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Novel chiral-diazepines function as specific, selective receptor agonists with variable coupling and species variability in human, mouse and rat BRS-3 receptor cells

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ABSTRACT

Bombesin receptor subtype-3 (BRS-3) is an orphan G-protein coupled receptor which is classified in the bombesin receptor (BnR) family with which it shares high homology. It is present widely in the central nervous system and peripheral tissues and primarily receptor-knockout studies suggest it is involved in metabolic-glucose-insulin homeostasis, feeding and other CNS behaviors, gastrointestinal motility and cancer growth. However, the role of BRS-3 physiologically or in pathologic disorders has been not well defined because the natural ligand is unknown. Until recently, no selective agonists/antagonists were available; however, recently synthetic high-affinity agonists, chiral-diazepines nonpeptide-analogs (3F, 9D, 9F, 9G) with low CNS penetrance, were described, but are not well-categorized pharmacologically or in different labarotory species. The present study characterizes the affinities, potencies, selectivities of the chiral-diazepine BRS-3 agonists in human and rodents (mice,rat). In human BRS-3 receptors, the relative affinities of the chiral-diazepines was 9G > 9D > 9F > 3F; each was selective for BRS-3. For stimulating PLC activity, in h-BRS-3 each of the four chiral diazepine analogs was fully efficacious and their relative potencies were: 9G (EC_{50}: 9 nM) > 9D (EC_{50}: 9.4 nM) > 9F (EC_{50}: 39 nM) > 3F (EC_{50}: 48 nM). None of the four chiral diazepine analogs activated r,m,h-GRPR/NMBR. The nonpeptide agonists showed marked differences from each other and a peptide agonist in receptor-coupling-stiochiometry and in affinities/potencies in different species. These results demonstrate that chiral diazepine analogs (9G, 9D, 9F, 3F) have high/affinity/potency for the BRS-3 receptor in human and rodent cells, but different coupling-relationships and species differences from a peptide agonist.

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1. Introduction

Bombesin receptor subtype-3 (BRS-3) is an orphan G-protein-coupled orphan receptor present in the central nervous systems and peripheral tissues. Because of its 51% and 47% amino acid homology with the two human Bombesin (Bn) receptors, gastrin-releasing peptide receptor (GRPR) and neuromedin B receptor (NMBR), it is classified in the bombesin receptor (BnR) family [10,22,43].

BRS-3 is receiving increased attention because studies, primarily using BRS-3 receptor knockout mice, suggest it is involved in insulin, glucose, lipid and metabolic homeostasis [13,17,40,52]; appetite and regulation of food intake [13,25,40], behavioral regulation [13,67,68]; gastrointestinal motility [22]; lung development and injury [55,59] and regulation of growth of normal and neoplastic tissues, as well as oncogene expression [13,24,37,57,59,65,66]. This has resulted in BRS-3 receiving considerable attention for a

Abbreviations: AR42J cells, rat pancreatic acinar cells; BALB 3T3, mouse MIC embryonic fibroblast cells; Bn, bombesin; BnR, bombesin receptors; BRS-3, bombesin receptor subtype-3; BSA, bovine serum albumin fraction V; CNS, central nervous system; DMEM, Dulbecco's minimum essential medium; h, human; EC_{50}, concentration causing half-stimulation; FBS, fetal bovine serum; GPCR, G-protein-coupled receptor; GRP, gastrin-releasing peptide; GRPR, gastrin-releasing peptide receptor; IC_{50}, half maximal inhibitor concentration; IBMX, 3-isobutyl-1-methylxanthine; IP, inositol phosphate; m, mouse; MK-5046, nonpeptide BRS3 agonist; NMB, neuromedin B; NMBR, neuromedin B receptor; peptide #1, [D-Tyr^6, β-Ala^{11}, Phe^{13}, Nle^{14}]Bn-(6–14); PBS, phosphate buffered saline; PLC, phospholipase C; r, rat.

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possible therapeutic role in treatment of obesity and/or diabetes mellitus [13,29]. However, the exact role of BRS-3 in physiological and pathologic disorders has not been well defined, because the natural ligand is unknown [22], and, in contrast to the other Bn receptors, until recently, no selective agonists/antagonists were available [13,19,22,29,43,62,64].

