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Development of a potent and selective GPR7 ($NPBW_1$) agonist: a systematic structure–activity study of neuropeptide B

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Abstract: Neuropeptide B (NPB) has been recently identified as an endogenous ligand for GPR7 (NPBW1) and GPR8 (NPBW2) and has been shown to possess a relatively high selectivity for GPR7. In order to identify useful experimental tools to address physiological roles of GPR7, we synthesized a series of NPB analogs based on modification of an unbrominated form of 23 amino acids with amidated *C*-terminal, Br(–)NPB-23-NH_2. We confirmed that truncation of the *N*-terminal Trp residue resulted in almost complete loss of the binding affinity of NPB for GPR7 and GPR8, supporting the special importance of this residue for binding. Br(–)NPB-23-NH_2 analogs in which each amino acid in positions 4, 5, 7, 8, 9, 10, 12 and 21 was replaced with alanine or glycine exhibited potent binding affinity comparable to the parent peptide. In contrast, replacement of Tyr^{11} with alanine reduced the binding affinity for both GPR7 and GPR8 four fold. Of particular interest, several NPB analogs in which the consecutive amino acids from Pro^4 to Val^{13} were replaced with several units of 5-aminovaleric acid (Ava) linkers retained their potent affinity for GPR7. Furthermore, these Ava-substituted NPB analogs exhibited potent agonistic activities for GPR7 expressed in HEK293 cells. Among the Ava-substituted NPB analogs, analog 15 (Ava-5) and 17 (Ava-3) exhibited potency comparable to the parent peptide for GPR7 with significantly reduced activity for GPR8, resulting in high selectivity for GPR7. These highly potent and selective NPB analogs may be useful pharmacological tools to investigate the physiological and pharmacological roles of GPR7. Copyright © 2007 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: neuropeptide B; orphan GPCR; GPR7; GPR8

INTRODUCTION

Neuropeptide B (NPB) and neuropeptide W (NPW) belong to the newly identified neuropeptide family as endogenous ligands for GPR7 and GPR8, and are also known as NPBW1 and NPBW2 respectively [1]. The distribution of NPB mRNA was similar to NPW mRNA, but with higher expression with the central nervous system (CNS). Furthermore, unlike NPW, NPB mRNA was detected in the thalamus and hypothalamus of human brain, suggesting the important role of NPB in the brain [1–5]. Although NPB and NPW share 61% sequence homology at the amino acid level, NPB has a higher affinity for GPR7, while NPW binds to GPR7 and GPR8 with similar affinities [2,3]. Regarding the structure–activity relation (SAR) information of NPW, it is reported that NPW has two functional forms of peptide ligands with length of 23 and 30 amino acid residues and that the Trp^1 is essential for the interaction of NPW to GPR8 [6]. It is also reported that endogenous NPB-29 is modified with bromine at the *N*-terminal Trp residue [5]. However, the brominated and unbrominated forms of NPB show comparable functional activities to both GPR7 and GPR8, and physiological role of the bromination of Trp^1 of NPB has remained unclear so far (Figure 1).

GPR7 and GPR8 were originally identified as orphan G-protein coupled receptors (GPCRs) that share 64% amino acid identity [7]. These receptors were initially cloned from human genomic DNA on the basis of on their homology with the δ-opioid and somatostatin receptors. Although GPR7 is the only receptor identified in rodents, both GPR7 and GPR8 are known to be expressed in humans and other higher species. Recent publications have reported that GPR7-deficient mice showed adult-onset obesity, similar to that reported in NPB-deficient mice, supporting the idea that the NPB-GPR7 pathway is involved in the regulation of feeding and energy expenditure [8,9]. In addition, several reports showed that NPB/NPW-GPR7 plays regulatory roles in the endocrine system and inflammatory pain response [9–11]. However, species difference of the NPB/NPW-GPR7/GPR8 system makes it difficult to elucidate the physiological roles of GPR7 in higher species particularly in humans.

In the present study, we synthesized a series of unbrominated NPB analogs by alanine scanning, *N*- or *C*-terminal truncations and the replacement

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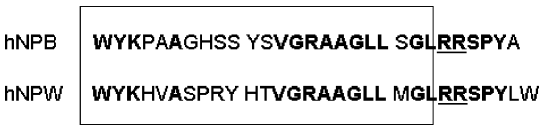

Figure 1 Amino acid sequences of mature human NPB and NPW. The box marks the core functional peptide sequences consisting of 23 amino acid residues. Underlines mark dibasic amino acid residues constituting a typical motif for a proteolytic cleavage site. Amino acid identities between NPB and NPW are shown in bold.

of consecutive amino acids with different length of 5-aminovaleric acid (Ava) linkers. Through a systematic SAR study of NPB analogs, we identified potent and selective GPR7 agonists that are useful to elucidate physiological roles of GPR7 in several species.

