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Discovery of Potent and Selective Peptide Agonists at the GRP-preferring Bombesin Receptor (BB_2)

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Abstract: Analogues of the nonselective bombesin receptor synthetic agonist H-D-Phe-Gln-Trp-Ala-Val-βAla-His-Phe-Nle-NH_2 were prepared and their biological activity assessed at the NMB-preferring/bombesin receptor (NMB-R; BB_1), the GRP-preferring/bombesin receptor (GRP-R; BB_2) and the orphan receptor bombesin receptor subtype-3 (BRS-3; BB_3). Progressive *N*-terminal deletions identified the minimum *C*-terminal sequences required for maintaining a significant agonist effect, whilst an alanine scan, targeted changes in stereochemistry and other pertinent substitutions identified key side-chain and stereochemical requirements for activation. Key structural elements required for functional potency at BB_1 BB_2 and BB_3, and for selectivity between these receptor subtypes were established. Synthetic peptides were discovered, which were highly potent agonists at BB_2 and extremely selective over both BB_1 and BB_3. Copyright © 2001 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: bombesin; gastrin-releasing peptide/bombesin receptor peptide agonist; structure–activity relationship

INTRODUCTION

Bombesin (Bn) is a tetradecapeptide isolated from amphibians [1] that has an *N*-terminal pyroglutamate residue and *C*-terminal amidation. The mammalian peptides neuromedin B (NMB) [2] and gastrin-releasing peptide (GRP) [3] contain 10 and 27 amino acids respectively and possess structural homology with the *C*-terminal octapeptide of Bn (see Table 1).

Three receptors that are able to bind bombesin have been isolated from human cells: the NMB-preferring receptor (NMB-R; BB_1) [4,5], the GRP-preferring receptor (GRP-R; BB_2) [6,7] and the orphan receptor bombesin receptor subtype-3 (BRS-3; BB_3) [8,9]. BB_1 and BB_2 are present in human lung carcinoma cells [10], the gastrointestinal (GI) tract and central nervous system (CNS), whilst BB_3 is found in the CNS as well as testis and lung cancer cells. A fourth member of the bombesin receptor family (BB_4) was isolated from frog brain [11] and, to date, any mammalian equivalent has not been identified. All four receptors are members of the G-protein coupled receptor superfamily. The mammalian bombesin-like peptides elicit a wide range of biological responses [12] in the CNS and in peripheral tissues. Their actions include stimulation of GI hormone release [13–15] and activity as growth factors in human small-cell lung cancer [16–18]. In the CNS, it is proposed these peptides have a role in feeding, grooming behaviour, the regulation of homeostasis, thermoregulation and metabolism.

The synthetic nonapeptide [D-Phe^6, βAla^{11}, Phe^{13}, Nle^{14}]Bn(6–14) (**1**), containing three unnatural residues and amidated at the *C*-terminus, was originally identified as a high affinity agonist at BB_3 [19]. This sequence and an analogue amenable to radiolabelling, [^{125}I-D-Tyr^6, βAla^{11}, Phe^{13}, Nle^{14}]Bn(6–14)

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Table 1 Aligned Sequences of Bn, NMB, GRP and the Synthetic Peptide [D-Phe^{6}, βAla^{11}, Phe^{13}, Nle^{14}]Bn(6–14) (**1**)

Peptide	No. from Bn *N*-terminus								
	6	7	8	9	10	11	12	13	14
Bn(6–14)	Asn	Gln	Trp	Ala	Val	Gly	His	Leu	Met-NH_2
NMB(2–10)	Asn	Leu	Trp	Ala	Thr	Gly	His	Phe	Met-NH2
GRP(19–27)	Asn	His	Trp	Ala	Val	Gly	His	Leu	Met-NH2
1	D-Phe	Gln	Trp	Ala	Val	β-Ala	His	Phe	Nle-NH2

Table 2 Functional Potency of [D-Phe^{6}, βAla^{11}, Phe^{13}, Nle^{14}]Bn(6–14) (**1**) and Natural Agonists at BB_1, BB_2 and BB_3

No.	Peptide	pEC_{50}		
		BB_1	BB_2	BB_3
1	[D-Phe^{6}, βAla^{11}, Phe^{13}, Nle^{14}]Bn(6–14) amide	8.06 ± 0.20	8.69 ± 0.14	6.71 ± 0.14
3	Bn	8.46 ± 0.07	8.89 ± 0.04	Inactive (@ 10 μM)
4	NMB	9.02 ± 0.06	7.95 ± 0.08	Inactive (@ 10 μM)
5	GRP	8.11 ± 0.05	9.12 ± 0.058	Inactive (@ 10 μM)

(^{125}I-**2**), were shown [20] to have a high affinity for all four of the bombesin receptor subtypes.

