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The design of a new potent and selective ligand for the orphan bombesin receptor subtype 3 (BRS3)

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Abstract: Extensive SAR studies on the unselective BRS3 agonist, [H-D-Phe^6,β-Ala^{11},Phe^{13},Nle^{14}]-bombesin-(6-14)-nonapeptide amide, have highlighted structural features important for BRS3 activity and have provided guidance as to the design of selective agonists. A radically modified heptapeptide agonist, maintaining only the Trp-Ala moiety of the parent [H-D-Phe^6,βAla^{11},Phe^{13},Nle^{14}]-peptide amide, and with a very different carboxyl terminal region, has been produced which was potent at BRS3 and essentially had no NMB or GRP receptor activity. Its structure is Ac-Phe-Trp-Ala-His(τBzl)-Nip-Gly-Arg-NH_2.

Keywords: selective agonist; bombesin receptor subtype 3 (BRS3); bombesin (Bn); neuromedin B (NMB); gastrin releasing peptide (GRP)

INTRODUCTION

The orphan bombesin receptor subtype 3 (BRS3) has been known for some time [1,2] and the biological relevance is currently being investigated in various disease states including obesity, hypertension, diabetes, respiration and cancer [3–5]. However, as yet, endogenous ligands remain unknown and the exact physiological function of BRS3 also remains to be established. The original ligands for BRS3 were synthetic bombesin (Bn) analogues: [H-D-Phe^6,β-Ala^{11},Phe^{13},Nle^{14}]-nonapeptide amide, agonist **1** and the related [H-D-Phe^6,Phe^{13}]-nonapeptide propyl amide, agonist **2** [6,7] (see Table 1). BRS3 has been shown [7] to have a unique pharmacology with low affinity for all known natural bombesin peptides.

Since agonist peptides **1** and **2** show low BRS3 agonist potency, compared with potency at the closely similar NMB and GRP receptors, there is a need for potent and more selective BRS3 agonists, and several groups have already made some progress in this direction [8–12]. In addition, some smaller molecular weight agonists are now known [13]. The purpose of our work was to identify the chief features within the agonist **1** sequence for activity at BRS3, to design a short, potent, selective BRS3 agonist, and then to use this information to aid non-peptide drug design. A brief summary is given of initial SAR results from over 500 peptide analogues, and investigations leading to a potent new selective BRS3 agonist with a radically altered structure is described.

Table 1 Sequences and Numbering for Bn Agonist 1 and Agonist 2

Bn
Pyr^1-Gln^2-Arg^3-Leu^4-Gly^5-Asn^6-Gln^7-Trp^8-Ala^9-Val^{10}-Gly^{11}-His^{12}-Leu^{13}-Met^{14}-NH_2
Agonist **1**
H-D-Phe^6-Gln^7-Trp^8-Ala^9-Val^{10}-Ala^{11}-His^{12}-Phe^{13}-Nle^{14}-NH_2
Agonist **2**
H-D-Phe-Gln-Trp-Ala-Val-Gly-His-Phe-Met-NH-$CH_2CH_2CH_3$

RESULTS AND DISCUSSION

For potency at BRS3, the initial SAR studies indicated that no essential amino acid residue was present within agonist **1**, although some residues had more influence on potency than others (Table 2). Good BRS3 agonist activity was retained for singly modified analogues of agonist **1** when the D-Phe in position 6 was replaced

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Table 2 Analogues of Agonist 1: H-D-Phe^{6}-Gln^{7}-Trp^{8}-Ala^{9}-Val^{10}-Ala^{11}-His^{12}-Phe^{13}-Nle^{14}-NH_2

Analogue number	Substituent and position	BRS3 activity		NMBR activity		GRPR activity	
		% control 1 μM	EC_{50} nM	% control 30 nM	EC_{50} nM	% control 30 nM	EC_{50} nM
1	Agonist **1**	100	5.7	100	0.041	100	0.093
3	D-Val^{6},Ile^{7}		2.4		0.11		0.06
4	2-Nal^{8}		135				
5	His(τBzl)^{10}	103	5	96		104	
6	His^{10}		13				
7	Phg^{10}	99	12.8	102		96	
8	Nip^{11}	94	3.6	100		98	
9	Gly^{12}	85	3.6	78		79	
10	Thr^{12}	83	3.6	83		78	
11	Ser^{12}	91	8.5	93		94	
12	Cys(Bzl)^{13}		13.8	83		86	
13	Trp^{14}	105	2.58	101	0.853	83	0.08
14	Nle-OH^{14}	120		96		97	
15	14-Norleucinol	109		96		92	
16	MePhe^{13}	91	8.5	95		94	
17	Pro^{11}	70		10		38	
18	Ala^{12}	94	4.2	14		71	
19	Phe^{12}	93		13		45	
20	Trp^{9}	82	3.5	49		94	
21	Phe^{9}	85	4.4	50		93	
22	Ile^{13}	99	3.6	49		96	
23	2-Nal^{14}	96	2.1	55		94	
24	MeAla^{11}	65	82.6	92	1.84	22	185