In contrast to other Bn receptors, BRS-3 has a low affinity for all natural occurring Bn-related peptides [13,34,48,49]. The only known high-affinity peptide agonist for the h-BRS-3 is the synthetic peptide agonist [Tyr^6, β-Ala^{11}, Phe^{13}, Nle^{14}] Bn-(6–14), however this Bn-analog also has high affinity for GRPR and NMBR in different species [22,34,41,50,61] and has low affinity for the rat and mouse BRS-3 (r,m-BRS-3) [27]. Recent studies report that the non-peptide, MK-5046 is a potent and selective agonist in different species [18,44,54] and it also has potent effects on glucose/energy homeostasis [13,17,18,54]. However, in animal and human studies, MK-5046 in addition to having the desired effects on body weight, appetite and glucose/metabolic control, had cardiovascular and body temperature/thermogenesis side-effects [13,17,26,44,54]. It was proposed that these side effects could be due to a CNS action of MK-5046, because it is CNS permeable and BRS-3 receptors are widely distributed in the CNS [17,21,51,69]. To investigate this possibility further, recently, chiral-diazepine nonpeptide analogs with low CNS penetrance (3F, 9D, 9F, 9G) have been reported which are potent agonists for the human BRS-3 [35,36]. However, a full pharmacological characterization of their selectivity and affinities for human and rodent Bn receptors (mouse, rat) has not been done which is needed before detailed *in vivo* studies are performed. This characterization is particularly important with Bn-receptor agonists, because previously studies demonstrate this class of receptors show considerable species variation with agonists, as well as antagonists [8,22,28,61,63,64]. Furthermore, until recently, similar to many other gastrointestinal hormone/neurotransmitter G-protein-coupled receptors [12,20,38], no BRS-3-nonpeptide agonists existed and at present for those recently described, little is known of their pharmacology at these receptors. Therefore, in the present study, we have investigated the affinity and potency of these novel chiral-diazepine analogs for in human, rat and mouse BnR's (h,r,m-BnRs).

2. Materials and methods

2.1. Materials

BALB 3T3 cells (mouse fibroblast) and AR42J cells (rat pancreatic acinar cells) were from American Type Culture Collection (ATCC), Rockville, MD; Dulbecco's minimum essential medium (DMEM), phosphate-buffered saline (PBS), G418 sulfate, fetal bovine serum (FBS), penicillin, streptomycin and sodium pyruvate from Gibco Life Technology (Grand Island, NY); bacitracin, soybean trypsin inhibitor, 3-isobutyl-1-methylxanthine (IBMX), formic acid, ammonium formate, disodium tetraborate, and alumina were obtained from Sigma–Aldrich (St. Louis, MO); iodine-125 (100 mCi/ml) and *myo*-[2-3H]inositol were from PerkinElmer Life Sciences (Boston, MA); 1,2,4,6-tetrachloro-3-6-diphenylglycouril (Iodo-Gen) from Pierce Chemical Co. (Rockford, IL); AG 1-X8 resin from BIO-RAD (Richmond, CA); human brain cDNA and mouse hypothalamic and brain cDNA library were from Zyagen (San Diego, CA). Standard protected amino acids and other synthetic reagents were obtained from Bachem Bioscience Inc. (King of Prussia, PA). MK-5046,(2S)-1,1,1-trifluoro-2-[4-(1H-pyrazol-1-yl)phenyl]-3-(4-[1-(trifluoromethyl) cyclopropyl]methyl]-1H-imidazol-2-yl) propan-2-ol [54] was a gift from Merk, Sharp and Dohme (West Point, PA); 9f, [(5R)-4-((3-[(6-methylpyridin-3-yl)oxy]phenyl)acetyl)-8-(trifluoromethyl)-2,3,4,5-tetrahydro-1H-pyrido[2,3-e][1,4]diazepin-5-yl]acetic acid [36]; 9g, [(5R)-4-([3-(2-methylpropoxy)phenyl]acetyl)-8-(trifluoromethyl)-2,3,4,5-tetrahydro-1H-pyrido[2,3-e][1,4]diazepin-5-yl]acetic acid [36], 3F and 9D were gifts from Daiichi Sankyo Co. (Tokyo, Japan).