H-Xaa-Gln-Trp-Ala-Val-βAla-His-Phe-Nle-NH_2

1 : Xaa = D-Phe

2 : Xaa = D-Tyr

The functional potency of **1** at BB_1, BB_2 and BB_3 is shown in Table 2, and is compared with the biological activities of the natural agonists. BB_2 exhibits selectivity for GRP whereas BB_1 shows selectivity for NMB. Both NMB and GRP are inactive at BB_3.

Previous studies [21–24] have shown that even though there is a close homology between NMB and GRP, and their respective receptors (55%), their structure–activity relationships are markedly different. Key elements that are important for binding affinity and functional activity in natural agonists at both BB_1 and BB_2 have been identified [21–24]. Site directed mutagenesis studies [25,26] have also identified residues in BB_1 and BB_2 that are critical for high affinity agonist binding. BB_3, although structurally related to BB_1 and BB_2 (47% and 51% sequence homology respectively), may have a natural agonist that is structurally unrelated to bombesin, GRP and NMB. Previous studies [27] have identified residues in NMB, which are important for optimal activity at BB_3.

We report here the biological evaluation of a number of analogues of the synthetic agonist **1** in order to identify the key structural elements of this peptide required for functional potency at BB_1, BB_2 and BB_3, and for selectivity between these receptor subtypes. The major part of this study was an alanine scan, in which each (non-alanine) residue (except for D-Phe^{6} and β-Ala^{11}) was systematically replaced with L-alanine. The *N*-terminal D-residue was replaced with D-alanine. *N*-terminally deleted peptides were utilized to enhance our knowledge of the minimum *C*-terminal fragments required for both full and partial activity. In order to probe certain stereochemical requirements, the phenylalanine residues at the *N*-terminus and adjacent to the *C*-terminal norleucine residue were each separately replaced with their corresponding D-enantiomer, as was norleucine itself. A number of other pertinent substitutions were also carried out. The phenylalanine residue in position 13 was replaced with leucine, the residue that occupies this position in the aligned sequences of GRP and Bn (see Table 1). Similarly, threonine, found in the analogous position in NMB, was substituted for Val^{10}. Glycine, found in the corresponding position

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in Bn, GRP and NMB was substituted for βAla[11]. Finally, the effect of altering the *C*-terminal amide to an acid functionality was investigated.

The intracellular signalling pathways of BB_1, BB_2 and BB_3 have been well described. Ligand binding results in a stimulation of phospholipase C [28–32], an increase in intracellular calcium ($[Ca^{2+}]_i$) [27–30,32] and tyrosine phosphorylation of intracellular proteins [27,32–36]. Peptide activity was determined by recording functional potency, as reflected by changes in $[Ca^{2+}]_i$ measured using fluorometric imaging technology.

MATERIALS AND METHODS

Peptides

[D-Phe[6], βAla[11], Phe[13], Nle[14]]Bn(6–14) (**1**) was obtained by contract synthesis from California Peptide Research Inc., USA. Bn, NMB and GRP were purchased from Bachem, UK. Peptides **6**–**22** were obtained by contract synthesis from AFFINITI Research Products Limited, UK. All peptides showed a purity of >95% by analytical reversed phase high performance liquid chromatography and peptide identities were confirmed by mass spectrometry.

Measurement of Intracellular Calcium

Cell culture. Human embryonic kidney (HEK)-293 BB_1 and HEK-293 BB_2 cells were routinely grown as monolayers in MEM containing Earle's Salts supplemented with 10% fetal bovine serum, 1% MEM non-essential amino acids, 1% L-glutamine and 500 µg ml G418. Rat basophil leukaemia (RBL)-2H3 BB_3 cells were grown in MEM containing Earle's salts supplemented with 10% fetal bovine serum, 1% MEM non-essential amino acids and 400 µg ml G418. All were maintained under 95%/5% O_2/CO_2 at 37 °C. Cells were passaged every 3–4 days and the highest passage number used was 24.

