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Discovery and optimization of a novel Neuromedin B receptor antagonist

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ABSTRACT

The discovery and parallel synthesis of potent, small molecule antagonists of Neuromedin B receptor based on the ary-hexahydro-dibenzodiazepin-1-one core is described.

In mammals, the bombesin family of G protein-coupled receptors consists of three subtypes:[1] Neuromedin B receptor (NMBR, BB_1),[2] Gastrin-releasing peptide receptor (GRPR, BB_2),[3] and BRS-3.[4] While high affinity endogenous ligands for BRS-3 are unknown, Neuromedin B (NMB) and Gastrin-releasing peptide (GRP) were discovered in the early 1980s.[5,6] NMB and GRP share sequence homology in the C-terminal region and mediate a range of biological mechanisms via action at their receptors. These include CNS-related responses such as thermoregulation,[7] satiety,[8] control of circadian rhythm,[9] and modulation of fear and anxiety responses,[10,11] as well as peripheral functions including macrophage activation[12] and gastrointestinal hormone release.[13] In addition, there is considerable literature suggesting a role in control of cellular proliferation.[14]

Previously, we and others described the discovery of small molecule ligands for NMBR.[15] In an effort to develop tool compounds to define how these receptors influence pathological processes, we report here the identification and optimization of potent and selective NMBR antagonists. The initial lead in this series, benzodiazepine **1** (Fig. 1), was identified as an NMBR antagonist from a high-throughput screen (HTS). Compound **1** displayed encouraging potency in a NMB displacement assay (IC_{50} = 300 nM)[16] and was selective (10 μM or greater) against a panel of other GPCR targets, including GRPR and other neuropeptide receptors. The benzodiazepine scaffold is highly amenable to diversification using parallel synthesis, and we were able to rapidly prepare and evaluate over 200 compounds as part of a series of small libraries to facilitate the discovery of a novel, potent spirocyclic derivative. Data on the most representative compounds are presented herein.

The synthesis of benzodiazepines is well documented.[17] **A** series of libraries based on **1** was prepared as described in Scheme 1 using the modified method reported by Blache et al.[17b] The synthesis began with a condensation between a 2-nitroaniline (**A**) and a 1,3-cycloalkane dione. The resulting enamine (**B**) was either methylated or left unchanged before reduction to the diamine (**C**).[18] Formation of the seven-member ring was accomplished via a Mannich-type cyclization between the diamine and an aryl aldehyde, this cyclization was carried out at room temperature in ethanol containing a catalytic amount of acetic acid.[19] Alkylation of the resulting secondary amine (D) was easily effected by treatment with an aldehyde and $Na(AcO)_3BH$.[20] All of the final products were purified by preparative HPLC.[21]

An analysis of the preliminary SAR derived from the HTS data suggested that the methyl substituent on the fused aryl of **1** is re-

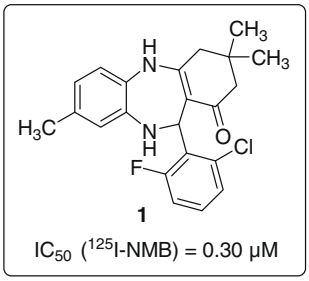

Figure 1.

