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The identification of nonpeptide neurotensin receptor partial agonists from the potent antagonist SR48692 using a calcium mobilization assay

James B. Thomas *, Hernán Navarro, Keith R. Warner, Brian Gilmour

Organic and Medicinal Chemistry, Research Triangle Institute, PO Box 12194, Research Triangle Park, NC 27709-2194, USA

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ABSTRACT

In a search for nonpeptide agonists for the neurotensin receptor (NTR1), we replaced the adamantyl amino acid moiety found in the antagonist SR48692 (**1a**) with leucine and related α-alkylamino acids found in peptide agonists. When tested in a calcium mobilization assay, we found that both D- and L-leucine confer partial agonist activity to the pyrazole scaffold with the L-enantiomer (**3a**) providing a significantly greater response. A brief SAR survey demonstrated that the observed agonist activity was resilient to changes made to the dimethoxyaryl ring in **3a**. The resulting compounds were less potent relative to **3a** but showed greater agonist responses. The partial agonist activity was extinguished when the chloroquinoline ring was replaced with naphthalene. Thus, while L-leucine appears to possess a powerful agonist directing affect for the NTR1 receptor, its presence alone in the molecular architecture is not sufficient to insure agonist behavior.

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Abuse of methamphetamine represents a major and increasing threat to public health.[1] Yet despite many years of research, no pharmacotherapies have been identified for psychostimulant abuse. Neurotensin (NT, pGlu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu), a tridecapeptide identified over 30 years ago[2] is widely distributed in the central and peripheral nervous system and functions as both a neurotransmitter and neuromodulator.[3–6] It is a key player with regard to dopamine control in an area of the brain that is central to the mediation of reward behavior. It is co-localized with mesolimbic dopamine and modulates its transmission,[7–9] functionally antagonizing dopamine in the mesolimbic system while increasing dopaminergic transmission in the nigrastriatal system.[10,11] It modulates both dopaminergic and glutamatergic inputs to the nucleus accumbens, a region critical to the brains response to psychostimulants.[12–14] Such an ability to modulate dopamine has drawn the attention of researchers evaluating the role that NT plays in a number of maladies including schizophrenia and abuse of psychostimulants.[15,16] NT receptor peptide agonists and nonpeptide antagonists have both received attention in these efforts. Along similar lines, our interest in identifying pharmacotherapies for methamphetamine abuse directed us to the discovery of nonpeptide small-molecule NT receptor agonists, an area of research that has received little attention over the years.

Neurotensin achieves its effects via three receptor proteins, NTR1, NTR2, and NTR3.[17–22] The first two are seven-transmembrane domain G-protein-coupled receptors (GPCRs) while the third is a single-transmembrane domain sorting protein. Despite the fact that this receptor system was identified many years ago, very few nonpeptide ligands have been described for any NT receptor.[23–25] For NTR1, the most widely studied small-molecule ligands are those related to the antagonist SR48692 (**1a**, Chart 1) that shows

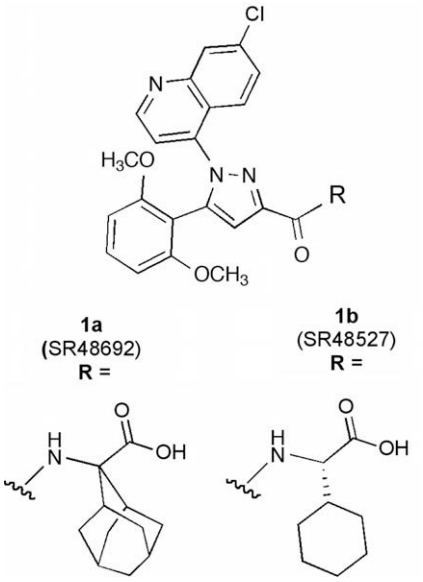

Chart 1. Standard antagonists for the NTR1 receptor.

* Corresponding author. Tel.: +1 919 541 6375; fax: +1 919 541 8868.
E-mail address: jbthomas@rti.org (J.B. Thomas).