by a range of D- or L-residues, and the Gln in position 7 was replaced by certain L-residues. Whereas, within the sequence of agonist **1**, residues 6 and 7 are not essential for activity, they do strongly contribute to the potency. The disubstituted agonist having D-Val and Ile in positions 6 and 7, respectively (**3**), maintained good BRS3 agonist potency. *N*-terminal acetylation of agonist **1** resulted in decreased BRS3 activity. The Trp residue in position 8 was far more sensitive to change, and no replacement analogues were found with as good a potency as the parent, although weak agonist activity was observed for certain L-substituents with a hydrophobic aromatic side chain separated from the backbone by one methylene group, for example the [2-Nal^{8}]-agonist **4** (2-Nal: β-(naphtha-2-yl)-alanine). Substitution at the Ala position 9 was also very sensitive to change, and none of the analogues retained good agonist activity at the 0.1 μM dosage. Val in position 10 could be substituted in a variety of ways, and the His(τBzl)^{10}, His^{10} and Phg^{10} analogues (**5**, **6** and **7** respectively) appeared to have comparable activity to agonist **1** itself. The βAla position 11 seems to be more sensitive to substitution with good activity only being retained in analogues having small side chains, but the cyclic Nip^{11} residue **8** (Nip: piperidine-3-carboxylic acid) was more potent than agonist **1**. The His side chain at position 12 could be replaced by fairly small alternatives and increased potency was observed for the Gly^{12}, Thr^{12} and Ser^{12}, analogues **9**, **10** and **11** respectively; large hydrophobic, acidic or basic side chains at this position gave decreased potencies. The Phe residue in position 13 could tolerate many substitutions well, including replacements by Ile^{13}, Cys(Bzl)^{13} and MePhe^{13}, **22**, **12** and **16** respectively.[†‡] The terminal Nle residue in position 14 also tolerated structural variation, with both the Trp^{14} analogue **13** and the 2-Nal^{14} analogue **23** being at least as potent as agonist **1**. Good BRS3 agonist activity was retained when the Nle^{14}-amide was replaced by norleucinol (**15**) or by Nle-OH (**14**). Whereas most of the simple analogues of agonist **1** were not selective to BRS3, a few did show some signs of improved selectivity, although this was usually at the expense of a large drop in BRS3 agonist potency. At the NMBR agonist potency was significantly reduced, compared with BRS3 activity, by making suitable substitutions at the 11- and 12-positions, for example, with Pro at position 11 (**17**), or

[†] In relation to the importance of backbone NH functions and potential turns in agonist **1**, good activity was retained in analogues with D-Pro in position 6 and with Pro in positions 7 and 12, and also with *N*-methylated residues in positions 6, 11 and 14.

[‡] No cyclic peptides had BRS3 activity.

Ala or Phe in position 12 (**18** and **19** respectively), and, to a lesser extent, by placing Trp or Phe at position 9, or Ile or 2-Nal in positions 13 and 14 respectively (**20**, **21**, **22** and **23**). GRPR agonist potency was reduced with MeAla in position 11 (**24**) and, to a lesser extent, with Phe in position 12 (**19**). Of the peptides showing the highest agonist potencies at the BRS3 receptor, the singly substituted Gly^{12}, Thr^{12} and Trp^{14} nonapeptides (**9**, **10** and **13**) did not have significantly increased BRS3 selectivity.