MATERIALS AND METHODS

Peptide Synthesis

NPB and NPB analogs were synthesized on solid support using an Fmoc/tBu strategy on a Pioneer peptide synthesizer (Applied Biosystems, Foster City, CA). Fmoc-Leu-NovaSyn TGR, Fmoc-Ala-NovaSyn TGR, Fmoc-Trp(Boc)-NovaSyn TGR or Fmoc-NovaSyn TGR resin (Novabiochem, Laufelfingen, Switzerland) was used as a starting material. All crude peptides were cleaved from resins with TFA/thioanisole/ethandithiol/*m*-cresol within 2 h, followed by the addition of cold diethyl ester for solidification. The resulting crude peptides, obtained after centrifugation, were purified with preparative reversed-phase HPLC (Hitachi D-6000 system, Hitachi, San Jose, CA) with YMC-Pack ODS-AQ column (20 × 250 mm; YMC, Kyoto, Japan). All peptides were characterized by a Waters LC-MS system (Waters 2690, Waters 996 and Waters Platform ZMD2000, Waters, Millford, MA) with YMC-Pack ODS-AQ column (2.0 × 150 mm; YMC, Kyoto, Japan).

Molecular Cloning, Transfection and Cell Cultures

The cDNAs of human GPR7 (Acc. U22491) and GPR8 (Acc. U22492) were cloned by PCR from human genomic DNA. Corresponding expression vectors were constructed and transfected into the HEK293-CRE-BLA cells containing a CRE (cAMP-responsible element)-β-lactamase reporter [12]. The HEK293-CRE-BLA cell lines stably expressing the human GPR7 and GPR8 were isolated under pressure of hygromycin B and used for the study of radioligand binding and cellular functional assays.

The HEK293 cells were cultured in Dulbecco's modified Eagle's medium : nutrient mixture F-12 (D-MEM/F12) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, 100 μg/ml streptomycin, 0.5 mg/ml G418 and 250 units/ml hygromycin B in a humidified atmosphere consisting of 5% CO_2 and 95% air at 37 °C.

Radioligand Binding Assays

HEK293 cells expressing GPR7 and GPR8 were collected by spinning at 500 *g* for 10 min and resuspended in lysis buffer (20% sucrose, 154 mM NaCl, 10 mM KCl, 0.8 mM $CaCl_2$, 10 mM MOPS, pH 7.4). The cell suspension was homogenized with the Polytron and centrifuged at 1000 *g* for 15 min at 4 °C. The supernatant was then centrifuged at 100000 *g* for 60 min at 4 °C. The pellets were resuspended in buffer A (5 mM HEPES, 5 mM Tris, pH 7.4) and centrifuged again at 10000 *g* for 60 min at 4 °C. The pellets were then resuspended in an appropriate volume of buffer A. The protein concentration of the membrane fraction was determined by the bicinchoninic acid assay method with bovine serum albumin as the standard (Pierce). The membrane homogenate was aliquoted and frozen at −80 °C until use.

The binding affinities of the synthetic peptides were assessed in a binding competition assay using membrane homogenate derived from HEK293 cells expressing recombinant GPR7 or GPR8 and [^{125}I]-NPW-23 (2200 Ci/mmol, PerkinElmer). Each assay tube contained 2–5 μg/assay membrane protein, a series of concentrations of the synthetic peptide being assayed and 30 pM [^{125}I]-NPW-23 in buffer B (25 mM Tris-HCl buffer containing 5 mM EDTA, 0.1% BSA, 0.05% CHAPS and 150 mM NaCl, pH 7.4). Nonspecific binding was determined in the presence of an excess amount of nonlabeled NPW-23 (final concentration 1 μM). After 1 h incubation at room temperature, the binding reactions were terminated by filtration through Whatman GF/C filters and followed by four washes with 0.5 ml ice-cold buffer B. Bound radioactivity was measured by scintillation counting in a TopCount, and binding data were analyzed with GraphPad Prism ver 3.03 (GraphPad Software, San Diego, CA). The binding affinity of each synthetic peptide was estimated as the K_i value. The K_i values of each peptide were calculated on the basis of the following formula from the IC_{50} values of each peptide and the K_d index of [^{125}I]-NPW-23, which is determined from the ligand saturation binding assay;