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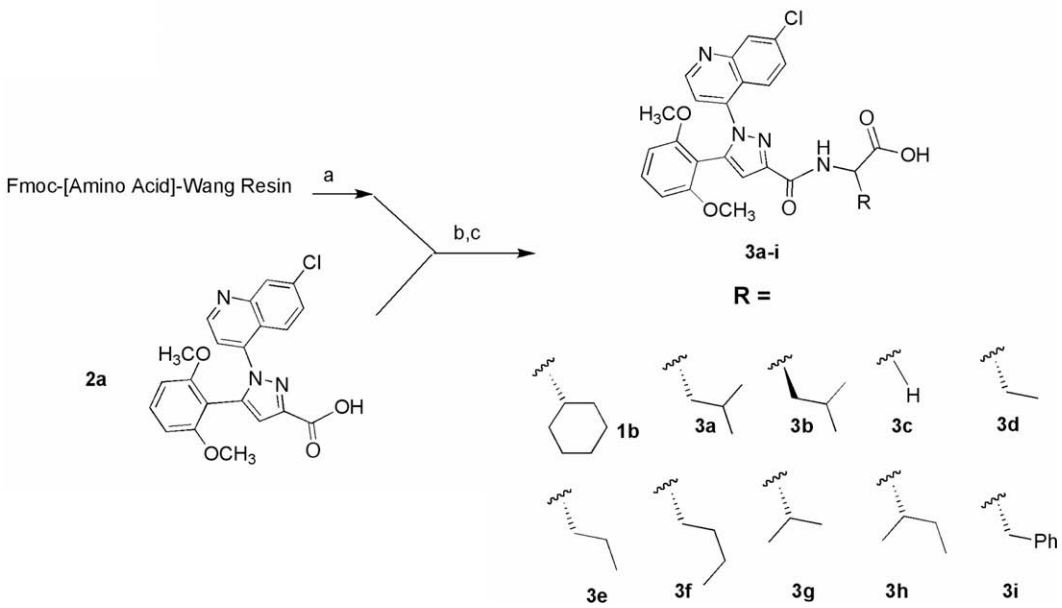
potent antagonist activity at NTR1 and good selectivity versus NTR2.[20,26] Despite the paucity of information regarding nonpeptide NT receptor compounds, much information supporting their design was available. Two research groups using point mutation studies have reported ligand-binding site models for the NTR1 receptor.[27–30] The model put forth by Barroso et al. and Labbe-Jullie et al. was of particular importance for the development of NTR1 nonpeptide small-molecule agonists as they proposed overlapping binding sites for the peptide agonist neurotensin-(8–13) and the nonpeptide antagonist **1a**. This suggested that it might be possible to obtain nonpeptide agonists through modification of the nonpeptide small-molecule antagonists.[27–29]

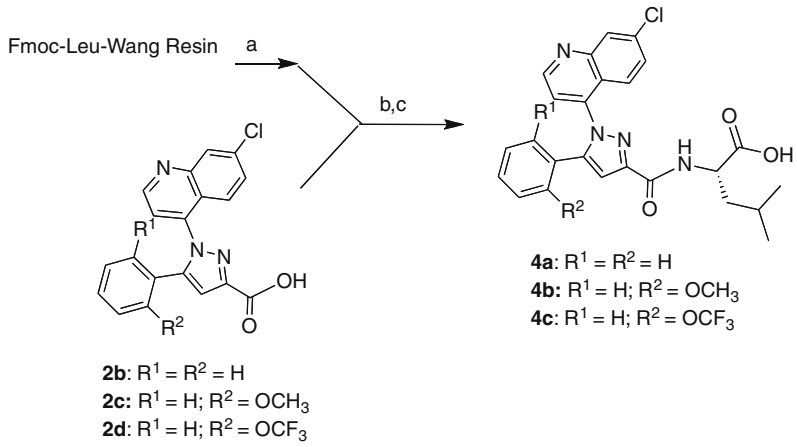
All of the receptor modeling studies to date have demonstrated that the terminal amino acid in both the agonist and antagonist ligands is of primary importance to the ligand/receptor interaction. Both ligand types were proposed to anchor on three key residues Arg^{327} Met,[208] and Phe.[331] The Arg^{327} residue was suggested to bind the Leu^{13} terminal residue in the peptide agonist and the carboxyl group in the nonpeptide antagonist **1a**. The side chains of the amino acids were proposed to interact with Met^{208} and Phe.[331] Taken together, these findings strongly suggested that replacement of the amino acids in the small-molecule antagonists (**1a** and **1b)** with those found in peptide agonists, L-leucine and similar L-alkyl-amino acids, was a reasonable strategy for discovery of small-molecule nonpeptide agonists.

