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Systematic Optimization of a Lead-Structure Identities for a Selective Short Peptide Agonist for the Human Orphan Receptor BRS-3

DIRK WEBER,[a] CLAUDIA BERGER,[b] TIMO HEINRICH,[b,1] PETER EICKELMANN,[b] JOCHEN ANTEL[b] and HORST KESSLER[a]*

[a] Institut für Organische Chemie und Biochemie, Technische Universität München, Lichtenbergstrasse 4, 85747 Garching, Germany
[b] Solvay Pharmaceuticals GmbH, Hans-Böckler-Allee 20, 30173 Hannover, Germany

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Abstract: The orphan receptor, human bombesin receptor subtype 3 (BRS-3) was assigned to the G-protein coupled bombesin receptor family because of its high sequence homology with the neuromedin B receptor (NMB-R) and gastrin-releasing peptide receptor (GRP-R). Since its pharmacology is still unknown, new highly potent and selective tool-substances are needed, that may be able to elucidate its possible role in obesity and cancer.

We have performed structure activity relationship studies on the high affinity peptide agonists [D-Phe^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn(6–14) and [D-Phe^6,Phe^{13}]Bn(6–13)propylamide, using their ability to mobilize intracellular calcium in BRS-3 transfected CHOGα-16 cells combined with receptor binding studies. It was demonstrated that for [D-Phe^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn(6–14) the side chains of the residues Trp^8 and Phe^{13}, and to a smaller extent β-Ala^{11}, are the important amino acid side chains for receptor activation and binding, however for [D-Phe^6,Phe^{13}]Bn(6–13) propylamide His^{12} seems to be more important than Phe^{13}. *C*- and *N*-terminal deletions and amino acid substitutions allowed further understanding. It was demonstrated that substitution of His^{12} by Tyr leads to a high selectivity towards GRP-R. Using the acquired information, a small tetrapeptide library was designed with compounds presenting Trp and Phe at varying stereochemistry and distances, which led to the discovery of the lead-structure H-D-Phe-Gln-D-Trp-Phe-NH_2. Systematic SAR revealed the important structural features of this peptide, *C*-terminal optimization resulted in the highly active and selective BRS-3 agonist H-D-Phe-Gln-D-Trp-1-(2-phenylethyl)amide. In summary, the size of the peptide was reduced from 8 or 9 amino acids to a tripeptide for BRS-3.

Keywords: BRS-3 ligands; orphan receptor; tetrapeptide mini-library; selective short peptide agonist

Abbreviations: Apa, (R,S)-3-amino-3-phenylpropionic acid; BB-R_4, bombesin receptor subtype 4; BLP, bombesin-like peptide; Bn, bombesin; BRS-3, bombesin receptor subtype 3; $[Ca^{2+}]_i$, intracellular calcium; CHO, chinese hamster ovary; DCE, dichloroethane; FLIPR, fluorometric imaging plate reader; FMPE, 2-(4-formyl-3-methoxyphenoxy)ethyl; GI, gastrointestinal; GPCR, G-protein coupled receptor; GRP, gastrin-releasing peptide; GRP-R gastrin-releasing peptide receptor; NMB, neuromedin B; NMB-R, neuromedin B receptor; propylamide, n-propylamide; SAR, structure activity relation; SCLC, small cell lung cancer; TIPS, triisopropylsilyl; TMOF, trimethylorthoformate.

* Correspondence to: Horst Kessler, Lichtenbergstrasse 4, D-85747 Garching, Germany; e-mail: kessler@ch.tum.de

[1] Present Address: Merck KGaA, Frankfurter Strasse 250, 64293 Darmstadt, Germany.

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INTRODUCTION

The orphan receptor, bombesin receptor subtype 3 (BRS-3) was identified as a member of the bombesin (Bn) receptor family since it shares 51% and 47% amino acid sequence homology with the two mammalian bombesin receptors, neuromedin B receptor (NMB-R) and gastrin releasing peptide receptor (GRP-R), respectively [1,2].

Unlike NMB-R and GRP-R, which are widely expressed in rat brain and gastrointestinal tract [3–7], the distribution of BRS-3 is much more limited. It has only been found in pregnant guinea-pig uterus, to a low degree in guinea-pig brain, on secondary spermatocytes in testis of rats, in a specific brain region of mice, and in human lung, ductal breast and epidermal carcinoma cell lines [1,2,8,9].

Although the orphan receptor strategy [10] has led to the identification and assignment of several neuropeptides to orphan GPCRs, in the case of BRS-3 the natural ligand still remains unknown, and the physiological and pathological function is still not understood. A first hint about the biological importance of BRS-3 was obtained from BRS-3 deficient mice, produced by targeted disruption. They developed a mild obesity, hypertension and diabetes [11,12]. Therefore it was concluded that BRS-3 is important for the regulation of energy balance, body weight and blood pressure. It is known that bombesin-like peptides (BLPs) are involved in the growth regulation of various cancers, especially small cell lung cancer (SCLC) cell lines [13,14]. Like NMB-R and GRP-R, BRS-3 was found on human lung cancer cells [1,15–17] and ovarian cancer [18], however, at a lower level of expression. Very recently, BRS-3 was connected with the treatment of neurological disorders [19].

So far two peptidic high affinity BRS-3 agonists have been described, [D-Phe^6,Phe^{13}]Bn(6–13) propylamide [20] and the non-selective high affinity agonist [D-Tyr^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn(6–14) and its D-Phe^6 analogue [21,22], which was further developed into the more selective (R)-Apa^{11} and (S)-Apa^{11} analogues [23]. They were derived synthetically from BLP fragments by substitution of amino acids and *C*-terminal modification. Receptor pharmacology studies have become possible for the first time, since to date no known natural agonist or antagonist of the Bn receptor family has shown high affinity on BRS-3 [2,20,21,24]. Studies with the agonist [D-Tyr^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn(6–14) showed that the BRS-3 receptor has a unique pharmacology compared with the other bombesin receptors, NMB-R, GRP-R and BB-R$_4$ [21,24,25]. Additional difficulties were encountered by the fact that no natural cell lines could be found to express BRS-3 in a sufficient level for study [20,25]. Therefore BRS-3 pharmacology studies were carried out using BRS-3 transfected BALB 3T3 or NCI-H1299 non-small cell lung cancer cells [20,25,26]. These studies [20,25], as well as others [27,28], gave insight in the signalling pathway of BRS-3. Receptor activation can be measured by an increase in the level of intracellular calcium ($[Ca^{2+}]_i$) caused by phospholipase C activation. Furthermore, activation of BRS-3 leads to increases in inositol phosphates and an increase in tyrosine phosphorylation of p125^{FAK} [25].