Measurement of $(Ca^{2+})_i$ using the FLIPR. HEK-293 BB_1, HEK-293 BB_2 and RBL-2H3 BB_3 cells were seeded into black walled clear-base 96 well plates (Costar UK) at a density of 25 000 cells per well in MEM-Alpha medium, supplemented as above and cultured overnight. The cells were then incubated with MEM-Alpha medium containing the cytoplasmic calcium indicator, Fluo-3AM (4 µM; Teflabs, Austin, Texas) and 2.5 mM probenecid at 37 °C for 60 min. The cells were washed four times with, and finally resuspended in, Tyrode's medium containing 2.5 mM probenecid. The plates were then placed into a fluorometric imaging plate reader (FLIPR; Molecular Devices, UK) to monitor cell fluorescence ($\lambda_{EX} = 488$ nm, $\lambda_{EM} = 540$ nm) [37] before and after the addition of various peptides (10 pM–10 µM).

RESULTS

N-Terminal Deletion

Table 3 shows the functional potencies at BB_1, BB_2 and BB_3 of analogues of **1** truncated at the amino terminus. Most significantly, removal of the *N*-terminal phenylalanine residue and cyclization of the resultant *N*-terminus to a pyroglutamic (Glp) residue (**6**) resulted in a 150-fold increase in functional potency at BB_2 whilst a ten-fold drop in biological activity was observed at both BB_1 and BB_3. Truncation to the *C*-terminal hexapeptide (**7**) afforded a further and more substantial drop in potency at BB_1, a 150-fold drop at BB_2 compared with the parent nonapeptide, and loss of activity at BB_3. Further

Table 3 Functional Potency of *N*-terminally Deleted Analogues of [D-Phe[6], βAla[11], Phe[13], Nle[14]]Bn(6–14) (**1**) at BB_1, BB_2 and BB_3

No.	Peptide	pEC$_{50}$		
		BB_1	BB_2	BB_3
1	[D-Phe[6], βAla[11], Phe[13], Nle[14]]Bn(6–14) amide	8.06 ± 0.20	8.69 ± 0.14	6.71 ± 0.14
6	[Glp[7], βAla[11], Phe[13], Nle[14]]Bn(7–14) amide	7.11 ± 0.13	10.85 ± 1.09	5.73 ± 0.28
7	[βAla[11], Phe[13], Nle[14]]Bn(9–14) amide	5.23 ± 0.06	6.50 ± 0.18	Inactive (@ 10 µM)
8	[βAla[11], Phe[13], Nle[14]]Bn(10–14) amide	Inactive (@ 10 µM)	5.37 ± 0.20	Inactive (@ 10 µM)

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deletion to the *C*-terminal pentapeptide (**8**) led to the loss of activity at BB_1 and a much reduced agonist response at BB_2.

Alanine Scan

The functional potencies of the peptides comprising the alanine scan of **1** against BB_1, BB_2 and BB_3 are given in Table 4. In the case of BB_1, the data showed that only substitution of alanine for phenylalanine in position 13 (compound **14**) resulted in a significant drop in functional potency from the parent ligand **1**. Four analogues exhibited a two-fold to ten-fold loss in activity at this receptor; the substituted residues in this group were Gln^7, Val^{10}, His^{12} and Nle^{14} (compounds **10**, **12**, **13** and **15**.). Substitution of alanine for Trp^8 and D-alanine for the D-phenylalanine at the *N*-terminus resulted in sequences which maintained functional potency (compounds **11** and **9**).

At BB_2, replacement of D-Phe^6 with D-alanine afforded a very considerable increase in functional potency. Replacement of the valine in position 10 with alanine increased potency some 80-fold whilst substitution of His^{13}gave a sequence that showed a 5-fold increase in activity Substitution of Trp^8 gave a peptide of similar potency to **1**. Three analogues gave a marginally reduced potency at BB_2; the substituted residues in this group were Gln^7, Phe^{13} and Nle^{14}.

