Published in final edited form as:

Biochem Biophys Res Commun. 2009 September 18; 387(2): 283–288. doi:10.1016/j.bbrc.2009.07.006.

Pharmacological characterization of a selective agonist for Bombesin Receptor Subtype - 3

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Abstract

Bombesin receptor subtype-3 (BRS-3) is an orphan G protein-coupled receptor in the bombesin receptor family that still awaits identification of its natural ligand. BRS-3 deficient mice develop a mild late-onset obesity with metabolic defects, implicating BRS-3 plays a role in feeding and metabolism. We describe here the pharmacological characterization of a synthetic compound, 16a, which serves as a potent agonist for BRS-3. This compound is selective for BRS-3 as it does not activate neuromedin B or gastrin-releasing peptide receptors, two most closely related bombesin receptors, as well as a series of other GPCRs. We assessed the receptor trafficking of BRS-3 and found that compound 16a promoted β -arrestin translocation to the cell membrane. Neither central nor peripheral administration of compound 16a affects locomotor activity in mice. Therefore compound 16a is a potential tool to study the function of the BRS-3 system *in vitro* and possibly *in vivo*.

Keywords

Bombesin receptor subtype-3; Agonist; Calcium mobilization; Receptor trafficking; β -Arrestin

1. Introduction

About 100 non-somatosensory G protein-coupled receptors (GPCRs) are “orphan receptors”: GPCRs whose endogenous ligands have not been identified [1] The physiological functions of these receptors are difficult to investigate directly due to our inability to activate them *in vivo*. Bombesin receptor subtype-3 (BRS-3, BB3) is a GPCR that has been resistant to deorphanization. First discovered in 1992 through homology screening approaches [2], BRS-3 was assigned to the bombesin receptor family for its high sequence similarity to two mammalian bombesin receptors: 47% to Neuromedin B receptor (NMB-R, BB1) and 51% to Gastrin-releasing peptide receptor (GRP-R, BB2) [2].

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Localization studies have shown that BRS-3 mRNA is present both in the central nervous system (CNS) and peripheral tissues [2,3,4,5]. In the periphery, BRS-3 mRNA is restricted to a few tissues, including the rat testis [2]. In the CNS, highest levels of expression were detected in the rat hypothalamus [5]. BRS-3-deficient mice develop a mild late-onset obesity, associated with metabolic defects [3], suggesting the BRS-3 system is involved in the regulation of feeding and metabolism.

Since BRS-3 does not interact with high affinity with any of the known natural agonists for other bombesin receptors, attempts were made trying to develop synthetic agonists [6]. One of the first compounds showing high affinity and efficacy for BRS-3 is [D-Tyr⁶, β-Ala¹¹, Phe¹³, Nle¹⁴]Bombesin-(6–14) (dY-Bn(6–14)), which made it possible to study the pharmacological profile of BRS-3 in detail [7]. It is now known that activation of BRS-3 leads to coupling with G_{q/11}-type proteins and activation of phospholipase C and D, eliciting downstream calcium mobilization, mitogen-activated protein kinase phosphorylation, Elk-1 activation, immediate nuclear oncogene activation, and activation of other tyrosine kinases [6].

dY-Bn(6–14) is, however, a non-specific agonist that activates also NMB-R and GRP-R with comparable affinity [6]. In 2003, Weber and colleagues synthesized a library of small molecular weight peptidomimetic compounds, many of which exhibited improved affinities and selectivity when screened against human BRS-3 (hBRS-3) [8]. Here we describe a detailed pharmacological characterization of one of their compounds, 16a, whose structure was identified as N1-(2-Phenylethyl)-(2R)-2-{[(1S)-1-(benzylcarboxamido)ethyl]carboxamido}-3-(1H-3-indolyl)propanamide, as shown in Fig. 1A [8].