Unfortunately, the described agonists are not useful tool-substances for the closer understanding of receptor binding and activation due to their length and linearity, which results in high flexibility of the peptide backbone. Discussions about the bioactive, receptor bound conformation of bombesin analogues resulted in a type II β-bend conformation [23,29–33] with Gly^{11} in a pivotal position, not least because of structural parallels to somatostatin analogues. More recently, for Ac-Bn-(7–14)-peptide a conformation of three consecutive γ-turns followed by a bend and finishing with two γ-turns was proposed [34]. However, so far none of these proposals has been elaborated into a working model. It is our general opinion that only conformationally restricted analogues can be used to elucidate indirectly the receptor bound conformation [35]. There are many cases where induced fit of receptors and ligands have changed ligand conformation drastically [36]. Therefore, it was decided to look for more information about the bioactive conformation and pharmacophors in a systematic SAR approach. Our final goal was to obtain proteolytically more stable small molecules.

In a first step we tried to elucidate the importance of each amino acid side chain in [D-Phe^6,Phe^{13}]Bn(6–13)propylamide and [D-Phe^6, β-Ala^{11}, Phe^{13},Nle^{14}]Bn(6–14) for receptor binding and activation via an alanine-scan, and to obtain information about the backbone conformation by replacing each amino acid by its stereoisomeric analogue. Earlier SAR studies for Bn and the fragment Ac-Bn(7–14) demonstrated the importance of Trp^8 and His^{12} [37], and Trp^8 and Leu^{13} [34], respectively. During the preparation of this work the alanine scan and analogues of [D-Phe^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn(6–14) were published [38]. The

difference to our findings will be discussed below in detail.

In a second step, with this information in hand, we applied a strategy similar to the 'peptoid' approach, which is a rationale and strategy for the design of small nonpeptide molecules using the chemical structure of mammalian neuropeptides as a starting point [39,40,41]. Peptide-derived antagonists have been described for several G-protein coupled receptors of gastrointestinal hormones/neurotransmitters (GI), including cholecystokinin [42–45], somatostatin [46], tachykinin [47–50] and Bn [51,52]. Because the dimension of a binding site is normally less than 15 Å [39], it was assumed that in the bioactive conformation of the peptide, the important amino acids for functional potency are close to each other. Therefore we tried to develop small peptides, which display only a few key amino acids. For these compounds we expected retained receptor binding and activation in the micromolar range, which then could be finally optimized in combinatorial SAR oriented medicinal chemistry. In this paper we describe how we have been able to rationally develop tripeptides that selectively activate BRS-3 in the nanomolar range. This represents the key step in the development of nonpeptide low molecular weight compounds for BRS-3.

MATERIALS AND METHODS

Peptide Synthesis

Fmoc-protected amino acids, 2-(4-formyl-3-methoxyphenoxy)ethyl polystyrene (FMPE) resin, Rink Amide MBHA resin and Sieber Amide resin were purchased from Novabiochem (Darmstadt, Germany). Tritylchloride-polystyrene-resin (TCP-resin) was obtained from PepChem (Tübingen, Germany), [D-Phe^{6},β-Ala^{11},Phe^{13},Nle^{14}]Bn(6–14) (**1**) was purchased from Polypeptide Laboratories (Wolfenbüttel, Germany), NMB (**2**) and GRP (**3**) were obtained from Bachem (Heidelberg, Germany), HATU and HOAT from PerSeptive Biosystems (Hamburg, Germany), TBTU and HOBt from Quantum Appligene (Heidelberg, Germany), TFA from Solvay (Hannover, Germany). All other chemicals were purchased from Aldrich (Deisenhofen, Germany) or Merck (Darmstadt, Germany).

Peptides **4**–**20** and **53** were synthesized on Rink Amide MBHA resin [53]. Peptides **21**–**36** and **67**–**74** were synthesized on FMPE resin [54], which was reductively aminated with the corresponding amine as previously described [55] and loaded with the appropriate Fmoc amino acid using HATU (2 eq)/HOAt (2 eq)/sym.-collidine (20 eq) activation. Peptides **37**–**49** and **54**–**65** were synthesized on Sieber Amide resin [56]. Peptides **50**–**52** and **66** were synthesized on TCP resin [57]. Chain extension for all peptides was carried out on a multiple peptide synthesizer SyRoll from MultiSynTech (Witten, Germany), using standard Fmoc protocols with TBTU/HOBt/DIPEA activation [58]. Fmoc group removals were carried out by treatment with 20% piperidine (v/v) in NMP. The *N*-terminal free amine of peptides **38**, **40**, **42**, **44**, **46**, **48**, **52** and **55** was acetylated on solid support using a mixture of 5.5% acetic acid anhydride and 9.5% DIPEA in NMP (v/v). Cleavage from solid support was achieved with 90% TFA/5% TIPS/5% H_2O (v/v), except for Sieber Amide resin, where 1% TFA in DCM (v/v) was used. For peptide purification and analytical purity determination RP-HPLC instruments from Amersham Pharmacia Biotech (Äkta Basic 100F/10F with pump system P-900 and detector UV-900) or Beckman (System Gold with solvent module 125 and detector module 166) were used equipped with Omicron YMC columns (semi-preparative: ODS-A C_{18}, 250 mm × 20 mm, 5 µm or 10 µm, flow rate: 8 ml/min; analytical: ODS-A C_{18}, 250 mm × 4.6 mm, 5 µm, flow rate: 1 ml/min). Peptides were eluted with linear gradients (30 min) of acetonitrile in water and 0.1% (v/v) trifluoroacetic acid. Purity of the peptides was 95% or higher except for **23** (93%) and **58** (92%), peptide identities were confirmed using a Finnigan LCQ mass spectrometer.

Molecular Cloning, Transfection and Cell Culture

The cDNA of the human BRS-3 receptor (Acc. L08893), of the human NMB receptor (Acc. M73482) and of the human GRP receptor (Acc. M73481) was kindly provided by Dr James Battey (NIDCD, NIH, USA) to Solvay Pharmaceuticals GmbH (Hannover, Germany). The cDNA was cut out of pGEM4 (Promega, Madison, USA), in the case of BRS-3 and GRP with Eco RI, in the case of NMB with Eco RI and Bam HI and cloned into the expression vector pcDNA3.1(-) (Invitrogen, Carlsbad, California).

