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Identification and optimization of novel partial agonists of Neuromedin B receptor using parallel synthesis

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Abstract—The design and parallel synthesis of potent, small molecule partial agonists of Neuromedin B receptor based on the 3-amino-2,3,4,9-tetrahydro-1*H*-carbazole-3-carboxylic acid amide core is described.
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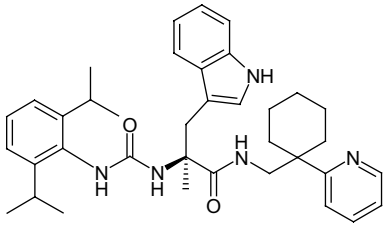
The bombesin family of G-protein coupled receptors consists of four subtypes:[1] Neuromedin B receptor (NMBR, BB_1),[2] Gastrin-Releasing Peptide Receptor (GRPR, BB_2),[3] BRS-3,[4] and BB_4.[5] Of these, the former two receptors have received the greatest level of attention in the scientific literature over recent years. NMB and GRP, the endogenous ligands for NMBR and GRPR, respectively, were discovered more than 20 years ago.[6,7] They display a seven amino acid C-terminal sequence homology, and mediate a range of biological mechanisms via action at their receptors. These include CNS-related responses such as thermoregulation,[8] satiety,[9] control of circadian rhythm,[10] and peripheral functions including macrophage activation,[11] and gastrointestinal hormone release.[12]

Examples of peptoid-based Neuromedin B receptor antagonists were disclosed several years ago;[13] however, there have been no other small molecule antagonists or agonists of NMBR reported in the literature. As part of our investigations into the discovery of novel heterocyclic ligands of NMBR, we chose to examine the published peptoid PD165929, **1** (Fig. 1), as a starting point for a targeted library.[13] Compound **1** was prepared according to literature precedent,[14] and was used in our biological assays as a reference compound. Interestingly, in our hands, **1** showed a lower binding affinity for



1

IC_{50} (^{125}I-NMB) = 0.35μM

Figure 1.

NMBR than had been reported previously in the literature.[15]

In an effort to identify a novel chemotype based on the structural features of **1**, we decided to prepare a conformationally-constrained derivative in the form of a 3-amino-2,3,4,9-tetrahydro-1*H*-carbazole-3-carboxylic acid amide, **2**. The synthesis of this compound was accomplished in four steps—incorporating a Fischer cyclization at the penultimate stage—starting from 8-amino-1,4-dioxa-spiro[4.5]decane-8-carboxylic acid, and proceeded in good overall yield (Scheme 1).[16]

We observed that **2** displayed a marginal increase in binding affinity for NMBR over **1**, but interestingly was also a highly selective, weak partial agonist, displaying no activity against a panel of GPCR targets encompassing members of the lipid and neuropeptide receptor families. Further, **2** showed no cytotoxicity when incubated with HeLa cells for 72 h (Fig. 2).[17]