The synthesis of the target compounds described in this study was accomplished by coupling pyrazole carboxylic acids **2a–e** with Fmoc-protected amino acids pre-loaded onto Wang resin (Schemes 1–3). The names of the amino acids used to prepare specific target compounds are listed in Table 1. The pyrazole acids **2a–e** were prepared according to the method previously described by Labeeuw et al.[31] In each synthesis, the resin was first Fmoc-deprotected with piperidine and then coupled to **2a–e** using benzotriazole-1-yl-oxy-tris-(dimethylamino)-phosphonium hexafluorophosphate (BOP) and triethylamine. Cleavage of the target compounds from the resin was accomplished using 95% trifluoroacetic acid in water to give the desired products **3a–i** (Scheme 1), **4a–c** (Scheme 2), and **5** (Scheme 3) in 60–80% yield.[32]

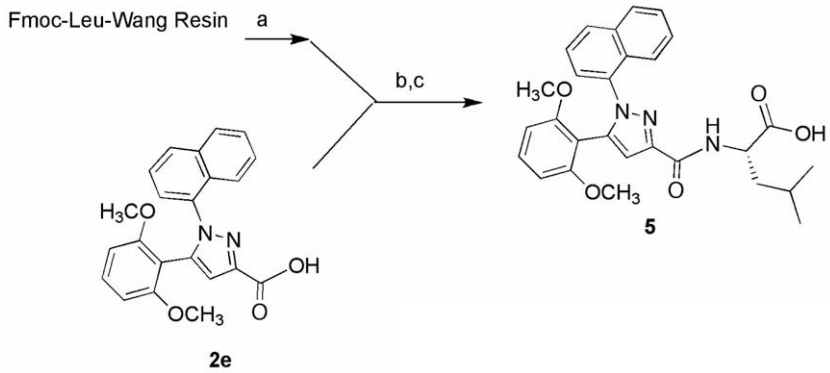
Instead of using a binding assay to test our compounds **3a–i, 4a–c**, and **5**, we chose to use a high-throughput functional assay. To this end, we generated a CHO-K1 cell line stably expressing NTR1.[33] The receptor specificity of agonist activity was determined



Scheme 1. Reagents: (a) 20% piperidine, NMP; (b) BOP, Et_3N, NMP, 95:5, TFA:H_2O.



Scheme 2. Reagents: (a) 20% piperdine, NMP; (b) BOP, Et_3N, NMP; (c) 95:5, TFA:H_2O.



Scheme 3. Reagents: (a) 20% piperdine, NMP; (b) BOP, Et_3N, NMP; (c) 95:5, TFA:H_2O.

Table 1
Fmoc-amino acid Wang resins used in target compound synthesis

Target compound	Fmoc-[amino acid]–Wang
1b	[L-Cyclohexylglycine]
3a, **4a–c**, **5**	[L-Leucine]
3b	[D-Leucine]
3c	[Glycine]
3d	[L-α-aminobutyric]
3e	[L-Norvaline]
3f	[L-Norleucine]
3g	[L-Valine]
3h	[L-Isoleucine]
3i	[L-Phenylalanine]

by the ability of the NTR1 antagonist **1a** to block test compound NTR1 activation. For these experiments, the cells were pre-incubated with 1 or 10 mM **1a** for 15 min at 37 °C prior to the addition of the test compound at its EC_{50}. The apparent affinity (K_e) of **1a** and **b** and **5** were determined by measuring the EC_{50} of neurotensin in their presence and absence.[34]

The screening data obtained from test compounds **3a–i** (Scheme 1) is shown in Table 2. As depicted, many of the compounds demonstrated agonist behavior while leucine, both L and D isomers, showed the greatest effect on calcium release. In contrast, the previously described antagonist, compound **1b** bearing the amino acid L-cyclohexylglycine, did not stimulate calcium release in our assay. Interestingly, the amount of activity observed varied with the structure of the side chain and the isobutyl group appeared to be preferred. The EC_{50} data was obtained for compounds **3a** and **3b**. Compound **3a** bearing the L-leucine amino acid, was found to be a partial agonist with an EC_{50} of 67 nM and an E_{max} of 51% of NT. Its enantiomer, **3b**, also showed partial agonist activity but was significantly less potent (EC_{50} = 986 nM). Both of these are considerably less potent than NT. Overall, this data set clearly demonstrates that the amino acid in the test compounds **1a**, **1b**, and **3a–i** has a powerful directing effect on the compounds intrinsic behavior and can confer either agonist or antagonist activity depending on its substructure. The NTR1 specificity of the observed calcium release for **3a** and **3b** was demonstrated by blocking with the NTR1 antagonist (**1a**) as described earlier (data not shown).