In the case of BB_3, removal of the phenyl ring in position 13 (compound **14**) eradicated the functional response. All other alanine substitutions resulted in small (two-fold maximum) reductions in activity at this receptor subtype.

Table 4 Functional Potency of Alanine Scan Analogues of [D-Phe^6, βAla^{11}, Phe^{13}, Nle^{14}]Bn(6–14) (1) at BB_1, BB_2 and BB_3

No.	Peptide	Substituted residue[a]	pEC$_{50}$		
			BB_1	BB_2	BB_3
1	[D-Phe^6, βAla^{11}, Phe^{13}, Nle^{14}]Bn(6–14) amide		8.06 ± 0.20	8.69 ± 0.14	6.71 ± 0.14
9	[D-Ala^6, βAla^{11}, Phe^{13}, Nle^{14}]Bn(6–14) amide	D-Phe^6	8.27 ± 0.05	11.19 ± 0.42	6.67 ± 0.42
10	[D-Phe^6, Ala^7, βAla^{11}, Phe^{13}, Nle^{14}]Bn(6–14) amide	Gln^7	7.37 ± 0.04	8.14 ± 0.11	6.38 ± 0.11
11	[D-Phe^6, Ala^8, βAla^{11}, Phe^{13}, Nle^{14}]Bn(6–14) amide	Trp^8	8.10 ± 0.11	8.81 ± 0.13	6.50 ± 0.13
12	[D-Phe^6, Ala^{10}, βAla^{11}, Phe^{13}, Nle^{14}]Bn(6–14) amide	Val^{10}	7.70 ± 0.06	10.60 ± 0.99	6.57 ± 0.99
13	[D-Phe^6, βAla^{11}, Ala^{12}, Phe^{13}, Nle^{14}]Bn(6–14) amide	His^{12}	7.13 ± 0.02	9.36 ± 0.46	6.46 ± 0.46
14	[D-Phe^6, βAla^{11}, Ala^{13}, Nle^{14}]Bn(6–14) amide	Phe^{13}	5.62 ± 0.13	8.18 ± 0.10	Inactive (@ 10 μM)
15	[D-Phe^6, βAla^{11}, Phe^{13}, Ala^{14}]Bn(6–14) amide	Nle^{14}	7.62 ± 0.07	8.26 ± 0.01	6.43 ± 0.01

[a] All residues substituted by Ala except for D-Phe^6, which was replaced with D-Ala.

Table 5 Functional Potency of Analogues of [D-Phe^6, βAla^{11}, Phe^{13}, Nle^{14}]Bn(6–14) (1) at BB_1, BB_2 and BB_3

No.	Peptide	Substitution	pEC$_{50}$		
			BB_1	BB_2	BB_3
1	[D-Phe^6, βAla^{11}, Phe^{13}, Nle^{14}]Bn(6–14) amide		8.06 ± 0.20	8.69 ± 0.14	6.71 ± 0.14
16	[Phe^6, βAla^{11}, Phe^{13}, Nle^{14}]Bn(6–14) amide	Phe for D-Phe^6	7.91 ± 0.06	8.55 ± 0.11	6.63 ± 0.11
17	[D-Phe^6, βAla^{11}, D-Phe^{13}, Nle^{14}]Bn(6–14) amide	D-Phe for Phe^{13}	6.23 ± 0.05	6.96 ± 0.06	Inactive (@ 10 μM)
18	[D-Phe^6, βAla^{11}, Phe^{13}, D-Nle^{14}]Bn(6–14) amide	D-Nle for Nle^{14}	6.73 ± 0.12	7.99 ± 0.21	6.08 ± 0.21
19	[D-Phe^6, Thr^{10}, βAla^{11}, Phe^{13}, Nle^{14}]Bn(6–14) amide	Thr for Val^{10}	5.87 ± 0.07	7.48 ± 0.14	Inactive (@ 10 μM)
20	[D-Phe^6, Phe^{13}, Nle^{14}]Bn(6–14) amide	Gly for βAla^{11}	7.63 ± 0.02	8.08 ± 0.11	Inactive (@ 10 μM)
21	[D-Phe^6, βAla^{11}, Nle^{14}]Bn(6–14) amide	Leu for Phe^{13}	7.23 ± 0.05	8.22 ± 0.04	6.39 ± 0.04
22	[D-Phe^6, βAla^{11}, Phe^{13}, Nle^{14}]Bn(6–14)	Nle-OH for Nle^{14}-NH_2	7.93 ± 0.05	8.74 ± 0.37	6.48 ± 0.37