$$K_i = IC_{50}[1 + (\text{radioligand})K_d^{-1}]^{-1}$$

Cellular Functional (β-lactamase reporter-gene) Assays

The β-lactamase reporter-gene assays were performed in 96-well plates as described previously [12] with slight modification. In brief, HEK293 cells transfected as described above were plated at 40000 cells/well in 50 μl Opti-MEM assay buffer containing 0.1% BSA in a 96-well plate on the day of the β-lactamase assay. Cells were stimulated with 2 μl of 10× final concentration of ligand and 50 μl of 2 μM forskolin buffer in a 96-well plate at 37 °C for 3 h. The 6× CCF2/AM substrate loading buffer was prepared as described previously [12] by mixing 12 μl of 1 mM CCF2/AM, 120 μl of 100 mg/ml pluronic F127 and 2 ml of 24% PEG400 with Enhanced Substrate Solution (ESS; Aurora Biosciences). After 3 h of ligand stimulation, 20 μl of the 6× CCF/AM loading buffer was added to the cells and the cells were incubated at room temperature for 1 h. The microplate was then read in a fluorescent plate reader with 405 nm excitation and 460 and 530 nm emissions via the bottom. The data are indicated as ratio of the emissions at 460 and 530 nm after subtraction of the background values. EC_{50} vales were calculated by GraphPad Prizm 3.03 using a nonlinear regression analysis where the maximum response was defined as 100% of the control.

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RESULTS AND DISCUSSION

Construction of Binding Assay Using (^{125}I)-NPW-23

Saturation and competition binding assays of [^{125}I]-NPW-23 were performed using membrane preparation from HEK cells expressing GPR7 and GPR8. Scatchard plot analysis showed that the membrane fractions of HEK293 cells expressing human GPR7 or GPR8 had a single class of high-affinity binding sites for [^{125}I]-NPW-23 at the dissociation constant (K_d) of 0.29 nM and 0.40 nM with maximal binding sites (B_{max}) of 2.60 and 2.66 pmol/mg protein, respectively (Figure 2). The K_d value for GPR7 (K_d = 0.29 nM) is in good agreement with that determined for the rat amygdala region by Singh *et al.* (K_d = 0.44 nM) [13].

Selection of a Benchmark form of NPB for the SAR Study

Because unbrominated NPB-29 [Br(−)NPB-29] had shown essentially the same potency and efficacy as the brominated form for both GPR7 and GPR8 in the melanophore cellular functional assays [5], we used the unbrominated NPB analogs in this study. Brezillon *et al.* reported that NPB-23, in which the six amino acid residues at the *C*-terminal are deleted from NPB-29, was still active on both GPR7 and GPR8 [4]. We assessed the role of the six amino acid residues at the *C*-terminal of NPB-29 using unbrominated, *C*-terminal amidated peptides, Br(−)NPB-29-NH_2 (analog **1**) and Br(−)NPB-23-NH_2 (analog **2**). As shown in Table 1, the *C*-terminal truncated Br(−)NPB-23-NH_2 (analog **2**) retained potent binding affinity for GPR7 (0.24 nM) and GPR8 (55 nM), and further exhibited comparable receptor selectivity (229-fold preference for GPR7) with Br(−)NPB-29-NH_2 (**1**) (207-fold preference for GPR7). These results confirmed that the six amino acids residues at the *C*-terminal of NPB-29 are not important for interaction with GPR7 and GPR8 [4] and that Br(−)NPB-23-NH_2 contains the essential domain to bind to GPR7 as well as GPR8. We tested the effect of the *C*-terminal amidation in a preliminary SAR study with NPB because *C*-terminal amidation often modulates ligand binding affinity and makes a peptide more resistant to proteolytic degradation, which gives a useful tool for an *in vivo* study. Consequently, we found that the *C*-terminal amidated form of Br(−)NPB-23 (i.e. analog **2**) exhibited increased binding affinity for both GPR7 and GPR8 by 5–6 fold as compared with the *C*-terminal-free form of Br(−)NPB-23 (analog **3**) (K_i; 0.24 nM *vs* 1.2 nM for GPR7, 55 nM *vs* 341 nM for GPR8). Therefore, we used Br(−)NPB-23-NH_2 as a benchmark peptide for SAR studies of NPB.