Table 2
Data obtained for target and standard compounds in an NTR1 agonist-mediated calcium mobilization assay

Compound	Screen[a] (10 μM)	EC_{50}[b] (nM ± SEM)	E_{max} (%NT ± SEM)	K_e (nM ± SEM)
NT		0.23 ± 0.04	100 ± 6	
1a				36 ± 10
1b	NA[c]			35 ± 9
3a	55 ± 12	67 ± 26	54 ± 18	
3b	57 ± 10	986 ± 496	37 ± 2	
3c	NA			
3d	11 ± 1			
3e	18 ± 4			
3f	20 ± 3			
3g	5 ± 1			
3h	NA			
3i	NA			
4a	62 ± 6	2043 ± 86	56 ± 7	
4b	86 ± 7	220 ± 42	81 ± 7	
4c	84 ± 7	1214 ± 197	91 ± 16	
5	NA			93 ± 32

[a] Test compounds were screened in triplicate at 10 μM and data is presented as percent stimulation relative to NT.
[b] Data represent means ± SE from at least two independent experiments.
[c] Not active.

Compounds **4a–c** were prepared to determine if the agonist directing effect of the L-leucine group in **3a** would be resilient to changes made to the substituents in its dimethoxyaryl ring (Scheme 2). Quéré had previously shown that these groups were essential to high affinity binding in **1a**.[35] As depicted in Table 2, analogs **4a–c** retained their partial agonist behavior and, **4b** and **4c** showed a higher agonist response relative to NT though they all had lower EC_{50} values. Compound **4a** with no methoxy groups was significantly less potent than **3a** and also less potent than **4b** with a single methoxy group. The lower potency of **4c** relative to **4b** suggests that the aryl ring prefers a greater degree of electron density. The NTR1 specificity of the observed calcium release for **4a–c** was demonstrated by blocking with the NTR1 antagonist (**1a**) as described earlier (data not shown).

Compound **5** was prepared to determine if the agonist directing effect of the L-leucine group in **3a** would be affected with changes made to its chloroquinoline ring (Scheme 3). We replaced the chloroquinoline group in **3a** with a naphthalene ring system since it is of similar size and had been shown to produce compounds with high binding affinity.[35] Interestingly, this compound (**5**) showed no agonist activity when screened at 10 mM but instead was found to be an antagonist with an apparent affinity (K_e) of 93 nM for the NTR1 receptor (Table 2). This finding illustrates that the presence of L-leucine in target molecules derived from **1a** is insufficient to guarantee that the resulting ligand will display agonist behavior for the NTR1 receptor. When considered together with the data from **4a–c**, the results from **5** suggest that each of the groups pendant to the pyrazole core can impact ligand activity which in turn suggests that each of these groups are interacting with the receptor protein, a notion consistent with the receptor modeling studies of Labbe-Jullie et al.[29] Taken together, this compound survey demonstrated that changing the amino acid residue in the antagonist **1a** to L-leucine (**3a**)

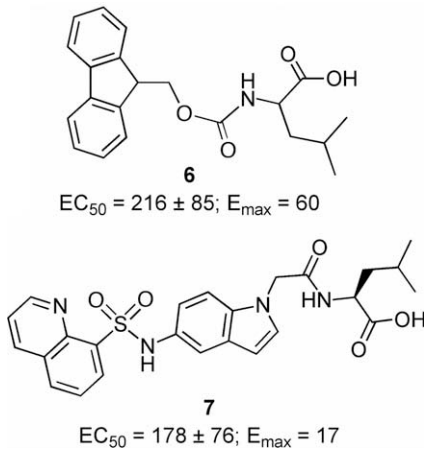


Figure 1. NTR1 receptor partial agonists disclosed by Wyeth Laboratories.

was sufficient to convert from antagonist to partial agonist activity. Beyond this we have shown that this agonist activity was resilient to some (**4a–c**) but not all (**5**) changes to the scaffold. We have also shown that the presence of L-leucine alone (**5**) is insufficient to guarantee partial agonist activity.