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Stereochemical Changes

Replacement of the *C*-terminal norleucine residue with D-norleucine (**18**; see Table 4) led to a >20-fold reduction in potency at BB_1 whereas activity dropped only five-fold at BB_2, and slightly less at BB_3. Replacement of the adjacent phenylalanine with its D-enantiomer (**17**) resulted in loss of biological activity at BB_3 and a reduction in activity of approximately 70-fold at BB_1, whilst only a slight reduction in activity was recorded at BB_2. Replacement of the *N*-terminal D-phenylalanine with its natural enantiomer (**16**) had little effect on activity.

Other Substitutions

Substitution of leucine for Phe^{13} (**21**) resulted in a small drop in potency at each receptor. However, substitution of Val^{10} with threonine (**19**) afforded an inactive peptide sequence at BB_3 and gave a >150-fold reduction in activity at BB_1, whereas only a 16-fold drop in potency was seen at BB_2. Replacement of the chain-extended β-alanine in position 11 with glycine (**20**) had very little effect on activity at BB_1 and BB_2, with only a small reduction in biological response observed at either receptor subtype. However, at BB_3 this alteration resulted in loss of activity. Changing the *C*-terminal amide group to an acid (**22**), resulted in little change in activity.

DISCUSSION

The synthetic peptide **1** is approximately four- and 100-fold selective at BB_2 over BB_1 and BB_3 respectively, whilst the natural agonist GRP is inactive at BB_3 (see Table 2). Removal of the *N*-terminal D-phenylalanine residue from **1** led to the identification of [Glp^7, βAla^{11}, Phe^{13}, Nle^{14}]Bn(7-14) amide (**6**) as a highly potent ligand for BB_2, which is >5000-fold selective over BB_1 and $>1.3 \times 10^5$ selective over BB_3. Substitution of D-alanine in position 6 also resulted in a peptide (**9**) of high potency and selectivity (>800-fold at BB_2 and $>3.3 \times 10^4$ at BB_3) whilst replacement with L-Phe (**16**) had little effect on activity. These results clearly demonstrate that the presence of the aromatic ring in position 6 is detrimental to the sub-nanomolar activity observed at BB_2, regardless of the orientation of the side chain at that position. The lack of a phenyl group at the *N*-terminus is playing an important role in determining receptor subtype selectivity between BB_1 and BB_2/BB_3. It is postulated that either the phenyl group's function is of great consequence in altering the conformation required for sub-nanomolar activity at BB_2, or that the steric requirements around position 6 are restricted for the peptide in its receptor-bound conformation at this receptor subtype. Further truncations resulted in a loss of activity or a marked decrease in activity at each receptor subtype. The minimum sequence needed for activation of BB_3 concurs well with the minimum size of NMB required for retention of full activity at BB_3 as previously reported [27].

The alanine scan showed that the other phenyl group, at position 13, is essential for a functional response at BB_3 and for the high functional potency of **1** at BB_1, but not at BB_2 (compound **14**). This result agrees well with a previous study [22] that shows this residue is important for receptor subtype selectivity between the NMB- and GRP-preferring Bn receptors.

Removal of the β-branched hydrophobic side chain in position 10 (compound **12**) resulted in a >80-fold increase in activity at BB_2, whereas a small drop in potency was recorded at BB_1 and BB_3. Thus, the presence of the valine side chain has a negative effect on potency at BB_2. Consequently, peptide **12** is 800-fold selective for BB_2 over BB_1 and $>1 \times 10^4$ selective over BB_3. Replacement of histidine in position 12 with alanine (compound **13**) reduced activity eight-fold at BB_1 and marginally at BB_3, but increased the response at BB_2 five-fold. In the case of the *C*-terminal octapeptide fragment of Bn, this polar aromatic residue has also been reported [23] to be more important for activity at BB_1 than BB_2.