Significant increases in selectivity for BRS3 over NMBR and GRPR were observed in the heptapeptide analogue **25**, which can be viewed as agonist **1**, simultaneously modified (a) by shortening at both terminals, (b) by acetylating the resultant terminal amino group and (c) by replacing Gln^7 with a hydrophobic aromatic Phe residue and replacing Phe^{13} with a charged polar Arg residue. Two more shortened and modified peptides, **26** and **27**, were also investigated: they are acylated carboxyl terminal fragments of agonist **1**. Analogues **26** and **27** showed increased selectivity compared with agonist **1** but were much less potent for BRS3. Within the sequence of peptide **25**, the substitution of Phe at position 7 and Arg at position 13 seems critical for the observed BRS3 selectivity§. See Tables 3 and 4 for the structures and activities of **25**–**27**.

Table 3 Sequence and Numbering

25
Ac-Phe^7-Trp^8-Ala^9-Val^{10}-β-Ala^{11}-His^{12}-Arg^{13}-NH_2
26
Tos-Gly-Leu-Val-β-Ala-His-Phe-Nle-NH_2
27
3-(Indol-3-yl)-propionyl-Leu-Val-β-Ala-His-Phe-Nle-NH_2

§A series of related peptides based on the sequence, Ac-Phe-Trp-Ala-Val-βAla-His-X-NH_2, gave the order of potency at the BRS3 receptor as X = Arg > Ser, Thr, His, Lys > Asp, Asn, Val. A related series based on the structure, Ac-X-Trp-Ala-Val-βAla-His-Arg-NH_2, showed the order as Phe > Ile, Ser, Trp, Asp, Lys > His, Arg. Also a series with dual changes, Ac-X-Trp-Ala-Val-β-Ala-His-Y-NH_2, produced the order for X/Y as Phe/Arg > Phe/His, Phe/Lys, Phe/Ser, Phe/Thr.

Multiple substituted sequences that combine the special features of agonist **25** with those substitutions at positions 10, 11 and 12 which increase BRS3 potency (see Table 2), produced the most potent and selective BRS3 agonists. Variation of the carboxyl terminal region of peptide **25** led to highly potent and very selective BRS3 agonists having essentially no activity at NMB or GRP receptors (Table 5). Replacement of Val in position 10 by His(τBzl) and βAla in position 11 by Nip (analogues **28** and **29** respectively), gave improved BRS3 potency and very high selectivity. The Nip^{11} analogue was synthesized with racemic nipecotic acid and preparative HPLC afforded both stereoisomers, **29A** and **29B**, which had comparable biological potencies. Slightly less potent, highly selective BRS3 agonists were also obtained with D-Arg or arginol in the terminal position 13 of **25** (analogues **30** and **31** respectively), or with Gly or Thr in position 12 (analogues **32** and **33**, respectively). Variation of the amino terminal region of **25** was not tolerated. Replacement of Phe^7 by D-Phe or Gln, or the Ala^9 by Leu (analogues **35, 36** and **37**, respectively), all resulted in the loss of BRS3 potency. Whereas the carboxyl terminus of **25** could be elongated with good retention of BRS3 potency and selectivity, as in analogues **41** and **42**, attempts to elongate **25** at the amino terminus (analogue **38**), or simultaneously at both termini (analogue **39**) led to the loss of BRS3 potency and a significant reduction of NMBR and GRPR potencies. It is conceivable that BRS3 selectivity has been induced by substitution of Arg and Phe at positions 13 and 7 respectively, and that the other substitutions at positions 10 to 12 contributed to the adoption of a better BRS3-binding conformation. In this study, the most selective NMBR and GRPR agonists appeared to be the nonapeptides **24** and **4**, respectively (Table 6), which have sequences more closely related to bombesins. The most potent and selective BRS3 agonist identified was the heptapeptide **34**, with an EC_{50} at BRS3 of 6.9 nM, and essentially no activity at NMB and GRP receptors. The increased BRS3 selectivity and potency of **34** was achieved by the combined effects of modifying **25** by (a) increasing the size of the hydrophobic side

Table 4 Selectivity Data for 25, 26, 27 and Agonist 1

Analogue number	BRS3 activity		NMBR activity		GRPR activity	
	% control 1 μM	EC_{50} nM	% control 30 nM	EC_{50} nM	% control 30 nM	EC_{50} nM
25	71	283	0	689	−1	1340
26	82	1630	4	913	0	1340
27	70	1960	8	328	1	702
Agonist **1**		5.7		0.041		0.093

Table 5 Analogues of 25: Ac-Phe^{7}-Trp^{8}-Ala^{9}-Val^{10}-βAla^{11}-His^{12}-Arg^{13}-NH_2