Keywords: Neuromedin B receptor; Partial agonist; Lead optimization; Parallel synthesis.
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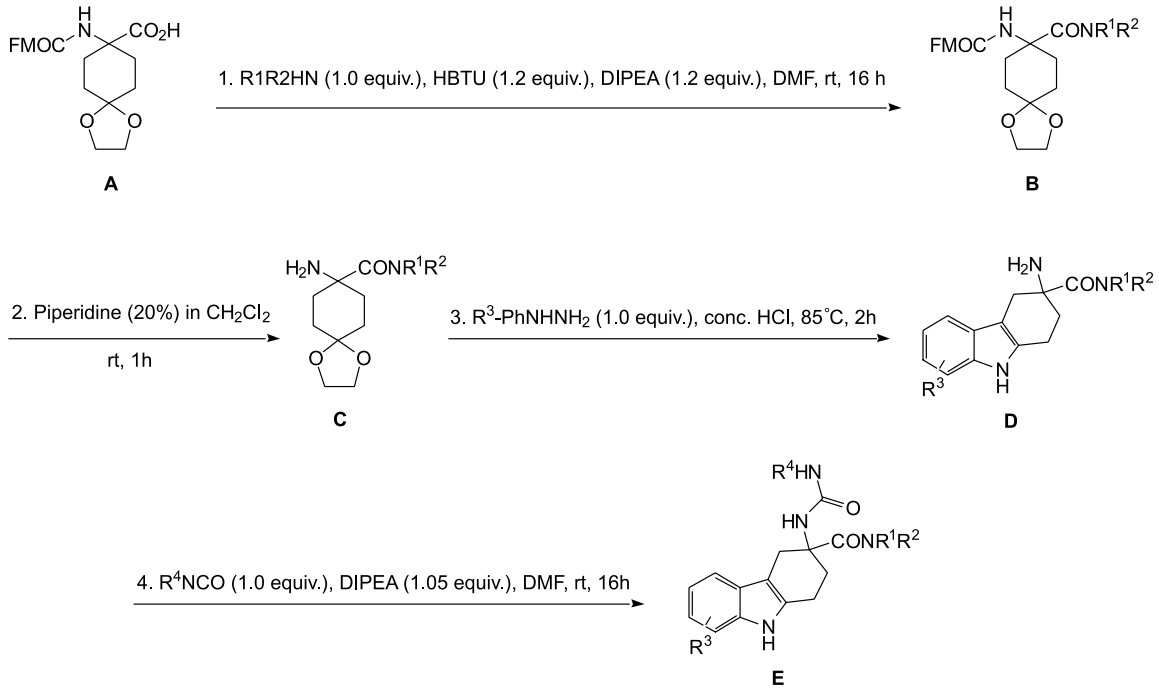
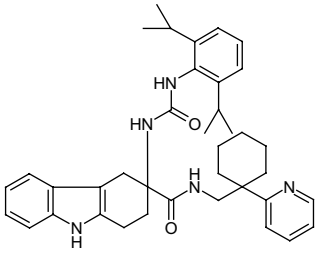


Scheme 1.



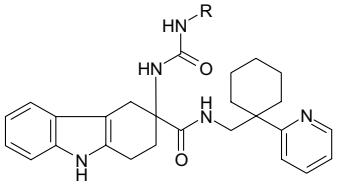
2

IC_{50} (^{125}I-NMB) = 0.324μM

Figure 2.

Encouraged by the preliminary data for **2**, we constructed a targeted library based upon this tetrahydrocarbazole core. The synthetic protocol used was identical to that illustrated in Scheme 1, and all of the analogues were prepared using our parallel synthesis modules and were purified by automated HPLC.[18] The library was assembled in four stages. Initially we conducted a thorough examination of the exocyclic nitrogen substitution pattern, and quickly concluded that a substituted phenylurea was essential for activity, with the 2,6-diisopropylphenyl urea being the optimal motif. In addition, we observed that the 4-nitrophenylurea derivative, **4**, also displayed sub-micromolar potency against the receptor; this nitrophenylurea motif was present in several of the most potent peptoid ligands based on compound **1** previously reported by Ashwood et al.[19] that were subsequently examined as part of a mutagenesis study to elucidate the role of the nitro substituent in hydrogen bonding to the receptor.[20] A representative set of data summarizing our results from this exercise is shown in Table 1.

Table 1.



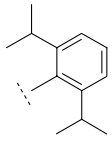
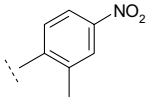
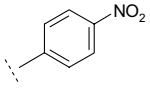
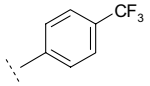
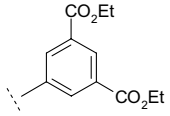
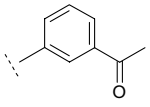
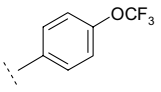
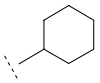
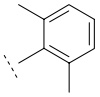
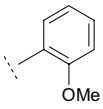
Compound #	R	IC_{50} (^{125}I-NMB), μM
2		0.32
3		0.62
4		0.80
5		1.11
6		1.85
7		2.27

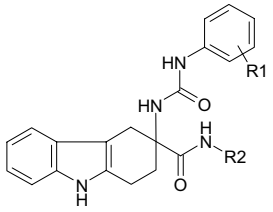
Table 1 (*continued*)

Compound #	R	IC_{50} (^{125}I-NMB), μM
8		3.04
9		3.92
10		5.49
11		8.72
12	Et	>10
13	H	>10

In the second phase of library construction, we focused on modifications to the carboxy terminus in order to determine the requirement for the *N*-(1-pyridin-2-yl-cyclohexylmethyl)-acetamide, a residue present in the most potent analogues in both the peptoid and tetrahydrocarbazole series. In this study, we retained the urea substituents as both 2,6-diisopropylphenyl and 4-nitrophenyl, and a range of amide derivatives of **2** were prepared. These encompassed examples representing subtle modifications to the lead structure, together with more random, structurally diverse analogues, prepared in an attempt to incorporate additional recognition elements and to reduce molecular weight. The majority of the analogues showed significantly diminished affinity for NMBR however, illustrating the importance of the pyridin-2-yl-cyclohexylmethyl motif in binding to the receptor; indeed the only derivatives that displayed an appreciable level of activity were the direct *gem*-dimethyl and cyclopropyl analogues, **14** and **15**, which adopt similar conformations to the parental compound (Table 2).