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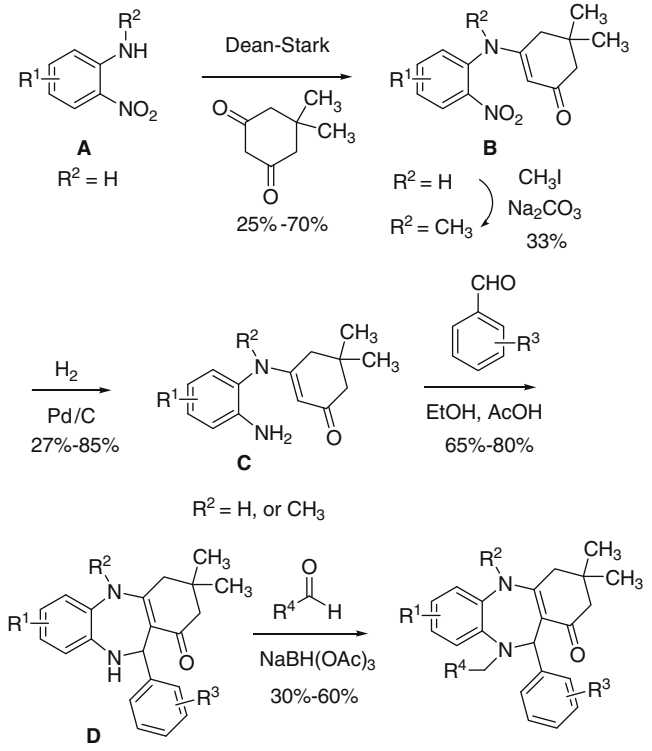
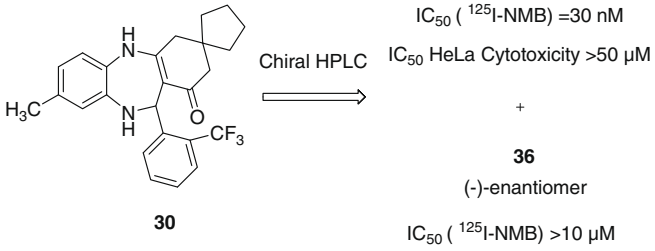


Scheme 1.

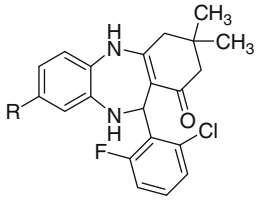


Scheme 2.

quired for binding affinity. Therefore, in the first library alternatives to the methyl group were examined. As the data in Table 1 show, efforts to replace the methyl group with polar functions yielded ligands with diminished binding affinity. However electron-deficient functions were tolerated, as exemplified by the CF_3 and Cl analogs **2** and **3**. For subsequent libraries, we elected to maintain CH_3 substitution as R.

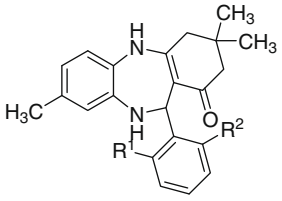
A second library focused on variants of the exocyclic phenyl motif. The data in Table 2 summarize the results of this study. Compounds **12**, **15** and **17** nicely illustrate the impact on binding affinity of the exocyclic aryl substituents of **1**. Compound **12**, for instance, shows that removal of the fluorine atom has a minimal effect on binding. However when the chlorine atom is removed, as in compound **15**, binding affinity is diminished fourfold. The reduced binding affinity of the difluoro compound **16** shows that the electron withdrawing nature of the substituents is not a key factor influencing binding. This point is further supported by compound **10**, which bears a space-filling substituent on a relatively electron rich aryl.

Table 1



ID #	R	IC $_{50}$ (^{125}I-NMB) (μM)
1	CH_3	0.30
2	CF_3	0.37
3	Cl	0.86
4	CF_3O	1.3
5	CN	1.3
6	CH_3O	2.2
7	COOH	10
8	OH	10
9	$CONH_2$	10

Table 2

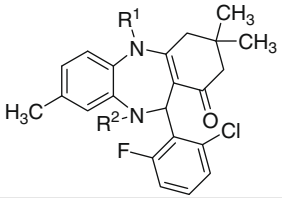


ID #	R^1	R^2	IC_{50} (^{125}I-NMB) (μM)
1	F	Cl	0.30
10	H	CH_3S	0.20
11	H	CF_3	0.25
12	H	Cl	0.28
13	H	NO_2	0.55
14	H	CH_3O	1.1
15	H	F	1.3
16	F	F	2.5
17	H	H	6.9

The next series of compounds was prepared to examine the effects of alkylating the benzodiazepine nitrogen atoms. The data, summarized in Table 3, show that methylation at R^1 resulted in a dramatic loss of affinity (**18**), while alkylation at R^2 conferred a marginal increase in potency (**19**, **20** and **21**).