Analogue number	Substituent and position in agonist **25**	BRS3 activity		NMBR activity		GRPR activity	
		% control 300 nM	EC_{50} nM	% control 300 nM	EC_{50} nM	% control 300 nM	EC_{50} nM
28	His(τBzl)^{10}	96	31	−2	>30 000	−1	>30 000
29A	Nip^{11} isomer A	89	92	−2		1	
29B	Nip^{11} isomer B	87	147	−2		−1	
30	D-Arg^{13}	76		−4		−3	
31	13-Arginol	79		−2		−1	
32	Gly^{12}	64		4		−1	
33	Thr^{12}	80		−3		−1	
35	D-Phe^{7}	−3		54[a]		75[a]	
36	Gln^{7}	−2		31[a]		38[a]	
37	Leu^{9}	13		47[a]		71[a]	
38	D-Phe-Phe^{7}	−2		35[a]		15[a]	
39	D-Phe-Phe^{7} Nle^{14}-NH_2	1		32[a]		69[a]	
41	Arg^{13}-Nle-NH_2	93	53	−1		−2	
42	Arg^{13}-Trp-NH_2	102	17	−4	>3000	−1	15 700

[a] at 30 μM.

Table 6 Comparison of the Most Selective Peptides for BRS3, NMBR and GRPR

Analogue number	Sequence	EC_{50} (nM)		
		BRS3	NMBR	GRPR
34[a]	Ac-Phe-Trp-Ala-His(τBzl)-Nip-Gly-Arg-NH_2	6.9	>30, 000	11 700
24	H-D-Phe-Gln-Trp-Ala-Val-MeAla-His-Phe-Nle-NH_2	82.6	1.84	185
4	H-D-Phe-Gln-2-Nal-Ala-Val-βAla-His-Phe-Nle-NH_2	>74	0.323	0.02

[a] Analogue **34** is a mixture of stereoisomers: see the discussion.

chain at position 10, (b) by substituting a cyclic residue at position 11 and (c) by shortening the side chain in position 12 by deletion of the polar imidazole group. In the case of **34** the analogue was obtained as a mixture of at least two stereoisomers because racemic nipecotic acid was used for the synthesis and separation by preparative HPLC was not possible. Furthermore, since the coupling of His(τBzl) is notoriously prone to racemization [14], **34** may also contain stereoisomers with the D-configuration at the His(τBzl) residue.

MODELLING STUDIES

Molecular modelling was used to attempt to rationalize the potency and selectivity of analogue **34**. The presence of Arg in position 13, e.g. in **25**, has been linked with good selectivity at the expense of potency, and an explanation of the potency of **34** was sought. The Nip^{11}-Gly^{12} motif of **34** strongly suggested a β-turn at this position, which would correspond to the βAla^{11}-His^{12} residues of **1**. The Chem-X software (Oxford Molecular Ltd, Oxford, UK) was used to construct models of agonist **1** and **34** in an anti-parallel β-sheet motif, with a β-turn for the Nip^{11}-Gly^{12} motif. When building the model of analogue **34** the S configuration of Nip was used. An overlay of these peptides suggested that the benzyl group of His(τBzl)^{10} in **34** could occupy the same position in 3D space as the benzyl group of Phe^{13} in agonist **1** and thereby be considered as one contributor to the activity of **34**. To verify this, conformational analysis was performed by rotating the four flexible bonds of the side-chain of His(τBzl)^{10} in **34** and the two flexible bonds of the side-chain of Phe^{13} in agonist **1** in 30° increments. Conformations were only accepted which made no unfavourable intra-peptide van der Waals interactions and where the centre of the benzyl ring of His(τBzl)^{10}