During the course of this investigation, Fan and coworkers disclosed the structures for two NTR1 partial agonists (**6** and **7**, Fig. 1).[36] These compounds arose from a campaign to identify NT small-molecule agonists using virtual screening techniques. The investigators confirmed the NTR1 receptor partial agonist activity of **6** and **7** in a calcium release (FLIPR) assay. We found this of interest, and based on our observations, do not believe that it is a coincidence that both of these compounds incorporate the amino acid leucine. Though there is much more to learn regarding small-molecule NTR1 compounds, it will be of interest to discover why leucine appears to be a privileged group for promoting agonist activity in NTR1 ligands.

In conclusion, we have demonstrated that replacement of the amino acid group in NTR1 antagonists such as **1a** or **1b** with leucine afforded small-molecule partial agonists (**3a** and **3b**) with the L-leucine enantiomer providing significantly higher potency. The propensity of this amino acid to confer agonist behavior was also observed in analogs of **3a** (**4a–c**). In addition to this, we have also shown that the presence of leucine as part of a molecular structure (**5**) is not enough to guarantee an NTR1 partial agonist. Finally, the results from this study also provide support for the receptor-binding models that proposed overlapping active sites for NT agonists and antagonists. Additional studies to identify potent NTR1 small-molecule agonists are currently in progress and will be reported in due course.

Acknowledgments

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32. All compounds showed satisfactory NMR and combustion data. The NMR spectrum of **3a** was recorded using a 300 MHz (Bruker AVANCE 300) spectrometer using tetramethylsilane as the internal standard. ^{1}H NMR ($CDCl_3$) δ 8.8 (d, 1H, J = 4.72 Hz), 8.14 (d, 1H, J = 1.81 Hz), 7.95 (d, 1H, J = 9.1), 7.5 (dd, 1H, J = 1.89 and 9.1 Hz), 7.4 (d, 1H, J = 7.95 Hz), 7.20 (t, 1H, J = 8.42 Hz), 7.10 (s, 1H), 7.08 (d, 1H, J = 4.72 Hz), 6.38 (d, 2H, J = 8.42 Hz), 4.81 (br s, 1H), 3.39 (s, 6H), 1.74 (m, 3H), 0.96 (dd, 6H, J = 5.49 and 10.21 Hz).
33. Agonist activity was measured in these cells by monitoring increases in internal calcium concentrations using the calcium-sensitive dye, calcium-4, and a FlexStation II^{384} fluorometric imaging plate reader (MDS Analytical Technologies, Sunnyvale, CA). Cells were plated in 96-well plates at 20,000 cells per well 24 h before assay. Prior to assay, the media was removed and the cells were washed 1× with 100 μl dye loading buffer (HBSS plus 20 mM HEPES, 2 mM probenecid) followed by addition of 100 μl dye loading buffer. Calcium-4 dye was reconstituted in dye loading buffer as per manufacturer's instructions and 100 μl dye was added to wells. Dye loading was performed for 1 h at 37 °C in 5% CO_2. Fluorescent intensities were measured for 17 s (baseline intensity) before and for 40 s after compound addition. Compound-induced fluorescence increases were measured as the difference between peak and baseline intensity for each well. Test compounds were initially screened at 10 μM final concentration and an EC_{50} was determined for compounds exhibiting at least 50% neurotensin E_{max}. EC_{50} values were determined from 10-point half-log concentration–response curves.
34. The assays were run as above except that during the last 15 min of the incubation the cells were pretreated (15 min, in duplicate) with **1a** (1 or 4 μM) or **1b** (1 or 10 μM) or 5 (1 or 10 μM) followed by the addition of one of eight different concentrations of neurotensin. Test compounds and neurotensin were dissolved in DMSO (1% final assay concentration). The K_e was determined using the equation: K_e = [antagonist]/((EC_{50} Ant/EC_{50}control) − 1). The EC_{50} values were determined using Prism (v 5.0, Graph Pad, San Diego, CA).
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