Human, rat and mouse BRS-3 display different pharmacological properties despite their high similarities in the amino acid sequences [9]. Like all other synthetic BRS-3 agonists developed so far, compound 16a was designed and modified for activating hBRS-3 [8]. Its activities for rat BRS-3 (rBRS-3) and mouse BRS-3 (mBRS-3), however, remained unclear. Using a calcium mobilization assay (FLIPR: Fluorometric Imaging Plate Reader), we proved that compound 16a is indeed a highly potent and specific agonist for rBRS-3 and mBRS-3. We also tested its effect on receptor trafficking. When a GPCR is activated by its cognate ligand, it can be phosphorylated at intracellular domains by G protein-coupled receptor kinases (GRKs), promoting the recruitment of β-arrestins, which in turn leads to uncoupling of the receptor from G proteins and downstream trafficking, including receptor internalization [10]. β-arrestins are important in mediating receptor internalization and desensitization, but their interaction with BRS-3 had not been previously studied. When expressed in the standard HEK293 cellular system, BRS-3 has affinity for β-arrestin2 prior to agonist stimulation which can be enhanced by addition of agonist. Further, BRS-3 is not internalized in this cellular model. The studies presented herein may prove useful for future ligand development and screening efforts targeting BRS-3.

2. Materials and Methods

Animals

Male C57Bl/6 mice (National Cancer Institute, Bethesda, MD), 8 to 12 weeks old, were group housed (four animals per cage) under controlled conditions (temperature 21 °C ± 2 °C; relative humidity 50%–60%; 12 h light-dark cycle, lights on 6:00 AM) with free access to water and food. All animal experiments were approved by the Institutional Animal Care and Use Committee, University of California, Irvine.

Drugs and Reagents

Compound 16a was purchased from Bachem (Torrance, CA). dY-Bn(6–14), bombesin, NMB, orexin, dopamine, kisspeptin, melatonin were purchased from Phoenix (Burlingame, CA). Human, rat and mouse BRS-3 cDNAs were cloned from brain hypothalamus by homologous RT-PCR based on published sequences.

Measurement of intracellular calcium mobilization

Lipofectamine transfection reagents (Invitrogen, Carlsbad, CA) were used to transiently transfect Human embryonic kidney 293T (HEK293T) cells with human, rat, and mouse BRS-3 cDNAs following the manufacturer's instructions. Transfected cells were cultured overnight before seeding for calcium assay as described before and agonist-induced intracellular calcium influx was measured as described before [11]. Dose response curves for agonist activation were calculated from peak fluorescence values of triplicates, and EC₅₀ values were calculated with Prism software (GraphPad, San Diego, CA).

β-Arrestin translocation and receptor internalization

HEK293 cells were grown in Eagle's minimal essential medium (MEM, Mediatech, Herndon, VA) supplemented with 10% FBS as previously described [12]. Cells were co-transfected with rBRS-3 (8 μg of cDNA) or an N-terminally tagged (haemagglutinin (HA)) rBRS-3 (8 μg) and β-arrestin2 tagged with green fluorescent protein (βarr2-GFP) (2ug) by electroporation using Gene Pulser II system (BIO-RAD, Hercules, CA) as described before [12]. In some studies, GRK2 was also expressed (2 μg). Cells were plated in collagen-coated 35 mm glass-bottomed culture dishes at approximately 0.5×10^6 cells / dish. The media was replaced with MEM lacking phenol red and serum for 30 min prior to each experiment. In some assays cells were treated with Alexafluor 488- or 568- conjugated anti-HA antibody (1:200, 15 min; Invitrogen) prior to agonist treatment to allow visualization of receptor internalization in live cells [12]. BRS-3 agonist compound 16a (1 μM) was added directly to the culture media. Cells were visualized under a confocal microscope with green-helium neon and argon lasers (Olympus, Tokyo, Japan). Images were collected sequentially using single-line excitation. Multiple cells were recorded per dish and representative cells are shown.