Ligand Binding Assay

N-terminal truncation. Since Tanaka *et al.* previously reported that Trp^1 of NPB-29 is essential for its functional activity to GPR7 [5], we examined the effect of *N*-terminal truncation of analog **2** on receptor binding affinity. Truncation of Trp^1 of Br(−)NPB-23-NH_2 (analog **4**) drastically reduced affinities for both GPR7 and GPR8, confirming that Trp^1 plays a critical role for receptor binding.

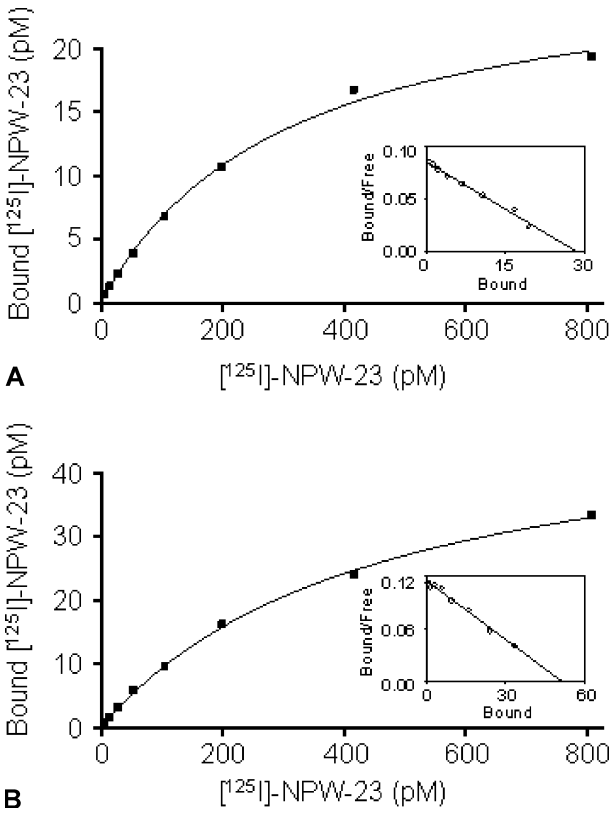

Figure 2 Saturation binding and Scatchard analysis of [^{125}I]-NPW-23. Saturation binding of [^{125}I]-NPW-23 using membrane fractions from HEK293 cells expressing GPR7(A) and GPR8(B). Insets: Scatchard analysis. Specific binding (■) was derived by subtracting the nonspecific binding from the total binding. Nonspecific binding was defined in the presence of 1 μM nonlabeled NPW-23. Data points are means of duplicate measurements in the representative experiment. The K_d/B_{max} values (mean ± SD) were 0.29 ± 0.06 nM/2.60 ± 0.36 pmol/mg protein and 0.40 ± 0.05 nM/2.66 ± 0.15 pmol/mg protein for GPR7 and GPR8, respectively (mean ± SD of duplicate determinations of the three separate saturation binding experiments).

Single amino acid positional scanning. Although both Br(−)NPB-23 and NPW-23 exhibited high GPR7 binding affinities, these ligands have only 61% amino acid identity (Figure 2) [2]. Alanine scanning studies of the nonconserved amino acids between NPB-23 and NPW-23 (positions 4, 7, 8, 9, 10, 11, 12 and 21) were performed to evaluate the respective roles of each amino acid for the interaction between Br(−)NPB-23-NH_2 and GPR7 and GPR8. In addition, the alanine residue at position 5 in Br(−)NPB-23-NH_2 was replaced with glycine. We evaluated the binding affinities of alanine-

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Table 1 Binding affinities of Br(−)NPB-29-NH_2 and Br(−)NPB-23-NH_2 analogs. The K_i values for GPR7 and GPR8 were determined by the competition binding assay using [^{125}I]-NPW-23. Selectivity for GPR7 over GPR8 is indicated as the ratio of K_i values. Each value represents the mean ± SD of three independent experiments