For transfection, CHO-K1 cells (Molecular Devices, Sunnyvale, California), stably transfected with the expression vector RD-HGA16 of the human Gα16-protein (Acc. M63904), were seeded into 24-well plates (2×10^4 cells/well) and cultured overnight under sterile conditions in a humidified Nuaire incubator from Zapf (Sarstedt, Germany) at 37 °C

and 5% CO_2 in Nut.Mix.F-12 (Ham) with Glutamax-I medium (GibcoBRL, Paisley, Scotland) supplemented with 10% fetal calf serum (inactivated at 56 °C for 1 h, origin: South America, GibcoBRL), 0.025 mg/ml gentamicin (GibcoBRL) and 0.2 mg/ml hygromycin B (GibcoBRL). The next day, the cells were transfected with 12 µl of a 0.3 µg/µl receptors DNA solution using the Effectene Transfection Reagent from Qiagen (Hilden, Germany) according to the manufacturer's instruction [59]. One day after transfection the medium was changed. From now on, cells were cultured under sterile conditions (37 °C, 5% CO_2) in Nut.Mix.F-12 (Ham) with Glutamax-I medium (GibcoBRL, Paisley, Scotland) supplemented with 10% fetal calf serum (GibcoBRL), 0.025 mg/ml gentamicin (GibcoBRL), 0.2 mg/ml hygromycin B (GibcoBRL) and 0.5 mg/ml geneticin (GibcoBRL). For cell test optimization, the cells with the highest receptor expression rate were selected. Therefore, transfected cells were diluted 1 : 30 000 with the above described medium and transferred into 96-well plates. After incubation (37 °C, 5% CO_2) overnight, wells containing a single cell were chosen and the cells were cultivated in 24-well plates, then 25 ml and finally 250 ml Costar plastic flasks (Corning, NY, USA). Then receptor expression was assessed by determination of the EC_{50} value of the corresponding endogenous ligand, NMB for NMB-R, GRP for GRP-R, or [D-Phe^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn(6–14) for BRS-3, respectively.

Selected cells were stored in aliquots of 1.8 ml (1×10^6 cells/ml) medium in 10% DMSO (v/v) at −80 °C. For cultivation, aliquots were warmed up to 37 °C, transferred into a Costar plastic flask (225 ml) from Corning (NY, USA) and diluted with 50 ml of supplemented medium. The medium was exchanged after 30 min of cultivation. Every following 1–3 days the medium was removed, the adherent cells (40%–95% confluency) were washed with PBS Dulbecco's (GibcoBRL) and then separated from the flask bottom by treatment with trypsin-EDTA (GibcoBRL) for 2 min at 37 °C. For further cultivation, the cells were transferred into a new plastic flask with fresh medium. For FLIPR measurements, the cells were seeded into Costar 96-well assay plates (clear bottom with lid, Corning) at a density of 1.2×10^4 cells/well.

FLIPR (Fluorometric Imaging Plate Reader) and Radioligand Binding Assays

CHO cells were cultivated 18–24 h in Costar 96-well assay plates (Corning) until they were confluent. Probenecid solution was prepared fresh every day, at a stock concentration of 250 mM. Therefore, probenecid (710 mg, 2.5 mmol) from Sigma (Seelze) was dissolved in 1N NaOH (5 ml) and diluted with HBSS without phenol red (GibcoBRL) with 20 mM HEPES (PAA Laboratories GmbH, Linz, Austria). A stock solution (2 mM) of fluorescent calcium indicator dye Fluo4 (Molecular Probes, OR, USA) was prepared from Fluo4 (1 mg) solubilized in DMSO (440 µl) and stored at at −20 °C. Immediately before use an aliquot of dye stock solution (22 µl) was mixed with an equal volume of 20% (w/v) pluronic acid F-127 (Sigma, Seelze) in DMSO. Then the cells were loaded for 45–60 min (37 °C, 5% CO_2) with 100 µl loading medium prepared from HBSS without phenol red (42 ml) with 60 mM HEPES and aliquots of stock solutions of probenecid (420 µl), Fluo4 (22 µl) and pluronic acid (22 µl). The cells were washed three times with 100 µl of HBSS with 20 mM HEPES and 2.5 mM probenecid in a Denley cell-washer (Labsystems). After the final wash, a 100 µl residual volume remained on the cells in each of the 96-well. Peptides were dissolved in DMSO as 10 mM stock solutions and diluted with HBSS with 20 mM HEPES into 96-well plates from Greiner (Frickenhausen, Germany). The highest concentration applied for measurements usually was 33 µM, in some cases as low as 1 µM. Well-to-well dilutions were 1 : 2, 1 : 3, 1 : 4 or 1 : 10 into 8 or 16 different wells depending on compound and receptor. For reference, each ligand microplate contained [D-Phe^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn(6–14) (**1**) in the case of measurements on BRS-3, NMB in the case of NMB-R and GRP in the case of GRP-R. The FLIPR from Molecular Devices (Sunnyvale, California) was programmed to measure background fluorescence for 30 s at 6 s intervals. Then 50 µl from each well of the ligand microplate were transferred to the cell plate and the fluorescence change in counts was recorded for 100 s at 1 s intervals and at 6 s intervals for the last 42 s. From each well the maximum fluorescence change was exported to Excel and normalized with the value of the reference compound at a concentration of maximal response, usually 16 µM. Dose response curves and EC_{50} values were calculated using Graphpad Prism (Version 3.00, Graphpad Software).

Affinity of the peptides to the BRS-3 receptor at a concentration of 10 µM was determined by CEREP (Celle L'Evescault, France) in a previously described radioligand binding assay [21] using Balb 3T3 cell membranes. Data is given

as the percent inhibition of the control specific binding of the reference compound [D-Tyr^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn(6–14) from two independent measurements.

RESULTS

Table 1 shows the functional potencies of **1** at NMB-R, GRP-R and BRS-3 in comparison with NMB and GRP. NMB-R showed a clear preference for NMB, GRP-R showed a preference, however less clearly, for GRP. Both peptides, NMB and GRP, showed relatively low activity on BRS-3. **1** showed functional potency at all three receptors with low selectivity.

Alanine Scans

The effect on calcium mobilization at NMB-R, GRP-R and BRS-3 and receptor affinity at BRS-3 of substitution of an individual amino acid against Ala or D-Ala, respectively, in **1** is shown in Table 2 and Figure 1.

In the case of BRS-3, replacement of Trp^8 or Phe^{13} reduced functional potency drastically by about 400-fold or 200-fold, respectively. This

Table 1 Mobilization of intracellular calcium by [D-Phe^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn(6–14) (**1**) and endogenous ligands in CHO cells transfected with the human bombesin receptors NMB-R, GRP-R and BRS-3. Functional potencies of the peptides are given in -pEC$_{50}$ ± SEM from 4–30 independent concentration-response curves

No.	Peptide	FLIPR-assay, -pEC$_{50}$		
		NMB-R	GRP-R	BRS-3
1	[D-Phe^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn(6–14)	6.51 ± 0.08	7.08 ± 0.09	7.21 ± 0.18
2	NMB	6.93 ± 0.11	7.38 ± 0.06	5.35 ± 0.04
3	GRP	5.49 ± 0.14	7.46 ± 0.15	5.20 ± 0.02

Table 2 Mobilization of intracellular calcium in CHO cells transfected with the human bombesin receptors NMB-R, GRP-R and BRS-3 and receptor affinity to the human bombesin receptor BRS-3 by analogues of [D-Phe^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn(6–14) (**1**) in which individual amino acids were replaced by Ala or D-Ala, respectively

