potencies for human GRP, NMB and BRS-3 receptors of [DTyr^{6}, βAla^{11},Phe^{13},Nle^{14}]Bn(6-14), and various short synthetic bombesin

	Bn-Receptor Transfected BALB 3T3 Cells					
	K_i (nM)			EC_{50}(nM)		
Peptide Number	hGRPR	hNMBR	hBRS-3	hGRPR	hNMBR	hBRS-3
1	0.53 ± 0.02	4.9 ± 0.2	0.54 ± 0.03	0.33 ± 0.01	0.18 ± 0.01	1.0 ± 0.1
2	0.63 ± 0.04	7.4 ± 0.6	1.4 ± 0.1	0.27 ± 0.01	0.50 ± 0.04	4.6 ± 0.3
18	9500 ± 450	>10000	5030 ± 65	>5000	>5000	>5000
19	>10000	>10000	>10000	>5000	>5000	>5000
20	>10000	>10000	5000 ± 90	>5000	>5000	>5000
21	151 ± 6	2400 ± 100	2.8 ± 0.1	54 ± 1	38.1± 1.2	6.0 ± 0.2
22	1900 ± 70	7200 ± 800	8.2 ± 1.1	320 ± 20	71 ± 2	3.5 ± 0.2
23	>5000	3800 ± 90	259 ± 6	1500 ± 90	630 ± 30	21 ± 1

The binding affinity (K_i) and the Ec_{50} for each peptide were determined as described in the legend for Table 2 and are from the data in Fig. 4. Structures of peptides are listed in Table 1.

Abbreviations: Nip, piperdine-3 carboxylic acid; Apa, aminopropionic acid; Ac, Acetyl; Bzl, Benzyl.

