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Pharmacological characterization of a selective agonist for bombesin receptor subtype-3

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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*.

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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].

Localization studies have shown that BRS-3 mRNA is present both in the central nervous system (CNS) and peripheral tissues [2–5]. 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-Tyr^6, β-Ala^{11}, Phe^{13}, Nle^{14}]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 [4]. 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

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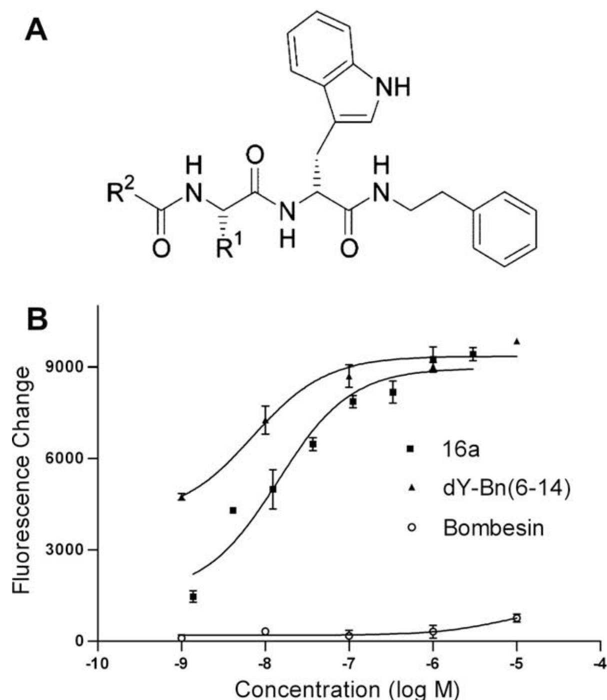

Fig. 1. (A) Structure of compound 16a [8]. R^1 = CH3, R^2 = Benzyl. (B) Comparison of the calcium mobilization effects of compound 16a, dY-Bn(6-14), and bombesin on HEK293T cells transiently transfected with hBRS-3. Compound 16a has comparable potency as dY-Bn(6-14). Bombesin activates hBRS-3 with low potency. All data represent at least three experiments in triplicates. Data are shown as means ± SEM. Dose–response curves were generated using Prism. See Table 1 for comparisons of EC_{50} values.

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 [9]. 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.

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 [10]. Dose–response curves for agonist activation were calculated from peak fluorescence values of triplicates, and EC_{50} 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 [11]. 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) (2 μg) by electroporation using Gene Pulser II system (BIO-RAD, Hercules, CA) as described before [11]. In come 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 [11]. 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 nmol compound 16a or vehicle (2 μl of 10% DMSO, 1% Tween 80) as described before [12]. 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.

Results

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 EC_{50} 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 EC_{50} of 7.10 ± 0.17 nM (Fig. 1B, Table 1). Bombesin, on the other hand, activates hBRS-3 very poorly (Table 1). NMB and

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