No.	Sequence	Inhib. (%)[a] BRS-3	FLIPR-assay, -pEC$_{50}$		
			NMB-R	GRP-R	BRS-3
4	**a**QWAVβAHF-Nle-NH$_2$	[b]	5.12 ± 0.59	8.28 ± 0.05	7.13 ± 0.09
5	f**A**WAVβAHF-Nle-NH$_2$	95	5.45 ± 0.07	7.63 ± 0.05	6.51 ± 0.05
6	fQ**A**AVβAHF-Nle-NH$_2$	47	Inactive	7.25 ± 0.04	4.62 ± 0.10
1	fQW**A**VβAHF-Nle-NH$_2$	96	6.01 ± 0.10	8.03 ± 0.07	7.21 ± 0.18
7	fQWA**A**βAHF-Nle-NH$_2$	97	5.41 ± 0.40	8.58 ± 0.08	6.78 ± 0.06
8	fQWAV**A**HF-Nle-NH$_2$	103	5.39 ± 0.10	7.22 ± 0.03	5.93 ± 0.02
9	fQWAVβA**A**F-Nle-NH$_2$	99	Inactive	8.31 ± 0.06	6.32 ± 0.24
10	fQWAVβAH**A-**Nle-NH$_2$	51	4.95 ± 0.22	8.24 ± 0.07	4.84 ± 0.07
11	fQWAVβAHF**A-**NH$_2$	100	Inactive	8.71 ± 0.07	6.60 ± 0.09

Functional potencies of the peptides were determined in the FLIPR-assay and are given in -pEC$_{50}$ ± SEM from 3–9 independent concentration-response curves. Receptor affinity at a concentration of 10 μM was determined by CEREP (Celle L'Evescault, France) in a radioligand binding assay [21]. It is given in percent inhibition of control specific binding of the reference compound [D-Tyr^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn(6–14) from two independent measurements.

[a] Of control specific binding at 10 μM.

[b] Not determined.

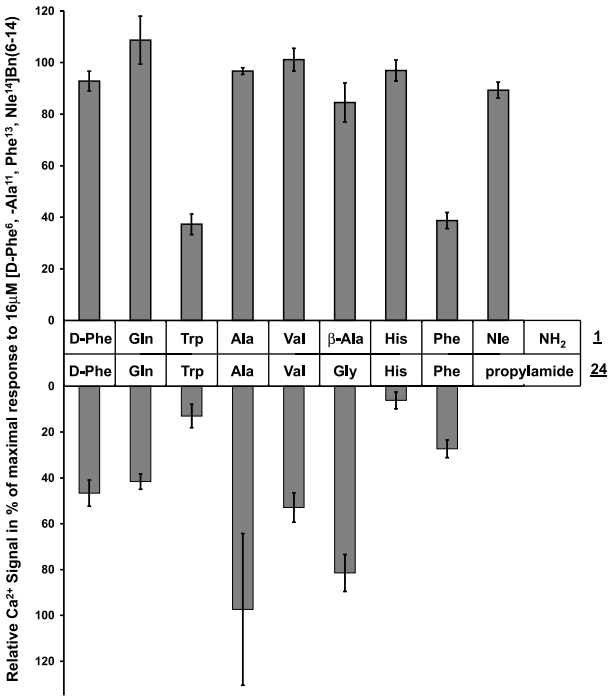


Figure 1 Effect on intracellular calcium mobilization of replacing single amino acids against Ala or D-Ala, respectively, in [D-Phe^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn(6–14) (**1**) compared with [D-Phe^6,Phe^{13}]Bn(6–13)propylamide (**24**) at 16 µM in BRS-3 transfected CHOGα16 cells. β-Ala was substituted with Ala. Functional potencies from 2–3 independent measurements are given in percent ± SEM relative to the Ca^{2+} response of **1**.

finding was consistent with a significant loss of affinity of analogues **6** and **10** towards the BRS-3 receptor as determined in the radioligand binding assay. A smaller, but still significant, drop in functional potency of about 20-fold was observed when substituting ß-Ala^{11}. Negligible effects were found when other amino acids were replaced against Ala. In the case of GRP-R, peptides showed smaller differences in potencies, a reduction of about 5–6 fold was found when Trp^8 and ß-Ala^{11} were exchanged. Marginal increases of about 4–5 fold could be observed when Val^{10} and Nle^{14} were substituted. At NMB-R, all replacements caused a loss of functional potency, alteration of Trp^8, His^{12} and Nle^{14} resulted in completely inactive peptides (analogues **6**, **9** and **11**). Smaller effects, about 4-fold loss of activity, could be observed for Gln^7, Val^{10}, and ß-Ala^{11}, whereas the functional potency of analogue **10** with substituted Phe^{13} dropped about 10-fold. The results of the alanine scan of **24** concerning calcium mobilization and receptor affinity at BRS-3 are given in Table 4 and Figure 1. The parent ligand **24** showed about 3-fold lower functional potency compared with **1**. Interestingly, the EC_{50} value rose about 1.5-fold, when Gly at position 11 was substituted (**26**). All other replacements in **24**, including *N*-terminal amino acids D-Phe^6 and Gln^7, led to a loss of functional potency. As in **1**, Trp^8 and Phe^{13} are key residues for receptor activation. Upon replacement, a significant drop of about 90-fold and 70-fold, respectively, was observed. However, contrary to **1**, substitution of His^{12} in **24** resulted in a completely inactive peptide. The importance of Trp^8 is underlined by the receptor affinity data, however, no significant impact was observed when His^{12} or Phe^{13} were altered.

D-Amino Acid Scans

The effect on calcium mobilization at NMB-R, GRP-R and BRS-3 and receptor affinity at BRS-3 of substitution of an individual amino acid against its stereoisomer in **1** is shown in Table 3 and Figure 2.

In the case of BRS-3, functional activity dropped about 400-fold when Phe^{13} was replaced. That the L-isomer is required at position 13 was also confirmed by radioligand binding data. Other substitutions also caused reductions in potency, especially those analogues with altered Ala^9, Val^{10} and ß-Ala^{11} displayed about 50–140 fold lower EC_{50} values. Relatively insensitive towards stereochemical changes were positions 6, 7 and, to a lesser degree also 12 and 14, where EC_{50} values dropped about 4–6 fold and about 20–30 fold, respectively. At GRP-R, functional potency dropped significantly when Trp^8, Ala^9, and also, to a lesser degree, the three *C*-terminal amino acids His^{12}, Phe^{13} and Nle^{14} were altered. Nevertheless, as for the replacement with alanine, the importance of Phe at position 13 was much lower at NMB-R compared with BRS-3 or GRP-R. Contrary to BRS-3, at NMB-R and GRP-R, position 11 showed low sensitivity concerning stereochemical changes with reductions of about 7-fold at NMB-R and approximately 3-fold at GRP-R. As for BRS-3, at both receptors NMB-R and GRP-R, *N*-terminal amino acids could be exchanged by their stereoisomeric counterparts without much impact on functional potency.