Spontaneous Locomotor Activity

Locomotion of mice was monitored in an automated activity system equipped with infrared (IR) sensors for both horizontal and vertical activity measurements (Versamax; AccuScan Instruments, Inc., Columbus, OH). Non-habituated mice were injected intraperitoneally (i.p.) with 10 mg/kg compound 16a or vehicle (10% Tween 20, 10 ml/kg). Locomotor activity was recorded for 90 min after i.p. injections. Horizontal activity represents IR beam breaks in the *x* and *y* dimensions, and stereotypic behavior is defined as repetitive breaks of a single beam that is not followed by a consecutive beam break of an adjacent sensor. Another group of mice were briefly anesthetized with halothane and then intracerebroventricularly (i.c.v.) injected with 5 nmole compound 16a or vehicle (2 μl of 10% DMSO, 1% Tween 80) as described before [13]. Recording of locomotor activity began 5 min after the injections and continued for 90 min.

Statistical Analysis

Statistics were performed using Prism software (GraphPad software, Inc., San Diego, CA). Locomotor behavior was analyzed by two-way analysis of variance (ANOVA), followed by Bonferroni's post-tests, where appropriate, *P* < 0.05 was considered statistically significant.

3. Results

3.1 Compound 16a activates human BRS-3 with high potency

BRS-3 is coupled to Gαq types of heterotrimeric G proteins, whose activation results in downstream calcium signaling. When applied to HEK293T cells transiently expressing hBRS-3, compound 16a caused a dose-dependent increase of intracellular free calcium concentration, with an EC₅₀ of 14.15 ± 0.13 nM (Fig. 1B, Table 1). As a positive control, dY-Bn(6–14) also showed potency at hBRS-3 with an EC₅₀ of 7.10 ± 0.17 nM (Fig. 1B, Table 1). Bombesin, on the other hand, activates hBRS-3 very poorly (Table 1). NMB and GRP were also tested but did not show activity even at 10 μM concentrations (Table 1).

3.2 Compound 16a activates rat and mouse BRS-3

The structure of compound 16a was optimized for activating hBRS-3 [8]. Its activity at rat and mouse BRS-3 was unknown. We show that, similar to what was observed with hBRS-3, compound 16a caused a dose-dependent increase in intracellular calcium concentration in HEK293T cells transiently expressing rBRS-3 (EC₅₀ = 109.90 ± 0.21 nM, Fig. 2A) and mBRS-3 (EC₅₀ = 33.30 ± 0.14 nM, Fig. 2B). Neither bombesin nor dY-Bn(6–14) showed activities comparable to that of compound 16a at the two rodent BRS-3 receptors (Table 1).

3.3 Compound 16a is selective for BRS-3

In order to investigate the specificity of compound 16a for BRS-3, we compared compound 16a-induced calcium mobilization on HEK293T cells transiently transfected with rNMB-R or hGRP-R. The quality of rNMB-R transfection was tested by its endogenous ligand NMB, which showed an EC₅₀ of 22.18 ± 0.15 nM in FLIPR (Fig. 2C). The quality of hGRP-R transfection was confirmed when bombesin, a potent agonist for hGRP-R, elicited a strong calcium response with an EC₅₀ of 1.82 ± 0.28 nM (Fig. 2D). In contrast, compound 16a did not cause any calcium release in these cells, even at 10 μM. Besides NMB-R and GRP-R, we also tested compound 16a on various other GPCRs: GPR54, MCH1 receptor, D₂ dopamine receptor, orexin receptor 1, MT₁ melatonin receptor. Compound 16a showed no activity at these receptors, as summarized in Table 2.

