Identification of 1-({[1-(4-Fluorophenyl)-5-(2-methoxyphenyl)-1H-pyrazol-3-yl]carbonyl}amino)cyclohexane Carboxylic Acid as a Selective Nonpeptide Neurotensin Receptor Type 2 Compound

James B. Thomas,*,[†] Angela M. Giddings,[†] Robert W. Wiethe,[†] Srinivas Olepu,[†] Keith R. Warner,[†] Philippe Sarret,[‡] Louis Gendron,[‡] Jean-Michel Longpre,[‡] Yanan Zhang,[†] Scott P. Runyon,[†] and Brian P. Gilmour[†]

[†]Center for Organic and Medicinal Chemistry, Research Triangle Institute, P.O. Box 12194, Research Triangle Park, North Carolina 27709, United States

[‡]Department of Physiology and Biophysics, Faculty of Medicine and Health Sciences, Université de Sherbrooke, 3001, 12th Avenue North, Sherbrooke, Quebec J1H 5N4, Canada

Ⓢ *Supporting Information*

ABSTRACT: Compounds active at neurotensin receptors (NTS1 and NTS2) exert analgesic effects on different types of nociceptive modalities, including thermal, mechanical, and chemical stimuli. The NTS2 preferring peptide JMV-431 (**2**) and the NTS2 selective nonpeptide compound levocabastine (**6**) have been shown to be effective in relieving the pain associated with peripheral neuropathies. With the aim of identifying novel nonpeptide compounds selective for NTS2, we examined analogues of SR48692 (**5a**) using a FLIPR calcium assay in CHO cells stably expressing rat NTS2. This led to the discovery of the NTS2 selective nonpeptide compound 1-({[1-(4-fluorophenyl)-5-(2-methoxyphenyl)-1H-pyrazol-3-yl]-carbonyl}amino)cyclohexane carboxylic acid (NTRC-739, **7b**) starting from the nonselective compound **5a**.
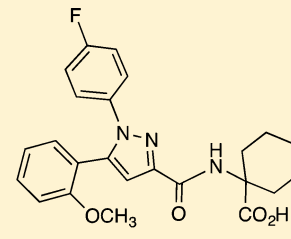
7b
Neurotensin Receptor Type 2 Selective

INTRODUCTION

Neurotensin (NT) is a tridecapeptide (Glu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu) that was identified 40 years ago from bovine hypothalamus.[1] NT functions as a neurotransmitter and neuromodulator demonstrating a range of biological actions.[2] In the CNS, NT is colocalized with and regulates the action of mesolimbic and nigrastriatal dopamine[3−7] as well as mediating nonopioid analgesia and hypothermia.[8,9] It is believed that NT operates primarily through interaction with two G-protein coupled receptors NTS1 and NTS2 (also referred to as NTR1, NTR2, NTRH, NTRL) to regulate these activities. A third receptor, NTS3, also known as sortilin, is a type I receptor bearing a single transmembrane domain.[10−14] The ability of the NT receptor system to regulate CNS dopamine led researchers to postulate that NT might be an endogenous neuroleptic[15] and that drugs acting via the NT system might therefore be useful as antipsychotic agents.[16,17] Methamphetamine also produces profound disruption of dopamine flow in the mesolimbic and nigrastriatal dopamine networks with chronic methamphetamine exposure, eliciting behaviors resembling schizophrenia.[18,19] It is thus no surprise that compounds acting via the NT network are now being investigated as potential treatments for methamphetamine abuse.[20,21]