Stereochemical changes in **24** basically resulted in inactive peptides at BRS-3 as shown in Table 4 and Figure 2. Only analogues **29** and **30** with

Table 3 Functional potencies from 3–6 independent concentration-response curves and receptor affinity of [D-Phe^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn(6–14) (**1**) analogues in which individual amino acids were replaced by their stereoisomeric counterparts. Additional experimental details are described in Table 2

No.	Sequence	Inhib. (%)[a] BRS-3	FLIPR-assay, -pEC$_{50}$		
			NMB-R	GRP-R	BRS-3
12	**F**QWAVβAHF-Nle-NH$_2$	100	5.54 ± 0.09	8.17 ± 0.06	6.60 ± 0.31
13	f**q**WAVβAHF-Nle-NH$_2$	100	5.17 ± 0.07	8.44 ± 0.07	6.46 ± 0.10
14	fQ**w**AVβAHF-Nle-NH$_2$	80	Inactive	6.11 ± 0.06	5.65 ± 0.10
15	fQW**a**VβAHF-Nle-NH$_2$	89	Inactive	6.22 ± 0.09	5.47 ± 0.04
16	fQWA**v**βAHF-Nle-NH$_2$	79	4.73 ± 0.08	7.19 ± 0.04	5.06 ± 0.04
17	fQWAV**a**HF-Nle-NH$_2$	74	5.18 ± 0.06	7.56 ± 0.07	5.40 ± 0.05
18	fQWAVβA**h**F-Nle-NH$_2$	94	4.78 ± 0.04	6.38 ± 0.03	5.74 ± 0.08
19	fQWAVβAH**f-**Nle-NH$_2$	46	Inactive	6.45 ± 0.06	4.60 ± 0.04
20	fQWAVβAHF-**D-Nle**-NH$_2$	92	4.74 ± 0.03	6.53 ± 0.02	5.84 ± 0.12

[a] Of control specific binding at 10 μM.

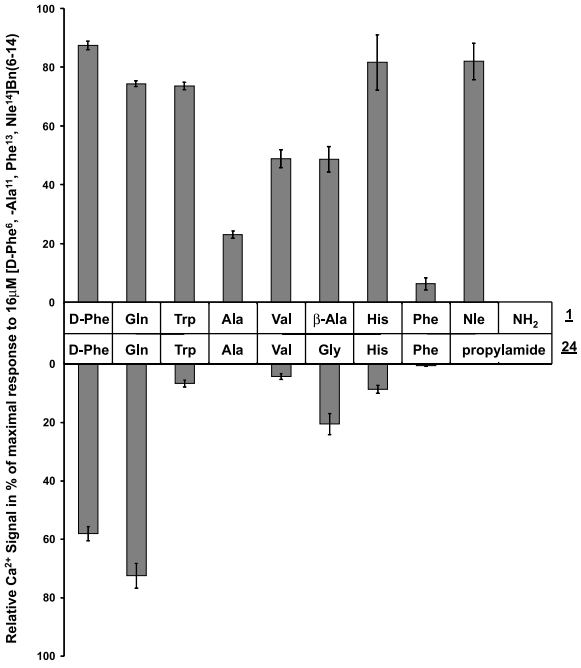


Figure 2 Effect on intracellular calcium mobilization of replacing individual amino acids against their stereoisomeric form in [D-Phe^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn(6–14) (**1**) compared with [D-Phe^6,Phe^{13}]Bn(6–13)propylamide (**24**) at 16 μM in BRS-3 transfected CHOGα16 cells. Gly and β-Ala were substituted with D-Ala. Functional potencies from three independent measurements are given in percent ± SEM relative to the Ca^{2+} response of **1**.

changed D-Phe^6 and Gln^7 retained modest functional activity.

Shortened Fragments and Substitutions

In a next step, *N*-terminal and/or *C*-terminal shortened fragments of **1** with acylated *N*-terminus and analogues, in which individual amino acids are substituted, were prepared (Table 5).

The effect of *N*-terminal deletion of D-Phe^6 and Gln^7 and simultaneous variation of position 11 by insertion of Gly, Ala, β-Ala and γ-amino-butyric acid was studied in a series of compounds, namely **37**–**44**. In order to avoid the possible negative influence of an *N*-terminal charge, each peptide analogue was additionally prepared in the *N*-terminal acetylated form (peptides **38**, **40**, **42** and **44**). All truncated fragments showed reduced functional activity with β-Ala > γ-amino-butyric acid > Gly > Ala on BRS-3 and, interestingly, also on GRP-R. At BRS-3, functional potency of **41** with β-Ala^{11} increased with *N*-terminal acetylation approximately 2.5-fold. This strategy had an opposite effect when applied to **43** with γ-amino-butyric acid at position 11, where activity dropped slightly, about 1.5-fold. Changing the stereochemistry at the *N*-terminal position 8 of analogues **41** and **42** resulted in peptides **45** and **46**, where functional potency was even slightly more reduced. Most interestingly, substitution of His^{12} by Tyr led to a selective GRP-R agonist **49**, which showed only about 2-fold reduced activity at GRP-R but almost eradicated functional response at NMB-R and BRS-3. *C*-terminal modification of the amide into a carboxylic acid (compound **50**) resulted in a huge loss of functional response on all three

Table 4 Functional potencies from 2–4 independent concentration-response curves and receptor affinity of [D-Phe^6,Phe^{13}]Bn(6–13)propylamide (**24**) analogues in which individual amino acids were replaced by Ala or D-Ala, respectively (**21**–**28**), and their stereoisomeric counterparts (**29**–**36**). Functional potencies on NMB-R and GRP-R were not determined. Additional experimental details are described in Table 2

No.	Sequence	Inhib. (%)[a]	-pEC$_{50}$[b]
		BRS-3	BRS-3
21	**a**QWAVGHF-propylamide	[c]	5.02 ± 0.09
22	f**A**WAVGHF-propylamide	93	5.43 ± 0.07
23	fQ**A**AVGHF-propylamide	42	4.70 ± 0.16
24	fQW**A**VGHF-propylamide	99	6.66 ± 0.18
25	fQWA**A**GHF-propylamide	99	5.36 ± 0.10
26	fQWAV**A**HF-propylamide	87	6.84 ± 0.08
27	fQWAVG**A**F-propylamide	89	Inactive
28	fQWAVGH**A-**propylamide	85	4.79 ± 0.05
29	**F**QWAVGHF-propylamide	[c]	5.44 ± 0.06
30	f**q**WAVGHF-propylamide	[c]	5.35 ± 0.02
31	fQ**w**AVGHF-propylamide	[c]	4.80 ± 0.12
32	fQW**a**VGHF-propylamide	[c]	Inactive
33	fQWA**v**GHF-propylamide	[c]	Inactive
34	fQWAV**a**HF-propylamide	[c]	4.96 ± 0.21
35	fQWAVG**h**F-propylamide	[c]	4.66 ± 0.49
36	fQWAVGH**f**-propylamide	[c]	Inactive

[a] Of control specific binding at 10 μM.
[b] FLIPR-assay.
[c] Not determined.

receptors, however, receptor affinity at BRS-3 was almost retained. Peptides **51** and **52** with truncated *C*-terminal Nle^{14}-amide and *N*-terminal D-Phe^6 and Gln^7 were completely inactive. Compound **53** was synthesized to further elucidate the importance of position 11 and for comparison between **1** and **24**. As shown by **53**, replacement of β-Ala^{11} by Gly led to a tremendous, about 35-fold reduction of functional potency at BRS-3 but only to small, about 2-fold declines at NMB-R and GRP-R.