3.4 Compound 16a promotes β-arrestin translocation to the cell membrane

To study the interactions between BRS-3 and β-arrestin, we co-transfected HEK293 cells with rBRS-3 or an HA-tagged rBRS-3 and a GFP-tagged β-arrestin2 construct with and without GRK2 overexpression. Agonist-induced β-arrestin2 translocation was assessed by confocal imaging of live cells and images were captured at the times indicated following addition of 1 μM of compound 16a. In many cells, β-arrestin2 was pre-associated with the cytoplasmic membranes when HA-rBRS-3 was co-expressed (Fig. 3A). A similar profile was observed for the un-tagged rBRS-3 (data not shown). In cells that did not reveal pre-association, agonist-induced translocation of βarr2-GFP into puncta at the cell membrane was enhanced over the 5 min treatment period. In an attempt to improve the robustness of the translocation assay, we overexpressed GRK2 with βarr2-GFP and the untagged rBRS-3 [10]. When GRK2 is over expressed, we still observed pre-association between rBRS-3 and βarr2-GFP and the addition of agonist produced a change in cell shape and increased the recruitment of increased the recruitment of β arr2-GFP over time (Fig. 3B). These observations suggest that the receptor has affinity for βarr2-GFP in the basal state and that the use of β-arrestin translocation assays in drug discovery may be difficult due to this high basal level of interaction.

To investigate whether BRS-3 is internalized after agonist treatment, we transiently transfected HEK293 cells with a rBRS-3 construct tagged at the N-terminus with an HA tag, allowing

antibody detection with an Alexafluor 488-conjugated anti-HA antibody in live cells. There was no visible internalization of the receptor following up to 2 hours of compound 16a treatment (Fig. 3C). Additional studies wherein immunohistochemical analysis was performed on fixed and permeabilized cells following agonist treatment also did not reveal receptor internalization (data not shown).

3.5 Central and peripheral injections of compound 16a do not affect locomotor activity

As a first test to determine whether compound 16a exhibits any adverse effects *in vivo*, we examined the locomotor activity of mice after compound 16a administration, for locomotion is one of the most direct indications of drug toxicity. Because compound 16a does not cross blood-brain barrier (data not shown), we injected mice either i.p. or i.c.v. to evaluate separately the central and peripheral effects of compound 16a. Mice injected i.p. with 10 mg/kg of compound 16a did not show any changes in horizontal or stereotypical movements compared to vehicle treated animals (Fig. 4A). We also i.c.v. injected 5 nmol of compound 16a or vehicle and observed no differences in distance traveled or stereotypical movements between the two treatment groups at any time points after injection (Fig. 4B). This indicates that compound 16a might not have evident adverse effects.

4. Discussion

The development of synthetic agonists can greatly expand our knowledge of novel receptor systems. In the case of the BRS-3 system most of these studies have focused on the signaling mechanisms of BRS-3 activation *in vitro*, using cell lines that express BRS-3 [6]. Direct assessment of the physiological effects of BRS-3 activation *in vivo* has not been reported. In this study, we characterized a new BRS-3 agonist, compound 16a, and compared its activities at the human, rat and mouse BRS-3.

In agreement with a previous report [8], we confirmed the high potency of compound 16a at hBRS-3, which is similar to that of dY-Bn(6–14). When we transiently transfected HEK293T cells with rat and mouse BRS-3, dY-Bn(6–14) showed more than 1000 fold reduction in its ability to induce cytosolic calcium mobilization, with EC₅₀ values in the micromolar range (Fig 2, Table 1). On the contrary, the calcium mobilization effects of compound 16a showed only a slight reduction when tested on rat and mouse BRS-3 (Fig. 2, Table 1). Therefore, compound 16a is a stronger agonist than dY-Bn(6–14) at rat and mouse BRS-3.

The species-dependent difference in the pharmacological profiles of BRS-3 has been reported earlier using dY-Bn(6–14) [4]. The difference in the binding affinities of dY-Bn(6–14) to rat and human BRS-3 was thought to result from differences in the third extracellular loops [4]. Due to the lack of a radiolabeled compound 16a analogue, it is not possible to assess its binding affinity to BRS-3. It would be interesting to use compound 16a in mutagenesis studies to identify which amino acids are key to its activity. It is possible that the binding site for compound 16a at rat and mouse BRS-3 is different from that for dY-Bn(6–14). But compound 16a's high potency at activating human, rat and mouse BRS-3 suggests that the differences in pharmacological profiles between human and rat BRS-3 as previously reported might be agonist-specific.