The third stage centered on our examining a broad range of substitution patterns within the indole ring. Table 3 summarizes key data obtained for the 2,6-diisopropylphenylurea derivatives prepared in this exercise. A general trend of improved affinity with

Table 2.



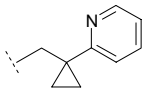
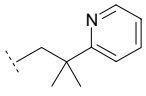
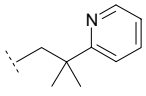
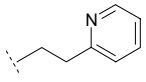
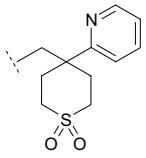
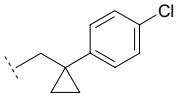
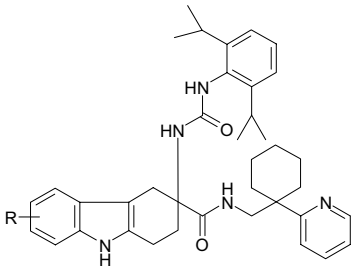
Compound #	R1	R2	IC_{50} (^{125}I-NMB), μM
14	2,6-Di-isopropyl		3.35
15	2,6-Di-isopropyl		4.15
16	4-NO_2		10
17	4-NO_2		>10
18	4-NO_2		>10
19	2,6-Di-isopropyl		>10

Table 3.



Compound #	R	IC_{50} (^{125}I-NMB), μM
20	5-OMe	0.25
21	5-Me	0.66
22	4,6-Di-Me	0.70
23	4,6-Di-Me	0.79
24	4,7-Di-F	1.01
25	5-OCF_3	1.89
26	7-Br	2.14
27	5-CF_3	3.66
28	5-NO_2	>10
29	5-CO_2H	>10

electron-rich indole systems was observed, with compound **20** being the most potent compound identified.

The final step of our study involved resolving the two enantiomers of **20**, which was accomplished by using preparative chiral HPLC.[21] We observed that the (+)-enantiomer, **30**, was the more potent of the two.[22] Further, this compound was very selective for NMBR over structurally-related GPCRs, and did not display any cytotoxic effects in HeLa cells (Scheme 2).

As mentioned above, compound **2** showed weak partial agonist activity in an inositol phosphate production SPA assay.[23] We subsequently observed that the level of partial agonism in this tetrahydrocarbazole series increased with improved binding affinity. Figure 3 illustrates the effects of the dose response curves of Neuromedin B upon treatment with increasing concentrations of **30**.

Figure 4 depicts the partial agonist effects of the key compounds prepared in this study, with levels of IP accumulation increasing with improved potency, as determined in the radioligand competition assay.

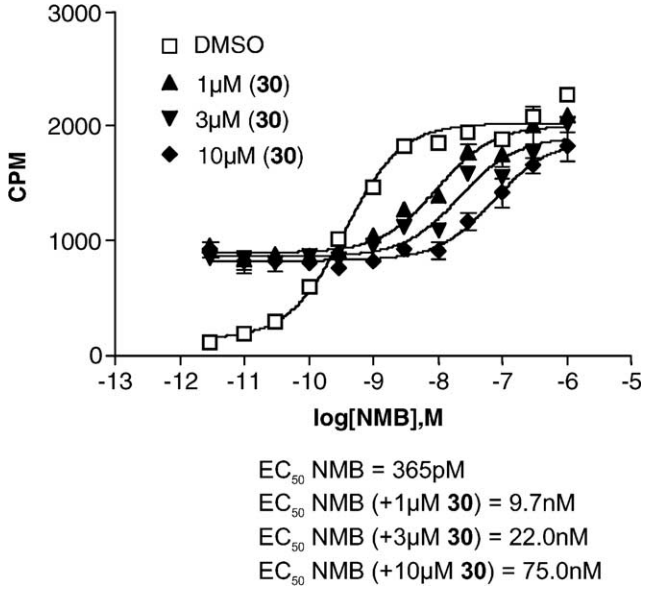


Figure 3.