2.2. Stable transfections

h-BRS-3, h,m-GRPR and h,r-NMBR stably transfected into BALB cells were made as described previously [2–5,48]. r-BRS-3 receptor (r-BRS-3) was obtained by PCR from a rat brain cDNA library from Zyagen (San Diego, CA) and the m-BRS-3 receptor (m-BRS-3) and m-NMB receptor (m-NMBR) from a mouse hypothalamic and brain cDNA library Zyagen (San Diego, CA). The PCR product for r,m-BRS-3 and m-NMBR were inserted in pCMV6-Entry (OriGene, Rockville, MD) and they then subcloned into pcDNA3 at the EcoRI site. BALB cells were stably transfected with r,m-BRS-3 or m-NMBR as previous described [2–5,48]. Three days after transfection, cells were split in a ratio of 1:3, and the selection antibiotic G418 (Life Technologies, Grand Island, NY) was added to the regular growth medium at a concentration of 800 μg/ml. Single colonies were isolated 2 weeks later and expanded in growth medium containing G418 (300 μg/ml). Colonies containing m-NMBR were selected by binding studies using ^{125}I-[D-Tyr^6, β-Ala^{11}, Phe^{13}, Nle^{14}]Bn-(6–14). Colonies containing m-BRS-3 or r-BRS-3 were selected by assessing MK-5046-stimulated charge in [^{3}H]inositol phosphates (IP) performed as described below.

2.3. Cell culture

h,r,m-BRS-3 stably transfected BALB cells; h,m-GRPR stably transfected BALB cells; h,r,m-NMBR stably transfected BALB cells were made grown in DMEM media supplemented with 10% FBS, 100 U/ml of penicillin, 100 μg/ml of streptomycin and 300 μg/ml of G418 sulfate. AR42J cells containing native r-GRPR [31,47] were grown in DMEM media supplemented with 10% FBS, 100 U/ml of penicillin and 100 μg/ml of streptomycin. All cells were incubated at 37 °C in a 5% CO_2 atmosphere.

2.4. Preparation of ^{125}i-labeled peptides

^{125}I-[D-Tyr^6, β-Ala^{11}, Phe^{13}, Nle^{14}]Bn-(6–14), with specific activity of 2200 Ci/mM, was prepared by a modification of methods described elsewhere [33,34]. In brief, 0.8 μg of IODO-GEN (in 0.02 mg/ml chloroform) was transferred to a vial, dried under a stream of nitrogen, and washed with 100 μl of 0.5 M KH_2PO_4, pH 7.4. To the reaction vial 20 μl of 0.5 M KH_2PO_4, pH 7.4, 8 μg of peptide in 4 μl of water, and 2 mCi (20 μl) Na^{125}I were added, mixed gently, and incubated at room temperature for 6 min The incubation was stopped by the addition of 100 μl of distilled water. Radiolabeled peptide was separated using a Sep-Pak (Waters Associates, Milford, MA) and high-performance liquid chromatography as described previously elsewhere [34,50]. The radioligand was stored with 0.5% BSA at 20 °C.

2.5. Binding studies

Binding studies with m-BRS-3 and r-BRS-3 could not be performed because they have low affinity for the universal Bn ligand ^{125}I-[D-Tyr^6, β-Ala^{11}, Phe^{13}, Nle^{14}]Bn-(6–14), and no other suitable ligand is available [22,27]. h-BRS-3/BALB (0.5×10^6 cells/ml), h-GRPR/BALB (0.5×10^6 cells/ml), r-GRPR/AR42J (1×10^6 cells/ml), m-GRPR/BALB (0.5×10^6 cells/ml), h-NMBR/BALB (0.05×10^6 cells/ml), r-NMBR/BALB (0.2×10^6 cells/ml) and m-NMBR/BALB (0.8×10^6 cells/ml) cells were incubated for 60 min at 21 °C with 50 pM ^{125}I-[D-Tyr^6,