There are no previous reports on the modulatory processes of BRS-3 signaling. Nothing is known about desensitization, down-regulation or internalization of the receptor after agonist activation. It is known that agonist-induced receptor activation can lead to the translocation of β -arrestin2 to the cell membrane and subsequent interaction with GPCRs [10]. Cells expressing BRS-3 exhibit β -arrestin2 translocation to the membrane prior to treatment. In some cells, translocation could be enhanced by the addition of compound 16a. The pre-association between β -arrestin2 and BSR-3 could indicate that BRS-3 may be constitutively active or desensitized.

Most GPCRs exist in a conformational equilibrium between active state and inactive state [14]. The addition of a cognate ligand stabilizes the active conformation, thus shifting the equilibrium. In the absence of a ligand, however, most GPCRs still promote a basal level of nucleotide exchange by the associated G proteins and therefore exhibit basal activities. In the case of BRS-3, the enhanced basal interaction with β -arrestin2 should be considered when developing assays to evaluate ligands at this particular receptor [15].

We did not detect BRS-3 internalization following compound 16a treatment. This was somewhat surprising considering that both NMB-R and GRP-R internalize rapidly after agonist activation [6]. However, the weak β -arrestin2 interaction following stimulation could contribute to this phenomenon, much like morphine's effects at the mu opioid receptor [16]. Since an HA tagged rBRS-3 was used in the study, it is possible that the HA tag fused to the N-terminus of the receptor locks the receptor in a certain conformation that prevents internalization, although HA-rBRS-3 responds to compound 16a in the calcium mobilization assay with similar potency as the wildtype receptor ($EC_{50} = 27.80 \pm 0.45$ nM, data not shown) and also recruits β -arrestin2 (Fig. 3A).

In conclusion, we have characterized pharmacologically the synthetic peptidomimetic compound 16a as a selective and highly potent agonist for human, rat and mouse BRS-3. It will be interesting to use compound 16a to assess whether *in vivo* activation of BRS-3 affects feeding behavior in rodents, and hopefully to unravel the mechanisms of how the absence of BRS-3 contributes to the obesity phenotype in BRS-3-deficient mice.

Acknowledgments

This work was supported by grants from National Institute of Health: MH60231, DK63001 and DA024746. We also thank Dr. Rainer Reinscheid at UC Irvine for providing us with the locomotion system and for critiquing the manuscript.

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A **B.** **Fig. 1.**

(A) Structure of compound 16a [8]. $R^1 = \text{CH}_3$, $R^2 = \text{Benzyl}$. (B) Comparison of the calcium mobilization effects of compound 16a, dY-Bn(6–14), and bombesin on HEK293T cells transiently transfected with hBRS-3. Compound 16a has comparable potency as dY-Bn(6–14). Bombesin activates hBRS-3 with low potency. All data represent at least three experiments in triplicates. Data are shown as mean $\pm$ S.E.M. Dose-response curves were generated using Prism. See Table 1 for comparisons of EC_{50} values.

Fig. 2. Comparison of the calcium mobilization effects of compound 16a on BRS-3, NMB-R and GRP-R. (A) and (B), compound 16a, dY-Bn(6–14), and bombesin at rat (A) and mouse (B) BRS-3 transiently transfected in HEK293T cells. Compound 16a shows greater potency than bombesin or dY-Bn(6–14). (C) HEK293T cells were transiently transfected with rNMB-R. At concentrations as high as 10 μ M, compound 16a failed to elicit intracellular calcium release, while NMB caused a dose-dependent increase in intracellular calcium. (D) HEK293T cells were transiently transfected with hGRP-R. Compound 16a had no effect on intracellular calcium release, even at 10 μ M, while bombesin, a potent agonist at GRP-R, produced a dose-dependent receptor activation. All data represent at least three experiments in triplicates. Data are shown as mean $\pm$ S.E.M. Dose-response curves were generated using Prism. See Table 1 for comparison of EC₅₀ values.