Tetrapeptide Lead-Structure

The evaluation of *N*- or *C*-terminal deleted fragments made clear that **1**, or its D-Phe^6 truncated fragment, already was a minimum active BLP-derived fragment concerning functional activity at BRS-3. Therefore a different approach was attempted to obtain smaller lead structures. Using the knowledge obtained from the results described above and considering the hypothesis that the spatial distance between Trp^8 and Phe^{13} in the bioactive conformation of **1** is small due to a turn induced around position 11 [23,29–34], a tetrapeptide was designed (Figure 3).

It consisted of the three *N*-terminal amino acids of **1** and Phe-NH$_2$ (compound **61**) and formed the basis of a four membered mini-library (compounds **54**, **61**–**63**). The peptides with permutated stereochemistry at the two *C*-terminal amino acids presented the residues Trp and Phe or their stereoisomers at two different distances.

Systematic SAR of Tetrapeptide Lead

Functional potency and receptor affinity at BRS-3 of the four membered mini-library (peptides **54**, **61**–**63**) and modified analogues are shown in Table 6. To our great surprise, compound **54** clearly showed rest-activity with an EC$_{50}$ value of about 10 μM in the FLIPR-assay.

Table 5 Functional potencies from 2–5 independent concentration-response curves and receptor affinity of [D-Phe^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn(6–14) (**1**) analogues in which *N*-terminal and/or *C*-terminal amino acids are deleted, individual amino acids are substituted (X = γ-amino-butyric acid) and the *N*-terminus is acetylated. Additional experimental details are described in Table 2

No.	Sequence	Inhib. (%)[a] BRS-3	FLIPR-assay, -pEC$_{50}$		
			NMB-R	GRP-R	BRS-3
37	WAV**G**HF-Nle-NH$_2$	60	Inactive	6.02 ± 0.04	4.93 ± 0.05
38	Ac-WAV**G**HF-Nle-NH$_2$	61	4.75 ± 0.07	6.22 ± 0.02	4.93 ± 0.02
39	WAV**A**HF-Nle-NH$_2$	24	Inactive	5.86 ± 0.05	4.76 ± 0.04
40	Ac-WAV**A**HF-Nle-NH$_2$	40	Inactive	5.75 ± 0.01	Inactive
41	WAVβAHF-Nle-NH$_2$	80	Inactive	6.89 ± 0.13	5.29 ± 0.06
42	Ac-WAVβAHF-Nle-NH$_2$	98	Inactive	7.45 ± 0.06	5.70 ± 0.10
43	WAV**X**HF-Nle-NH$_2$	82	Inactive	6.18 ± 0.03	5.33 ± 0.05
44	Ac-WAV**X**HF-Nle-NH$_2$	78	Inactive	6.45 ± 0.04	5.17 ± 0.06
45	**w**AVβAHF-Nle-NH$_2$	79	Inactive	6.86 ± 0.07	4.72 ± 0.01
46	Ac-**w**AVβAHF-Nle-NH$_2$	95	Inactive	6.70 ± 0.07	5.49 ± 0.10
47	WAVβA**Y**F-Nle-NH$_2$	<10	4.88 ± 0.01	5.85 ± 0.02	4.76 ± 0.02
48	Ac-WAVβA**Y**F-Nle-NH$_2$	<10	4.71 ± 0.12	6.17 ± 0.11	4.76 ± 0.01
49	fQWAVβA**Y**F-Nle-NH$_2$	<10	4.74 ± 0.04	7.78 ± 0.13	5.01 ± 0.10
50	fQWAVβAHF	92	Inactive	Inactive	4.77 ± 0.37
51	WAVβAHF	<10	Inactive	Inactive	Inactive
52	Ac-WAVβAHF	<10	Inactive	Inactive	Inactive
53	fQWAV**G**HF-Nle-NH$_2$	97	5.81 ± 0.08	7.72 ± 0.09	5.64 ± 0.06

[a] Of control specific binding at 10 μM.

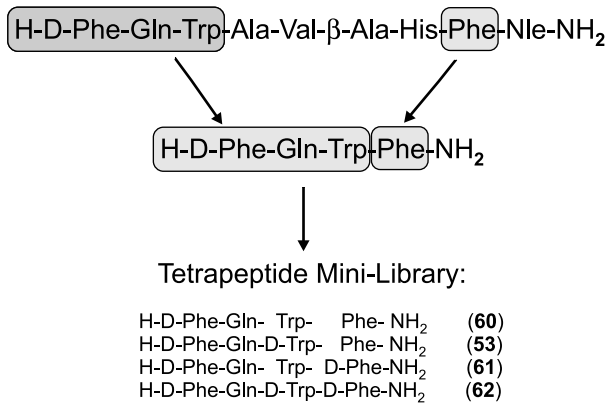


Figure 3 A tetrapeptide consisting of the three *N*-terminal amino acids of [D-Phe^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn(6–14) (**1**) and Phe-NH$_2$ formed the basis of a four membered mini-library. The peptides with permutated stereochemistry at the two *C*-terminal amino acids presented the residues Trp and Phe or their stereoisomers at two different distances.

N-terminal acetylation resulted in a loss of functional activity (**55**), however, enhanced receptor affinity was observed. Analogues of **54**, in which amino acids were replaced against alanine (**56**–**59**) indicated the importance of the three aromatic residues whereas removal of the side chain of Gln even slightly increased receptor affinity. Analogues with modified stereochemistry (compounds **60**–**63**) demonstrated the intolerance towards *C*-terminal alterations as well as the necessity of a D-conformation at the Trp-residue (**61**). On the other hand, functional potency was retained when stereochemistry was changed at the *N*-terminal D-Phe (**60**). As expected from the aforementioned results, Gln could be replaced by Asn (**64**). However, β-Ala was not tolerated (**65**). Again, *C*-terminal removal of the lipophilic amide resulted in a compound without functional response (**66**).