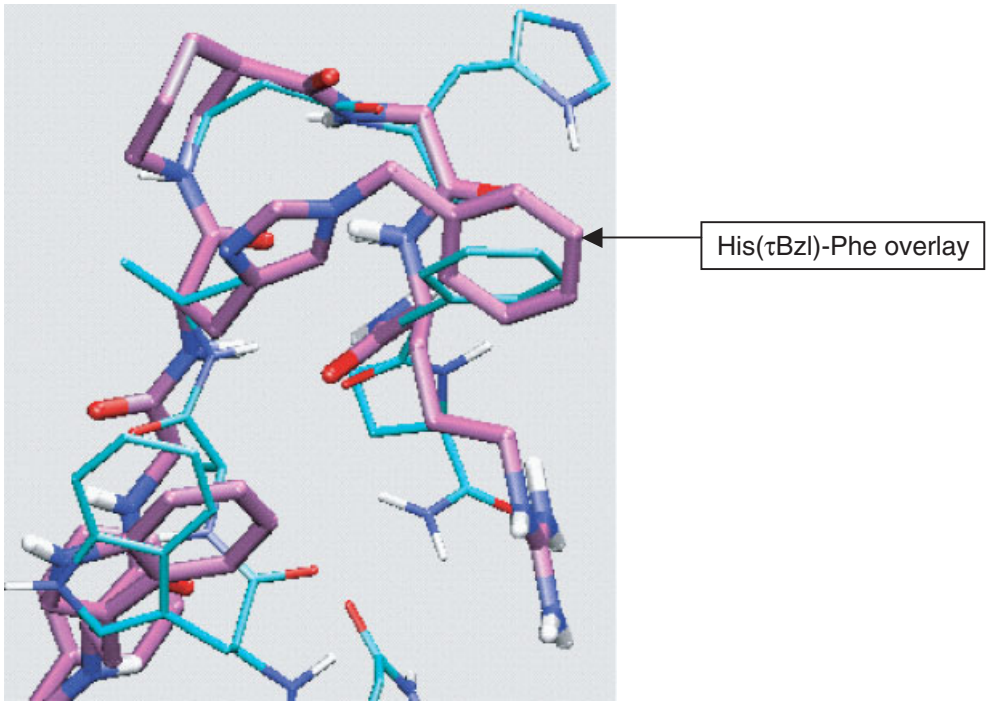

Figure 1 Overlaid β-turn conformations of **1** (reproduced in **cyan**) and **34** (reproduced in **magenta**). In the above modelling of **34** the His(τBzl) is presented in the S configuration.

of **34** was within 1.0 Å of the centre of the benzyl ring of Phe[13] of agonist **1**. The lowest energy conformations of these peptides are shown in Figure 1 revealing that there is a good overlay between the His(τBzl)[10] of **34** and the Phe[13] of agonist **1**. This model could suggest the design of further peptide sequences.

CONCLUSIONS

In summary, a group of potent and selective agonists for BRS3 were produced, of which the most selective was agonist **34**. This represents a heptapeptide in which only the Trp-Ala moiety of agonist **1** has been retained and therefore does not appear to be a direct bombesin-type analogue. It is possible that **34** could resemble the as yet unknown endogenous BRS3 ligand. It is believed that the structure of **34** is significantly different from other bombesin related BRS3 ligands already reported. Compound **34** will be useful as a biological standard for BRS3 and the results reported here should aid in the design of small non-peptide BRS3 agonists containing drug-like scaffolds.

MATERIALS AND METHODS

Peptide Synthesis

The peptides were synthesized in-house or by contract synthesis using standard Fmoc-solid phase procedures and purified by preparative HPLC usually to greater than 95% purity by HPLC (C-18, Vydac 218TP54, 250 × 4.6 mm) with mass confirmation by MALDI-MS mass spectrometry. Starting amino acids were of the L-configuration except where otherwise indicated.

Biological Assays

Stable transfection. Human BRS-3 cDNA in the mammalian expression vector pCD2 were transfected into Balb/3T3 cells with Lipofectamine (Invitrogen) and selected with 800 µg/ml of G418. Rat NMBR and mouse GRPR stable cells were isolated as previously described [15].

Measurement of intracellular calcium mobilization. The agonistic activity of peptides was measured by intracellular calcium mobilization. Cells were grown in Dulbeco's Modified Eagle's Medium (DMEM) containing 10% FCS. Cells were scraped, pelleted at 800 **g** for 5 min and resuspended with DMEM (without phenol red) containing 0.1% BSA. Fluo-3 AM and Pluronic F127 were added to the cells to give final concentrations of 10 µM and 0.05%, respectively. After an incubation for 1 h, extracellular dyes were removed and resuspended with DMEM (without phenol red) at a density of 3×10^5 cells/ml. After adding the peptide to cell, cell fluorescence was measured by F3000 Fluorescence Spectrophotometer (Hitachi). To determine the intracellular calcium concentration, maximum fluorescence was measured by treatment with calcium ionophore A23187.

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