Interestingly, functional potency was maintained (compound **11**) when alanine was substituted for Trp^8, a residue that has been shown in GRP, NMB and Bn (or its fragments and synthetic analogues) to be important for activity at both BB_1 and BB_2 [23,24,38–40]. All of the other alanine mutations had only a limited effect on functional potency.

In the case of analogues containing an amino acid of opposite stereochemistry (compounds **16**, **17** and **18**), the side chains remain chemically unchanged, and the overall hydrophobicity and dipole moment are only slightly altered, so that the orientation of the side chains may be studied. Replacement of Phe^{13} with its D-enantiomer resulted in loss of activity at BB_3 and a 70-fold reduction in activity at BB_1, whilst only a five-fold reduction was observed

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at BB_2, emphasizing the more important role this aromatic residue plays in BB_3 and, to a lesser extent, BB_1 recognition. The result at BB_3 agrees with the fact that Phe^9 of NMB has also been shown [27] to be critical for activity at the orphan receptor. A switch in the stereochemistry of the *C*-terminal norleucine residue (**18**) resulted in a 20-fold drop in activity at BB_1 whereas the reduction in potency at BB_2 and BB_3 was less significant. Incorporation of L-Phe for D-Phe at the *N*-terminus, as mentioned above, had only a limited effect at all three receptor subtypes.

It has been suggested [41] that bombesin agonists adopt a folded antiparallel β-sheet with a γ-turn at Val^{10}-Gly^{11}-His^{12}-Leu^{13}. A more recent study [23] proposed that Ac-Bn(7–14) in its binding conformation at BB_1 and BB_2 consists of three consecutive γ-turns followed by a bend and finishing with two γ-turns. Consequently, a number of conformationally restricted bombesin analogues have been tested, in which the dipeptide Val-Gly has been substituted by turn mimetics [42]. Substitution of glycine for β-alanine in **1** afforded a small drop in functional potency at both BB_1 and BB_2. However, at BB_3 this reduction in carbon chain length resulted in the loss of a functional response. Therefore, whether the principle effect of this substitution was to alter either the conformation of this putative turn region in **1** and/or simply shorten the length of the peptide backbone, the modification was not critical to biological activity at BB_1/BB_2 but was in the case of BB_3.

A difference between NMB and GRP is that the former peptide has a threonine and the latter a leucine at the position five residues from the *C*-terminus (see Table 1). The fact that substitution of threonine for valine at position 10 (**19**) gave >150-fold reduction in activity at BB_1, whereas only a 16-fold drop in potency was seen at BB_2, was therefore unexpected. In the case of BB_3, introduction of the β-hydroxyl group resulted in loss of activity. A second difference between NMB and GRP is that the former has phenylalanine in position 13 whereas the latter has a leucine residue. Replacement of phenylalanine in position 13 by leucine (**21**) resulted in a similar small drop in potency at each of the receptor subtypes.

CONCLUSION

In summary, we have identified a number of key structural features of **1** required for a significant functional response at BB_1, BB_2 and BB_3. Significantly, removal of the *N*-terminal phenylalanine residue and cyclization of the resultant *N*-terminus to a pyroglutamic residue afforded a potent BB_2 specific octapeptide agonist, [Glp^7, βAla^{11}, Phe^{13}, Nle^{14}]Bn(7–14) amide (**6**), which is >5000-fold selective over BB_1/BB_3. Substitution of D-alanine in position 6 or L-alanine in position 10 also resulted in sequences (**9** and **12** respectively) of high potency at BB_2 with a selectivity of $\geq$800-fold over BB_1/BB_3.

The minimum *C*-terminal fragments to show activity at BB_1, BB_2 and BB_3 were the heptapeptide (9–14), hexapeptide (10–14) and octapeptide (8–14) respectively. The structure–function studies conducted have confirmed that the presence of Phe^{13} and Gly^{11} are required for activity of **1** at BB_3 Removal of the phenyl and β-branched side-chains in positions 9 and 12 respectively gave an increased agonist response at BB_2.

The potent and selective synthetic peptide agonists reported in this manuscript may provide useful tools to study further the role of BB_2 in various biological processes. Molecular modelling studies, aligned with the design of further analogues of **1**, e.g. containing multiple substitutions such as D-Ala for D-Phe^6 and L-Ala for L-Val^{10}, will further elucidate the key structural requirements required for receptor subtype selectivity.

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