Analog #	Sequence	Ki ± SD (nM)		Selectivity
		GPR7	GPR8	
1	WYKPAAGHSSYSVGRAAGLLSGLRRSPYA-NH_2	0.14 ± 0.03	29 ± 4.8	207
2	WYKPAAGHSSYSVGRAAGLLSGL-NH_2	0.24 ± 0.05	55 ± 15	229
3	WYKPAAGHSSYSVGRAAGLLSGL	1.2 ± 0.38	341 ± 68	284
4	YKPAAGHSSYSVGRAAGLLSGL-NH_2	982 ± 99	N.D.	N.D.
14	WYKXAGHSSYSVGRAAGLLSGL-NH_2	0.21 ± 0.04	53 ± 12	252
15 Ava-5	WYKXXXXXGRAAGLLSGL-NH_2	1.3 ± 0.29	1011 ± 201	778
16 Ava-4	WYKXXXXGRAAGLLSGL-NH_2	0.73 ± 0.05	648 ± 8.0	888
17 Ava-3	WYKXXXGRAAGLLSGL-NH_2	0.40 ± 0.03	490 ± 52	1225
18 Ava-2	WYKXXGRAAGLLSGL-NH_2	0.84 ± 0.06	611 ± 46	727
19 Ava-1	WYKXGRAAGLLSGL-NH_2	261 ± 22	5619 ± 4018	22

X = 5-aminovaleric acid.
N.D. = not determined because of inactivity.

Table 2 Binding affinities of alanine- and glycine-substituted analogs of Br(−)NPB-23-NH_2. The K_i values of Br(−)NPB-23-NH_2 and its single-residue-substituted analogs for GPR7 and GPR8 are summarized. Each value represents the mean ± SD of three independent experiments

Analog #	Sequence	Ki ± SD (nM)	
		GPR7	GPR8
2	WYKPAAGHSS YSVGRAAGLL SGL-NH_2	0.24 ± 0.05	55 ± 15
5	WYK**A**AAGHSS YSVGRAAGLL SGL-NH_2	0.21 ± 0.04	34 ± 5.6
7	WYKPAA**A**HSS YSVGRAAGLL SGL-NH_2	0.42 ± 0.26	109 ± 5.8
8	WYKPAAG**A**SS YSVGRAAGLL SGL-NH_2	0.22 ± 0.06	23 ± 2.9
9	WYKPAAGH**A**S YSVGRAAGLL SGL-NH_2	0.24 ± 0.04	66 ± 7.3
10	WYKPAAGHS**A** YSVGRAAGLL SGL-NH_2	0.25 ± 0.05	55 ± 8.9
11	WYKPAAGHSS **A**SVGRAAGLL SGL-NH_2	1.1 ± 0.27	239 ± 26
12	WYKPAAGHSS Y**A**VGRAAGLL SGL-NH_2	0.17 ± 0.03	12 ± 0.93
13	WYKPAAGHSS YSVGRAAGLL **A**GL-NH_2	0.21 ± 0.05	53 ± 3.2
6	WYKP**G**AGHSSYSVGRAAGLLSGL-NH_2	0.30 ± 0.11	64 ± 6.3

or glycine-substituted analogs for GPR7 and GPR8 in comparison with analog **2**, the parent peptide (Table 2). Among the alanine-substituted analogs, analog **11**, in which Tyr^{11} was replaced with Ala, displayed decreased competition for binding by approximately five fold (1.1 nM for GPR7 and 239 nM for GPR8) as compared with analog **2**. In contrast to the decreased binding affinity of the alanine substitution, iodination of Tyr^{11} was reported as having little effect on the binding affinity of NPB [2]. Taken together, these observations suggest the possibility that the bulky side-chain moiety of Tyr^{11} of NPB is important for its binding affinity.

Unexpectedly, all of the other alanine-substituted analogs exhibited little or no decrease in binding for GPR7 (0.17–0.42 nM) as compared with analog **2**. Regarding GPR8, analog **11** again displayed four-fold decreased affinity (239 nM) while analog **7** showed two-fold decreased activity (109 nM) for GPR8. Analogs **5**, **8** and **12** displayed two- to four- fold increased affinities (34, 23 and 12 nM, respectively) whereas analog **6**, **9**, **10 and 13** showed comparable activity (64, 66, 55 and 53 nM, respectively) to analog **2** for GPR8.

Taken together, these observations suggest that Tyr^{11} of NPB is an essential amino acid for the interaction with both GPR7 and GPR8 and Gly^{7} of NPB plays a relatively important role for the interaction with GPR7 and GPR8.