C-Terminal Optimization

In a next step *C*-terminally modified analogues of the discovered tetrapeptide lead-structure **54** were

Table 6 Functional potencies from 2–5 independent concentration-response curves and receptor affinity of the tetrapeptide mini-library (peptides **54**, **61**–**63**) and analogues in which individual amino acids were substituted. All compounds are inactive on NMB-R and GRP-R in the FLIPR-assay except **54** with -pEC$_{50}$ of 4.76 ± 0.01 on GRP-R, functional activity of **55** on NMB-R and GRP-R was not determined. Additional experimental details are described in Table 2

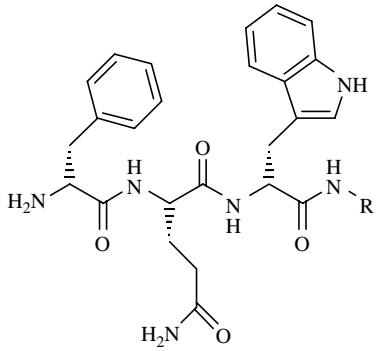
No.	Sequence	Inhib. (%)[a]	-pEC$_{50}$[b]
		BRS-3	BRS-3
54	fQwF-NH$_2$	15	5.00 ± 0.09
55	**Ac**-fQwF-NH$_2$	25	Inactive
56	**a**QwF-NH$_2$	<10	Inactive
57	f**A**wF-NH$_2$	26	4.82 ± 0.10
58	fQ**a**F-NH$_2$	<10	Inactive
59	fQ**WA**-NH$_2$	<10	Inactive
60	**F**QwF-NH$_2$	14	5.05 ± 0.03
61	fQ**W**F-NH$_2$	11	Inactive
62	fQ**Wf**-NH$_2$	<10	Inactive
63	fQw**f**-NH$_2$	19	Inactive
64	f**N**wF-NH$_2$	27	5.08 ± 0.08
65	fβ**A**wF-NH$_2$	13	Inactive
66	fQwF	<10	Inactive

[a] Of control specific binding at 10 μM.
[b] FLIPR-assay.

prepared (Table 7). Formally, the *C*-terminal Phe-NH$_2$ group was replaced by differently substituted arylamines.

Most importantly, incorporation of a 1-(2-phenylethyl)-residue (**68**) increased the functional potency about 15-fold. Insertion of a benzyl-moiety resulted in a loss of functional activity (**67**), which demonstrated that the carbon chain length is crucial. Aromatic substitutions (**69** and **70**) or replacement of phenyl- by 2-pyridyl- (**71**) did not further improve functional potency. Interestingly, compound **71** showed almost equal functional potency compared with **68** but much lower receptor affinity. Sterical constraints (**72** and **73**) reduced functional response about 2-fold compared with **68**, incorporation of a 1-(2,2-diphenylethyl)-residue (**74**) resulted in a completely inactive compound. Compounds **67**–**74** did not induce calcium mobilization on NMB-R and GRP-R.

Table 7 Functional potencies from 2–5 independent concentration-response curves and receptor affinity of *C*-terminally modified analogues of **54**. All compounds are inactive on NMB-R and GRP-R in the FLIPR-assay. Additional experimental details are described in Table 2



No.	R	Inhib. (%)[a]	-pEC$_{50}$[b]
		BRS-3	BRS-3
67	Benzyl-	49	Inactive
68	1-(2-Phenylethyl)	82	6.15 ± 0.08
69	1-[2-(3,4-Dimethoxyphenyl)ethyl]	<10	Inactive
70	1-[2-(4-Bromophenyl)ethyl]	58	5.68 ± 0.07
71	1-(Pyridine-2-ylethyl)	16	5.97 ± 0.06
72	1-[(R)-(+)-β-Methylphenylethyl]	59	5.63 ± 0.05
73	1-[(R)-(−)-β-Methylphenylethyl]	51	5.58 ± 0.09
74	1-(2,2-Diphenylethyl)	28	Inactive

[a] Of control specific binding at 10 μM.
[b] FLIPR-assay.

DISCUSSION

In recent studies [21,22], the affinity of [D-Phe^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn(6–14) (**1**) towards the bombesin receptors NMB-R, GRP-R, BRS-3 and BB-R$_4$ has been determined in radioligand binding assays. Later, the ability of **1**, NMB, GRP and Bn to induce calcium mobilization in a FLIPR-assay using HEK-293 and RBL-2H3 cells was assessed [38]. In accordance with these studies **1** showed high functional potency on all three bombesin receptors NMB-R, GRP-R and BRS-3 in our measurements (Table 1). However, in contrast to others [38], we found little functional activity for NMB and GRP on BRS-3 with NMB > GRP. Relatively low functional potency of NMB on BRS-3 was also reported by Wu

et al. [20]. Cell test sensitivities of the BRS-3 assay with an EC_{50} of 62 nM for **1** and of the GRP-R assay with EC_{50} of 35 nM for GRP were higher compared with the NMB-R assay where $EC_{50} = 117$ nM for NMB. In accordance with previous studies [38], functional selectivity of the BRS-3 receptor towards **1** was much higher than selectivity of the NMB-R receptor towards NMB and the GRP-R receptor towards GRP. However, in contrast to [38], we found that the functional potency of **1** > GRP on NMB-R and NMB > **1** on GRP-R.

Structure-activity studies revealed the importance of Trp^8 and His^{12} for biological activity of Bn [37], and Trp^8 and Leu^{13} for the activity of Ac-Bn(7–14) on NMB-R and GRP-R [34], respectively. However, in a very recently published work [38], functional potency was maintained on all three bombesin receptors, when Trp^8 was substituted by alanine in [D-Phe^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn(6–14). This contradicts our measurements, which showed in accordance with the studies mentioned above [34,37] that Trp^8 in **1**, as in other bombesin analogues, is crucial for receptor activation and affinity (Table 2, Figure 1), although a change in its configuration did not greatly affect functional potency (Table 3). This result was even more confirmed by the structure activity studies performed on [D-Phe^6,Phe^{13}]Bn(6–13)propylamide (**24**) (Table 4, Figure 1). On the other hand, the reported significant reduction of the biological response on NMB-R and BRS-3, but not on GRP-R by replacement of Phe^{13} with alanine in **1** [38] is consistent with our findings (Table 2, Figure 1). Furthermore, an L-conformation at position 13 is absolutely necessary for functional potency on BRS-3 but not on GRP-R (Table 3, Figure 2). A previous study [60] already pointed out that the residue in position 13 is important for selectivity of NMB-R over GRP-R. However in Ac-Bn(7–14), Leu^{13} was approximately equally important for both, NMB-R and GRP-R [34]. Interestingly, the absence of the imidazole ring at position 12 caused no major loss of functional potency on BRS-3 in **1** but, similar to the observation for Bn [37], in **24** (Tables 2 and 4, Figure 1). The explanation for this shift of importance probably lies within the one carbon atom elongated backbone structure at position 11 in **1** compared with **24** (β-Ala exchanged vs Gly). However contrary to that, Leu at position 13 was important for receptor affinity at NMB-R and GRP-R in Ac-Bn(7–14) [34]. The discovery of the highly potent BRS-3 agonist **1** [21] and the more selective analogues, which where obtained by manipulation of position 11 [23], also demonstrated the crucial role of this position in BLPs concerning BRS-3 activity and selectivity over the other bombesin receptors, NMB-R and GRP-R. Comparison of peptide **1** with β-Ala^{11}, peptide **8** with Ala^{11} and peptide **53** with Gly^{11} showed that functional potency decreased drastically in the order β-Ala > Ala > Gly on BRS-3, whereas functional potency concerning NMB-R and GRP-R is less affected. This corresponds very well with previous results [38]. Interestingly, a similar order for functional potency on BRS-3 was observed for peptides **24** and **26** with Ala > Gly. However, analogues of **24** with variation at position 11 showed higher functional potency compared with their corresponding analogues of **1**. Furthermore, *N*-terminally truncated compounds **37**–**44** also confirmed that position 11 is obviously optimized with β-Ala on BRS-3 and also on GRP-R (Table 5). However, for the truncated compounds the order for Ala and Gly was flipped with Gly > Ala. With the exception of positions 6 and 7, a change in the stereochemistry of **24** resulted in inactive peptides (Table 4, Figure 2). When compared with the corresponding D-amino acid scan analogues of **1** this negative trend can also be at least partially attributed to the difference in backbone length at position 11, which obviously makes the bioactive conformation of peptide **24** more sensitive towards stereochemical changes.