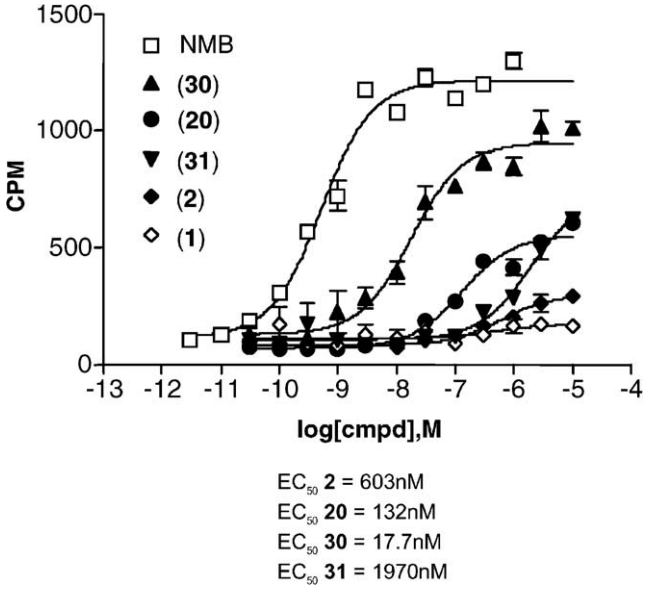
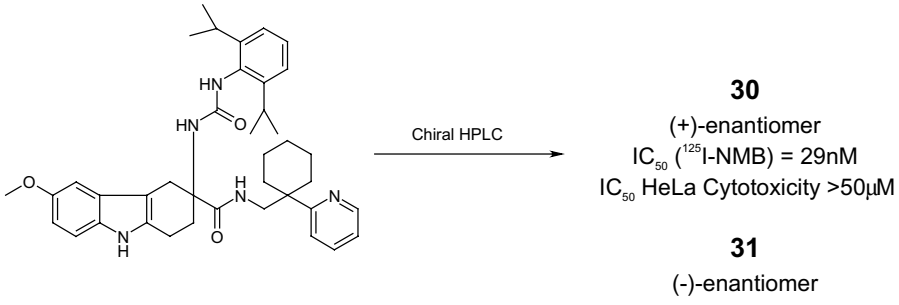


Figure 4.

In summary, a series of novel and potent NMBR partial agonists has been identified. The compounds were de-



Scheme 2.

signed on a conformationally constrained derivative of a known peptoid-based NMBR antagonist, and were produced in a matter of weeks using a rapid solution-based parallel synthesis approach.