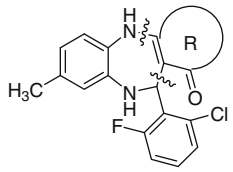
We next turned our attention to the alicyclic portion of the molecule (Table 4). Significant loss of affinity was observed for the unsubstituted (**25**) and ring contracted (**29**) analogs, indicating that the cyclohexyl ring and the geminal dimethyl are important contributors to binding. Moreover, no improvements were ob-

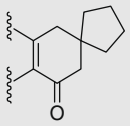
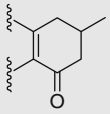
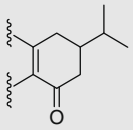
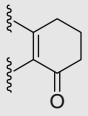
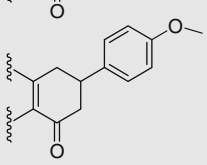
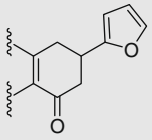
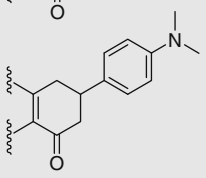
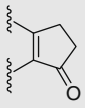
Table 3



ID #	R^1	R^2	IC_{50} (^{125}I-NMB) (μM)
1	H	H	0.30
18	CH_3	H	10
19	H	CH_3	0.17
20	H	C_2H_5	0.18
21	H	*i*-C_4H_9	0.15

Table 4



ID #	R	IC_{50} (^{125}I-NMB) (μM)
22		0.28
23		0.45
24		0.81
25		1.4
26		2.0
27		3.0
28		5.4
29		10

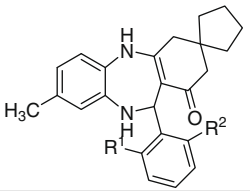
served for the mono-substituted analogs **23**, **24**, and **26**–**28**. Interestingly, spirocycle **22** showed slightly increased affinity, suggesting that a rigid structure may be favored in this region of the molecule.

Finally, we revisited exocyclic phenyl substitutions in the context of the spirocyclic derivative **22** (Table 5). Several potent analogues were identified, with compound **30** displaying a threefold increase in potency compared to the original lead compound **1**.

Compound **30** was resolved using preparative chiral HPLC to furnish two optically-pure enantiomers (Scheme 2) (**35** and **36**).[22,23] The (+)-enantiomer **35** was more potent than the racemic mixture, and 10-fold more potent than **1.** Compound **35** showed no cytotoxicity at concentrations up to 30 μM when incubated with HeLa or HEK293 cells for 72 h.[24] The (−)-enantiomer, **36**, was inactive.

Figure 2 illustrates that compound **35** behaved as an NMBR antagonist in a signaling assay measuring accumulation of inositol

Table 5



ID #	R_1	R_2	IC_{50} (^{125}I-NMB) (μM)
30	H	CF_3	0.096
31	H	Cl	0.24
32	H	CH_3S	0.25
33	F	CF_3	0.54
34	H	CF_3S	0.64

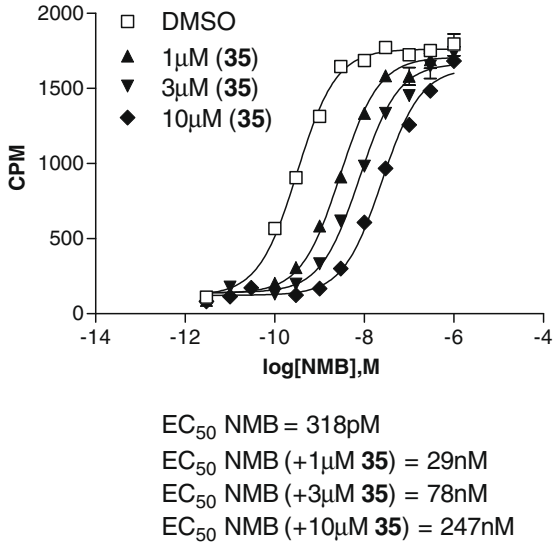


Figure 2.

phosphate[25], right-shifting a dose response curve of NMB. In addition, **35** reduced the activity of a sub-maximal dose of NMB, with an IC_{50} of 580 nM, a 10-fold improvement over **1** (Fig. 3).