5-Aminovaleric acid linker analogs. It is noteworthy that all the analogs except analog **11** retained GPR7 binding affinities that are comparable to or moderately decreased or increased compared with analog **2**, the parent peptide. These data suggest that the side chains

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of the amino acids in position 4–12, except Tyr^{11}, contribute little to the interaction between NPB and GPR7. These findings prompted us to design a series of Ava-linker analogs in which the Pro^4-Val^{13} segment was replaced with Ava. Ava linkers are suggested to mimic in length the main chain of a dipeptide unit [14]. Intriguingly, Bednarek *et al.* reported that the Ava linker affects the biological activity of peptides by enhancing their conformational freedom at the receptors [14].

In order to determine the effects of amino acid residues 4–13 on the binding profiles of NPB, we synthesized a series of Br(–)NPB-23-NH_2 analogs that have one to five Ava linkers. First, the substitution of Pro^4 and Ala^5 with a single Ava linker gave analog **14**, which possessed affinities comparable to the parent [Br(–)NPB-23-NH_2], with K_i values of 0.21 and 53 nM for GPR7 and GPR8, respectively (Table 1). This result indicates that Pro^4 and Ala^5 are not important for the interaction of NPB-23 with GPR7, supporting the result of the alanine-substitution study.

Incorporation of five Ava linkers in positions 4–13 gave analog **15** (Ava-5), which is expected to have greater conformational freedom than the parent while maintaining the same length. Analog **15** exhibited 5-fold lower GPR7 affinity and 18-fold lower GPR8 affinity, resulting in the increased selectivity for GPR7 over GPR8 (778-fold) than the parent (Table 1). Interestingly, Analog **15** retains none of the amino acids that differs between NPB-23 and NPW-23, except Ser^{21} (Figure 2). The results with analog **15** therefore suggest that the side chains of the nonconserved amino acids in positions 4–13 have minimal effects on the interaction with GPR7, while they play relatively important roles for the interaction with GPR8. In addition, these results also might suggest that the topological requirements of NPB for the formation of stable complexes with GPR7 are not more stringent than those with GPR8.

To examine the effects of the linker length on the Br(–)NPB-23-NH_2 binding profiles, we synthesized additional analogs by replacing amino acids 4–13 of Br(–)NPB-23-NH_2 with one to four Ava linkers. Interestingly, analog **16** (Ava-4) and analog **17** (Ava-3) retained GPR7 binding affinity comparable to the parent. These results indicate that the three *N*-terminal amino acid residues (WYK) and the ten *C*-terminal amino acid residues (GRAAGLLSGL) are sufficient for high affinity for GPR7, but the length between these domains is not essential for the interaction with GPR7.

Interestingly, analog **18** (Ava-2) exhibited a 4-fold decrease in GPR7 affinity and an 11-fold decrease in GPR8 affinity compared with the parent, whereas analog **19** (Ava-1) exhibited a 1088-fold decreased affinity for GPR7, suggesting that Ava linkers with less than three units are too short to maintain the conformational freedom of NPB analogs required for effective interaction with GPR7 and GPR8. Among the Ava-linked analogs tested so far, analog **17** (Ava-3) exhibited the highest affinity to GPR7 (K_i; 0.40 nM *vs* 0.24 nM for analog **2**) while showing a nine-fold decrease in affinity to GPR8 (K_i; 490 nM), giving the highest selectivity for GPR7 over GPR8 (1225-fold). These results also suggest that the conformational restriction and/or distance between WYK and GRAAGLLSGL differently affect affinities of NPB for GPR7 and GPR8. To our knowledge, this is the first report showing that modification of distance or flexibility between WYK and the *C*-terminal GRAAGLLSGL of NPB affects the affinity and selectivity for GPR7 and GPR8. Recently Lucyk *et al.* demonstrated that secondary structures of the *N*-termini of NPB and NPW significantly differ in that NPB forms a type II β-turn from residues Lys^3 to Ala^6, where Pro^4 highly favors the development of the turn owing to the constraint that Pro imposes on polypeptide backbone conformations [15]. Although analog **5** retains activity comparable to analog **2** for both GPR7 and GPR8, our results suggest the possibility that the destruction of the whole β-turn structure by replacement of these consecutive amino acid residues with Ava linkage (e.g. Ava-5) affects the interaction with GPR8 but not GPR7. These observations also support the idea that the secondary structure of the *N*-terminal portion of NPB is a critical factor for receptor selectivity.