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Fig. 1

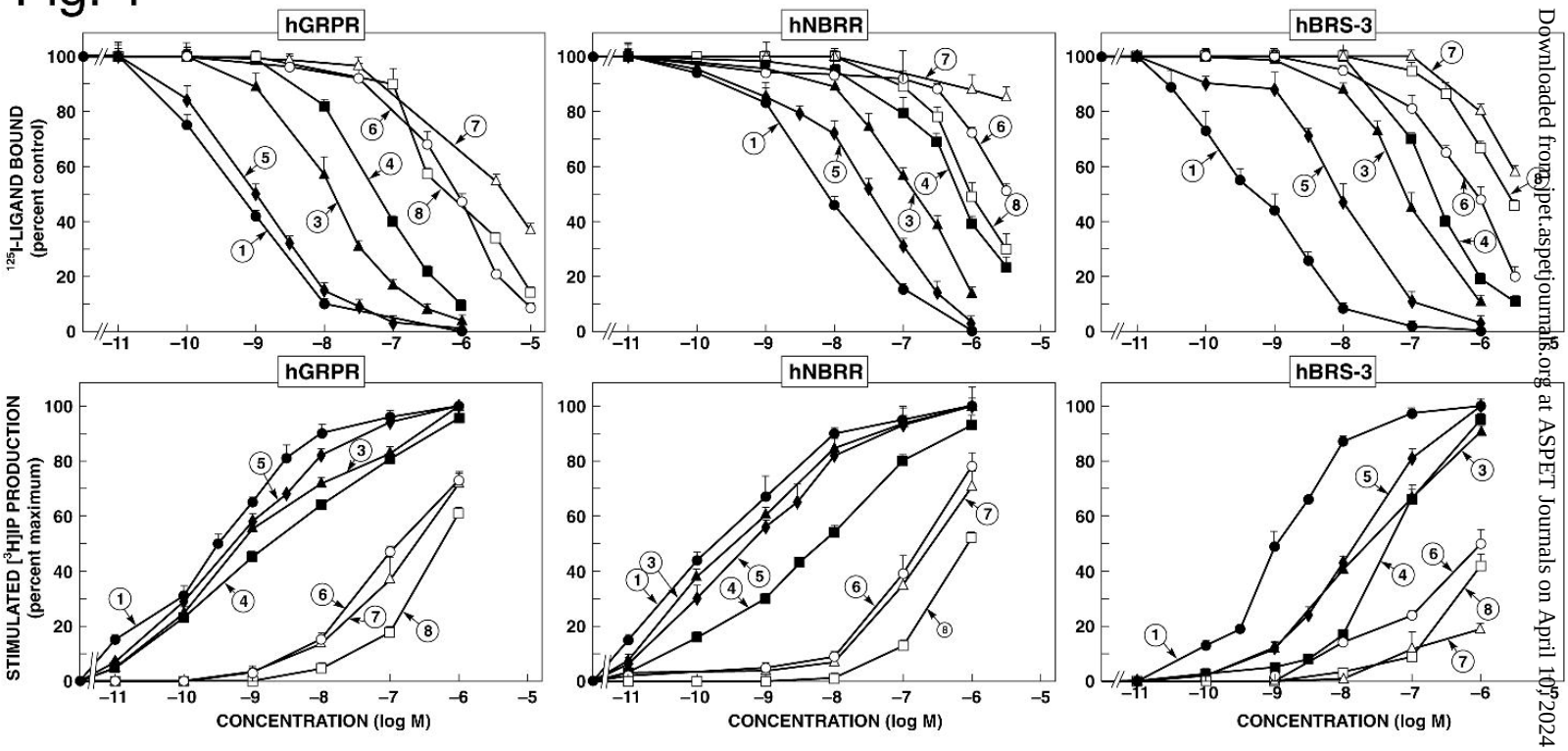

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Fig. 2