Contrary to a previous report [38] we could not observe enhanced functional potency on GRP-R when D-Phe^6 was replaced by alanine. However, there was a small increase when Val^{10} was substituted (Table 2). As for BRS-3, Trp^8 and β-Ala^{11} were found to be important for functional potency on GRP-R, but, most importantly and in good agreement with previous findings [38], not Phe^{13} (Table 2). Furthermore, besides the low significance of the presence of the phenyl-ring at position 13 for functional activity on GRP-R, a change in its orientation also had a low impact on activity (Tables 2 and 3). Interestingly, high selectivity for GRP-R of the unselective agonist **1** could be achieved by a single substitution of His^{12} against Tyr (Table 5). The reasons for this selectivity, as can be concluded from the aforementioned results, rather lie in the presence of the tyrosine sidechain than in the absence of the imidazole-ring of histidine. It can be speculated that the orientation of the adjacent phenyl ring at position 13, which is crucial for selectivity at GRP-R over NMB-R and BRS-3, is unfavourably affected by this substitution.

Although residues at positions 6, 7 and, with the exception of NMB-R, also 14 seemed not to be necessary for functional potency on all three receptors as determined by the alanine- and D-amino acid scans, it was not possible to obtain equally active shorter fragments by simple deletion (Table 5). Therefore it can be concluded, that **1** or **24**, respectively, or their one *N*-terminal amino acid shortened fragments are minimum active BLP-derived fragments. This agrees well with the minimum length of the NMB sequence that is required for retention of full BRS-3 activity [20,38]. Although there are different suggestions about the exact bioactive conformation of bombesin and related peptides [23,29–34], it is feasible that they adopt a bend-like structure. This is supported by the fact that a β-turn mimetic could successfully replace the dipeptide Val-Gly in conformationally constrained bombesin analogues [61]. Furthermore, compound **54** (Table 6) suggests that a relative proximity of residues Trp^8 and Phe^{13} in the bioactive conformation of **1** is very probable. The same conclusion can be drawn from the recently described antagonists for NMB-R, GRP-R [51,52] and related receptors [42–45,47–50]. Although these compounds have been developed by similar strategies [39–41], a successful shortening of the sequence required for BRS-3 activation, as represented by compound **54**, was very surprising. Systematic SAR of lead structure **54** revealed that all three aromatic residues are necessary for functional potency on BRS-3 (Table 6). Furthermore, a D-conformation for the Trp seemed to be mandatory (Table 6). Our attempts to optimize lead structure **54** were directed towards the *C*-terminus (Table 7). As expected, the transformation of the *C*-terminal amide group into a free carboxylic acid was unfavourable for functional potency on BRS-3 (Table 5 and 6) because most naturally occurring peptides including the bombesin-like peptides are *C*-terminally amidated. Moreover a comparison of compounds **24** and **53** showed that deletion of the *C*-terminal amide group even raised BRS-3 functional potency. Based on this observation we rationalized that further increased *C*-terminal lipophilicity by removal of the *C*-terminal amide group would lead to a substantial increase of functional activity of lead structure **54**. This concept worked very well, within our studies the unsubstituted 1-(2-phenylethyl)amide (compound **68**) proved to be the most suitable *C*-terminal structural moiety (Table 7). Structural constraints did not improve functional response, too lipophilic residues were not tolerated (Table 7). Gln was incorporated into the structure of compound **54** because it was part of the *N*-terminal tripeptide sequence of peptide **1** (Figure 3). In addition to that Gln served as a spacer between Phe and Trp. Therefore it was not very surprising that its sidechain was not necessary for functional potency on BRS-3 (Table 6). The fact that Gln might be replaceable gives a promising outlook for further structural modifications towards nonpeptide ligands for BRS-3.

CONCLUSION

In summary we have performed detailed structure activity studies on the peptides [D-Phe^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn(6–14) (**1**) and [D-Phe^6,Phe^{13}]Bn(6–13)propylamide (**24**) concerning functional potency on NMB-R, GRP-R and BRS-3. We demonstrated that for functional activity of **1** on BRS-3, the amino acids Trp^8, Phe^{13} and also β-Ala^{11} are important. However, in **24** the sidechain of His^{12} is crucial for BRS-3 functional activity. This shift of importance can at least be partially attributed to the difference in backbone length at position 11. Substitution of His^{12} by Tyr in **1** led to a high selectivity of GRP-R over BRS-3 and NMB-R: Functional potency on GRP-R was almost maintained, whereas functional response on BRS-3 was reduced about 150-fold and about 20-fold on NMB-R.

The knowledge derived from the structure activity studies was used to develop a small tetrapeptide library. Peptides, which comprised the three *N*-terminal amino acids of **1** and a *C*-terminally amidated Phe, were synthesized. By permutating the stereochemistry of the two *C*-terminal amino acids Trp and Phe, we demonstrated that only compound **54** was able to selectively activate BRS-3 with an EC_{50} value in the micromolar range. The BRS-3 functional potency of this lead structure could be increased markedly, i.e. about 15-fold, by removal of the *C*-terminal amide. We believe that the selective short peptide BRS-3 agonist H-D-Phe-Gln-D-Trp-1-(2-phenylethyl)amide (**68**) has the potential to serve as a structural template and starting point for the design of proteolytically more stable nonpeptide small molecules. These compounds might be useful to elucidate the physiological role of the human orphan receptor BRS-3. According to our data, efforts towards a further optimization of **68** can be directed towards a modification of the Gln.

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