Fig. 3.

Agonist-induced β -arrestin2-GFP translocation and BRS-3 internalization in HEK293 cells. (A) HEK293 cells were transiently transfected with HA-tagged rBRS-3 and GFP tagged β -arrestin2 (β arr2-GFP). Confocal microscopy was used to obtain real-time imaging of the cells before (first two panels at time 0 (T=0)) and after 1 μ M agonist treatment at room temperature (times indicated in minutes). The first panel represents cell surface staining of Alexafluor 568-conjugated anti-HA antibody to demonstrate that the receptor is co-expressed with the β arr2-GFP. The top row demonstrates how some cells show puncta formation prior to drug treatment, while other cells (inset enlarged 2X below) reveal translocation after compound 16a (1 μ M) treatment within 5 min (second row, white arrow, punctuate accumulation at membrane). (B) Overexpression of GRK2 improves the detection of β arr2-GFP translocation in HEK293 cells expressing untagged BRS-3 and β arr2-GFP. (C) Treatment with compound 16a (1 μ M, up to 2 h) does not lead to internalization of the N-terminally HA-tagged rBRS-3 as visualized by Alexafluor 488-conjugated anti-HA antibody labeling of live cells.

Fig. 4.

Effects of compound 16a administration on locomotor activity in mice. (A) Locomotion of mice after i.p. administration of vehicle (10% Tween 20) or 10 mg/kg of compound 16a. Distance traveled and stereotypic counts were monitored for 90 min after i.p. administration (each data point represents mean $\pm$ S.E.M., each treatment group $n=7$). (B) Effects of i.c.v. administration of compound 16a on distance traveled and stereotypic counts. Mice were monitored for 90 min after the administration of 2 μ l of vehicle (10% DMSO, 1% Tween 80, $n=4$) or 5 nmol of compound 16a ($n=5$).

Table 1

Comparison of Agonist Activity in Functional FLIPR Assays

Agonists	EC ₅₀ (nM) ± S.E.M.		
	Rat BRS-3	Mouse BRS-3	Human BRS-3
Compound 16a	109.90 ± 0.21	33.30 ± 0.14	14.15 ± 0.13
dY-Bn(6–14)	12490 ± 0.42	9112 ± 0.52	7.10 ± 0.17
Bombesin	> 10,000	> 10,000	> 10,000
GRP	> 10,000	> 10,000	> 10,000
NMB	> 10,000	>10,000	> 10,000

EC₅₀ values (nM) for stimulation of intracellular Ca²⁺ release by compound 16a, Bombesin, dY-Bn(6–14), GRP and NMB at rat, mouse, human BRS-3 receptors transiently transfected in HEK293T cells. EC₅₀ values were calculated from triplicate incubations. Data from representative experiments are shown.

Table 2

Selectivity profile of compound 16a

Receptor	Endogenous Agonist	Activity of compound 16a
rNMB-R	NMB	No Activity
hGRP-R	GRP	No Activity
rGPR54	Kisspeptin	No Activity
hMCH1 Receptor	MCH	No Activity
hOrexin Receptor-1	Orexin	No Activity
hD ₂ Dopamine Receptor	Dopamine	No Activity
hMT1 Melatonin Receptor	Melatonin	No Activity

No calcium mobilization effect of compound 16a up to 10 μ M was found when tested on various GPCRs. GPR54 was stably transfected in CHO cells. MCH1 receptor was transiently transfected in HEK293T cells. Orexin receptor-1, D₂ dopamine receptor and MT1 melatonin receptor were transiently transfected in HEK293T cells expressing the promiscuous G α 15 which couples to most GPCRs to activate calcium signaling. All data represent at least three experiments in triplicates.