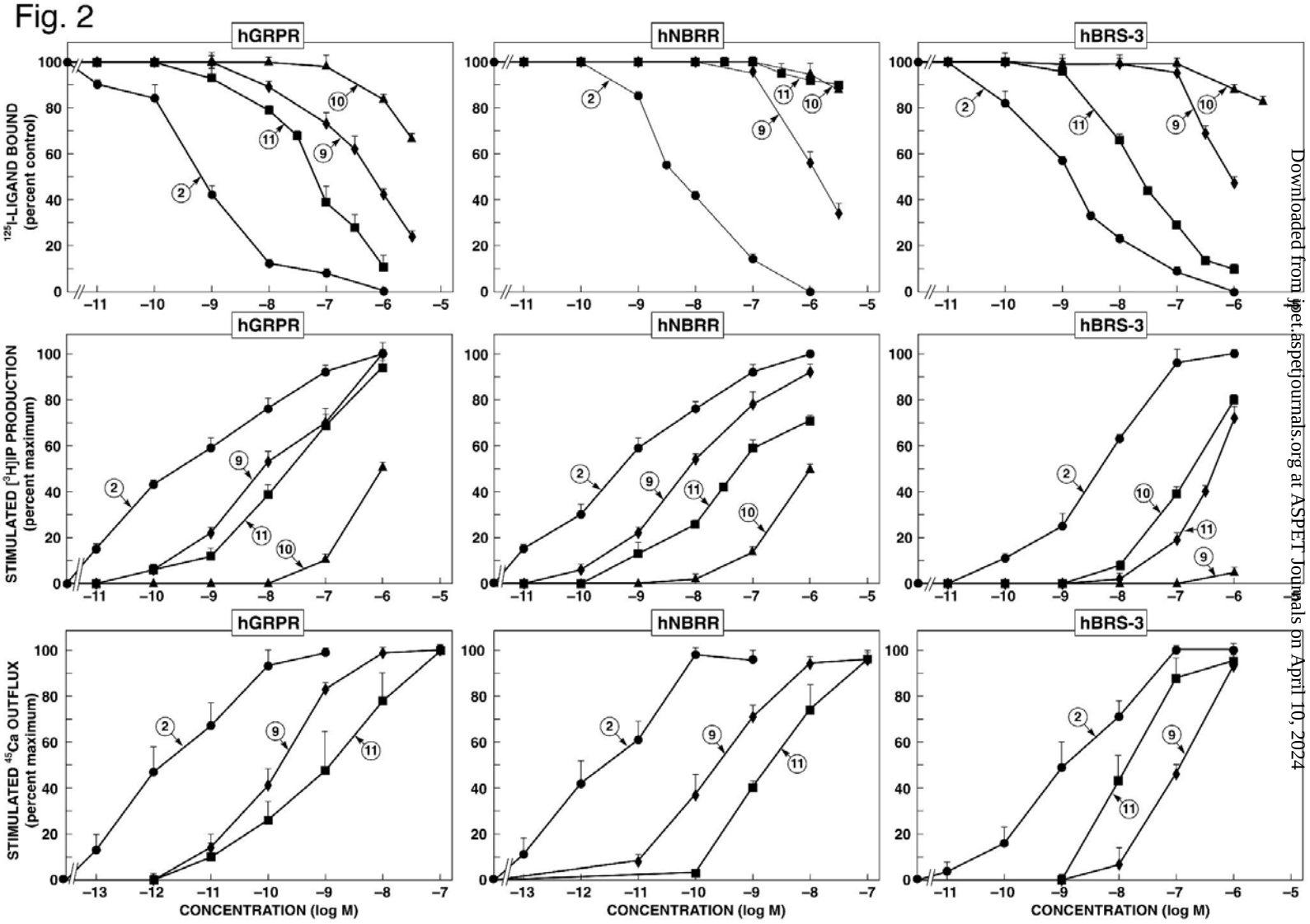

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Fig.3

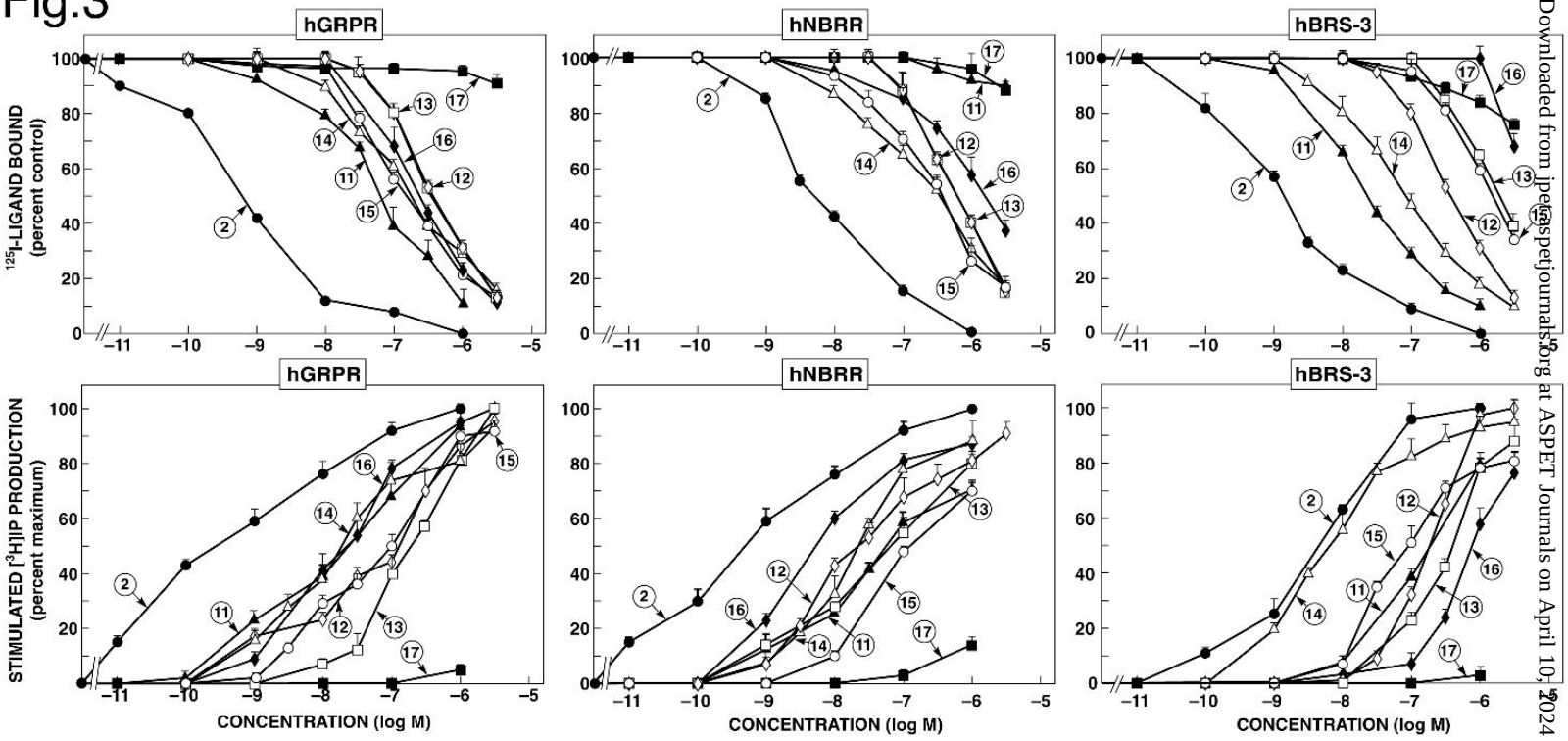

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Fig. 4