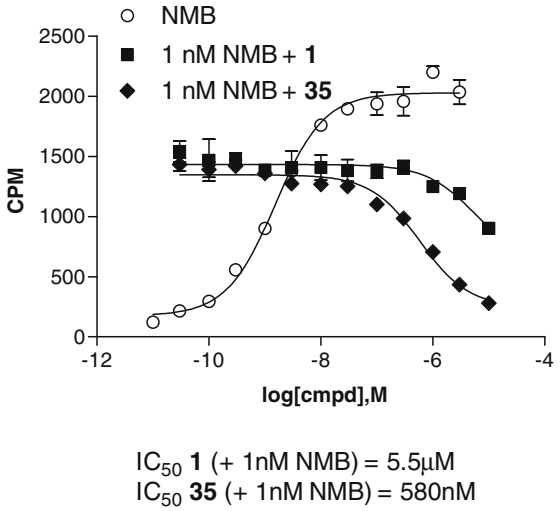


Figure 3.

In summary, we have identified a novel benzodiazepine-based NMBR antagonist (**35**) using a rapid parallel synthesis approach. This potent antagonist has potential as a tool compound to study the biology controlled by NMBR.

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16. 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). Standard error of the assay day-to-day was <30%; well-to-well variability was <10%.
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21. Purification was conducted using a Parallex Flex™ HPLC system purchased from Biotage Inc. Final products had 95% purity by HPLC and the structures were confirmed by LC–MS and ^{1}H NMR.
22. Resolution of **30** was accomplished using a Water 600 Tower system equipped with a Chiralpak AD column (Daicel Chemical Industrial # AD00CJ-DG008, 25 cm × 2 cm). Protocol: a solution of **30** (10 mg in 0.5 mL isopropyl alcohol) was injected and isocratic gradient of 60% isopropyl alcohol in hexanes at 20 mL/min was maintained. The fractions corresponding to the two enantiomers (**35** = 5 min; **36** = 20 min) were collected, and concentrated to afford **35** (2.5 mg) and **36** (2.5 mg). LC–MS analysis confirmed the desired molecular weights (M+1 = 427.5) of both enantiomers, 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 60% isopropyl alcohol in hexanes at 1 mL/min. Both **35** and **36** were determined to have enantiomeric excesses greater than 99%. Optical rotation were measured using a Jasco P-1020 polarimeter (**35**: $[\alpha]_D$ +49.8 (*c* 0.2, CH_3OH). Compound **36**: $[\alpha]_D$ −50.3 (*c* 0.2, CH_3OH).
23. *Analytical data for* **35**: δ_H (400 MHz, DMSO-d_6) 9.02 (1H, s), 7.65 (1H, *J* = 8.8 Hz, d), 7.23–7.30 (2 H, m), 6.99 (1 H, *J* = 10.1 Hz, d), 6.87 (1 H, *J* = 10.1 Hz, d), 6.50 (1 H, *J* = 10.1 Hz, d), 6.19 (1 H, s), 6.08 (1 H, *J* = 7.1 Hz, d), 4.85 (1 H, *J* = 6.5 Hz, d), 2.74 (1 H, *J* = 18.5 Hz, d), 2.60 (1 H, *J* = 18.5 Hz, d), 2.29 (1 H, *J* = 20.0 Hz, d), 2.13 (1 H, *J* = 20.0 Hz, d), 1.99 (3 H, s), 1.45–1.65 (6 H, m), 1.27 (1 H, m), 1.06 (2 H, *J* = 7.6 Hz, d). M+1 found 427.1; $C_{25}H_{25}F_3N_2O$ requires 426.5.
24. Protocol for cytotoxicity assay: HeLa cells were seeded at 5 k/well in a 96 well plate (6 plates-for triplicate 0 and 72 h readings). A threefold dilution series of each compound was generated, starting at 10 mM. The solution was diluted eight 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.
25. Protocol for the inositol phosphate (IP) accumulation assay: hNMBR-transfected HEK293 cells (2.5×10^4) were incubated in 96 well plates in DMEM High Glucose, w/o L-glutamine and 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.