Cellular Functional Assay

The functional profiles of the Ava-linked analogs (Ava-2, 3, 4 and 5) were examined by a cellular functional assay using HEK293 cells stably expressing GPR7 and GPR8 (Figure 3). All the Ava-linked analogs displayed full agonistic activities for GPR7 and GPR8. Regarding GPR7, all four analogs had activities comparable to Br(–)NPB-23-NH_2 (analog **2**), and the agonistic efficacies were roughly parallel to the binding affinity (Table 1). Regarding GPR8, Ava-linked analogs showed decreased agonistic activities compared with analog **2**, supporting the idea that the amino acid residues in positions 4–13 are important for the optimal functional conformation to bind and activate GPR8. Among them, Ava-5 (**15**) and Ava-3 (**17**) displayed drastically decreased activity (EC_{50}; 280 nM and 77 nM *vs* 8.6 nM for analog **2**) (Table 3). Consequently, Ava-5 (**15**) and Ava-3 (**17**) exhibited around five-fold higher selectivity for GPR7 over GPR8 compared with the parent peptide Br(–)NPB-23-NH_2.

CONCLUSIONS

Through systematic SAR studies, we found that the Trp^1 and Tyr^{11} residues of NPB play critical roles for the ligand–receptor interactions, whereas the side chains of the other amino acid residues, Pro^4, Ala^5, Gly^7, His^8, Ser^9, Ser^{10}, Ser^{12} and Ser^{21} are not

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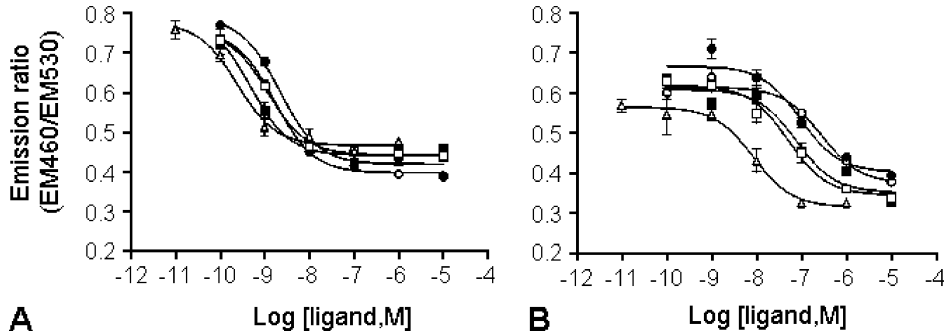

Figure 3 Comparison of the functional activities of Br(−)NPB-23-NH_2 and Ava-substituted analogs. The agonistic activities of Br(−)NPB-23-NH_2 and its Ava-linker-substituted analogs for GPR7 (**A**) and GPR8 (**B**) were examined by a cAMP-driven reporter-gene assay in the presence of 1 μM forskolin. (Δ) Br(−)NPB-23-NH_2, (□) Ava-2, (■) Ava-3, (●) Ava-4, (○) Ava-5. Sequences and EC_{50} values of the Ava-linker-substituted analogs are summarized in Table 3.

Table 3 Agonistic potency of Br(−)NPB-23-NH2 and its Ava-linkage analogs EC_{50} values of Br(−)NPB-23-NH_2 and its Ava-linker-substituted analogs for GPR7 and GPR8 (Figure 3)

Analog #	Sequence	EC_{50} (nm)		Selectivity
		GPR7	GPR8	
2	WYKPAAGHSSYSVGRAAGLLSGL-NH_2	0.24	8.6	36
15 Ava-5	WYKXXXXXGRAAGLLSGL-NH_2	1.7	280	165
16 Ava-4	WYKXXXXGRAAGLLSGL-NH_2	2.2	94	43
17 Ava-3	WYKXXXGRAAGLLSGL-NH_2	0.48	77	160
18 Ava-2	WYKXXGRAAGLLSGL-NH_2	1.2	51	43

X = 5-aminovaleric acid.

essential determining factors. Consistent with these results, examination of Ava-linker analogs revealed that *N*-terminal WYK and *C*-terminal GRAAGLLSGL sequences are essential for full binding and agonistic activity in ligand binding and functional assays. Finally, we identified analog **15** (Ava-5) and analog **17** (Ava-3) as novel synthetic NPB analogs with retained potency and enhanced selectivity for GPR7. Analogs **15** and **17** might be useful tools to further address the physiological roles of GPR7, especially in higher species where both GPR7 and GPR8 are expressed.

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