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15. Protocol for whole cell radioligand binding assay: Human embryonic kidney 293 (HEK293) cells were stably transfected with expression plasmids encoding hNMBR. Competition binding assays were performed for 1 h at room temperature in the presence of 150 pM ^{125}I-[D-Tyr0]NMB (2200 Ci/mmol, Perkin–Elmer Life Sciences) and 1×10^6 transfected cells in Dulbeco's Modified Eagle Media (Mediatech, Inc.) in 100 μL in 96 well plates. Bound ligand was separated from unbound ligand by filtration using a Filtermate (Packard) and total counts bound determined on a TopCount NTX reader (Packard).
16. Typical synthetic procedure for **2** and analogues thereof: A solution of *N*-Fmoc-amino-4-(ethylene ketal)cyclohexylcarboxylic acid, **A** (1.0 wt) in *N,N*-dimethylformamide was treated with amine (1.0 equiv), HBTU (1.2 equiv) and Hünig's base (1.2 equiv), and the reaction mixture was agitated at room temperature for 16 h. The solvent was removed using a Genevac centrifugal evaporator, and the residue partitioned between brine and dichloromethane. The organic phase containing **B** was isolated, and treated with piperidine (20% of overall volume); the reaction mixture was agitated for 1 h at room temperature, and was then concentrated to dryness. The residue, **C**, was then treated with 1 M HCl (aq), and the solution washed with dichloromethane. The aqueous phase was isolated and phenylhydrazine (1.0 equiv) was added, together with a few drops of concentrated HCl, and the reaction mixture was heated to 85 °C for 2 h. The solution was cooled, neutralized with saturated sodium bicarbonate solution, and the product, **D**, was extracted with dichloromethane. The overall yields for the synthesis of **D** typically exceeded 60%. For the final step, stock solutions of the primary amine **D** were treated with equal mole quantities of isocyanate in *N,N*-dimethylformamide, in the presence of Hünig's base. After 16 h stirring at room temperature, conversions in excess of 90% to the desired ureas **E** were determined by HPLC at 220 and 254 nm using an Agilent 1100 LC/MSD VL ESI system. The products were then purified directly using automated preparative HPLC (see Ref. 19). Note that for several examples, step 3 involved the use of 1,4,-dioxane as the solvent to circumvent problems with poor aqueous solubility of a number of phenylhydrazines used in the library synthesis.
17. Typical protocol for cytotoxicity assay: HeLa cells were seeded at 5k/well in a 96 well plate (6 plates-for triplicate 0 and 72 h readings). A three-fold dilution series of each compound was generated, starting at 10 mM. The solution was diluted 8 times to prepare 9 concentrations ranging from 10 mM to 150 nM. Compound was added to cells (1–100 μL total volume), and the final concentration of compound was 100 μM to 15 nM. Zero and 72 h time points were recorded as follows: (a) 10 μL Alamar Blue reagent was added to the wells, and the samples were incubated at 37 °C for 3 h; (b) fluorescence intensity was then recorded on an LJL Analyst. The relative growth was compared to a DMSO control well for each compound.
18. Synthesis was accomplished using 24-position 'Greenhouse' and 96-position METZ Heater-Shaker modules purchased from Radleys Ltd. (see www.radleys.com). Purification was conducted using a Parallex Flex™ HPLC System purchased from Biotage Inc. (see www.Biotage.com).
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21. Resolution of **20** was accomplished using a Waters 600 Tower system equipped with a Chiralpak AD column (Daicel Chemical Industries # AD00CJ-DG008, 25 cm × 2 cm). Protocol: a solution of **20** (8.0 mg in 500 mL isopropyl alcohol) was injected and an isocratic gradient of 8% isopropyl alcohol in hexanes at 20 mL/min was maintained. The fractions corresponding to the two enantiomers (**30** = 35 min; **31** = 27 min) were isolated, and were concentrated in vacuo to afford **30** (1.96 mg) and **31** (2.23 mg). LCMS analysis[17] confirmed the desired molecular weights (M + 1 = 636.5) of both products, and purities were determined by analytical HPLC using a Hewlett Packard series 1050 Tower system with a Daicel Chemical Industries Chiralpak AD-H column (# AD-H0CE-DC041, 25 cm × 0.4 cm), employing an isocratic gradient of 8% IPA in hexanes at 1 mL/min. Both **30** and **31** were determined to have enantiomeric excesses

greater than 99%. Optical rotations were measured using a Jasco P-1020 polarimeter (**30**: $[\alpha]_D^{27}$ +57.8 (*c* 0.2, CH_3OH). **31**: $[\alpha]_D^{27}$ −42.8 (*c* 0.2, CH_3OH)).

22. Analytical data for **30**: δ_H (400 MHz, DMSO-d_6) 10.90 (1H, s), 8.62 (1H, s), 7.80 (2H, m), 7.55–7.40 (4H, m), 7.20–6.95 (6H, m), 6.20 (1H, s), 3.73 (3H, s), 3.45–3.2 (5H, m), 2.90–2.70 (5H, m), 2.20–2.10 (2H, m), 2.00 (1H, m), 1.65–1.20 (7H, m), 1.20–1.00 (12H, m). M + 1 found 636.1; $C_{39}H_{49}N_5O_3$ requires 635.38.
23. Protocol for the inositol phosphate (IP) accumulation assay: hNMBR-transfected HEK293 cells (2.5×10^4 were incubated in 96 well plates in DMEM Eagle High Glucose, w/o L-Glutamine, I-Inositol (US Biological) supplemented with 2 g/L sodium bicarbonate, 25 mM Hepes, 2% glutamine, 10% dialyzed FBS (Gibco), and 1 μCi/mL myo-[^{3}H]Inositol (82.0 Ci/mmol, Amersham Pharmacia Biotech) overnight at 37 °C. Test compounds were then added in inositol-free DMEM containing 0.3% BSA (Sigma) and 10 mM LiCl (Sigma) for 60 min at 37 °C. The cells were lysed with 20 mM formic acid for 2 h at 4 °C and added to RNA-binding Ysi scintillation proximity assay beads (Amersham Pharmacia Biotech) for 30 min. Plates were stored overnight at room temperature in the dark and read the next day on a TopCount NTX.