The NT system also plays an important role in the pain processing network.[8,22] The nonopioid analgesia mediated by the NT system has also generated much interest over the years as a potential means of circumventing the side effects produced by opioid anal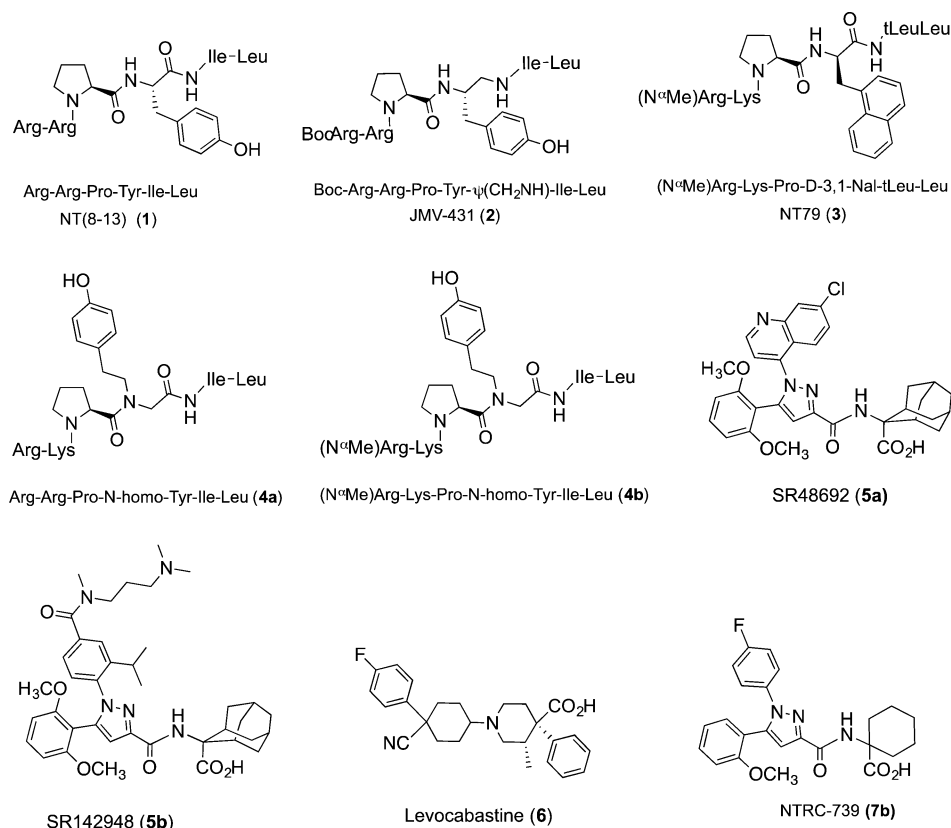gesics including addiction, constipation, and respiratory depression. NT-mediated analgesia has been demonstrated with compounds selective for both the NTS1 and NTS2 receptors as well as nonselective compounds.[23−28] Beyond this, there is now substantial evidence that both NTS1 and NTS2 can mediate relief from chronic or neuropathic pain.[29] This type of pain is difficult to treat with current drugs and does not always respond well to opioid therapy.[30] Taken together with their neuroleptic activity, it is easy to understand why the development of compounds acting via the NT network has engendered so much interest.

The NTS1 receptor has received the most attention since the discovery of the NT system as it binds NT with high affinity, hence the name NTRH (NT receptor high affinity), and is well behaved in heterologous expression systems. The NTS2 receptor, on the other hand, has received less attention by comparison, and as a consequence fewer compounds have thus far been identified that interact with this receptor. As is true for NTS1, the most potent and selective NTS2 compounds discovered to date have been obtained via modification of the NT(8−13) (**1**) fragment.[31] Discovery of potent and selective nonpeptide compounds however has proven elusive and remains as a significant challenge.

Several important milestone compounds from NT receptor research are depicted in Chart 1. The 8−13 fragment of NT, compound **1**, is as potent as the full length peptide, and several

Chart 1

Arg-Arg-Pro-Tyr-Ile-Leu
NT(8-13) (1)

Boc-Arg-Arg-Pro-Tyr-ψ(CH₂NH)-Ile-Leu
JMV-431 (2)

(NᵅMe)Arg-Lys-Pro-D-3,1-Nal-tLeu-Leu
NT79 (3)

Arg-Arg-Pro-N-homo-Tyr-Ile-Leu (4a)

(NᵅMe)Arg-Lys-Pro-N-homo-Tyr-Ile-Leu (4b)

SR48692 (5a)

SR142948 (5b)

Levocabastine (6)

NTRC-739 (7b)

hexapeptide variants of **1** have been reported over the years that either favor NTS2 over NTS1 or are selective for NTS2 versus NTS1. The first of these is JMV-431 (**2**) that was produced via reduction of the tyrosine 11 amide bond and shows a clear preference for NTS2.[32,33] While it is not bioavailable, it is active in several models of chronic pain when delivered intrathecally.[28,29] The peptide NT79 (**3**), reported more recently,[34] is selective for NTS2 (>100-fold) and has provided a wealth of information regarding the role of NTS2 in animal models of pain, antipsychotic activity, thermoregulation, and regulation of blood pressure.[34] Like peptide **2**, this compound attained NTS2 selectivity via modification of the tyrosine 11 residue. The most recent additions to the rolls of NTS2-selective compounds are the potent peptide–peptoid hybrids **4a** and **4b**.[35,36] These compounds are ultraselective for NTS2, with selectivity ratios reaching 12000- and 22000-fold, respectively, with **4b** also demonstrating excellent plasma stability.
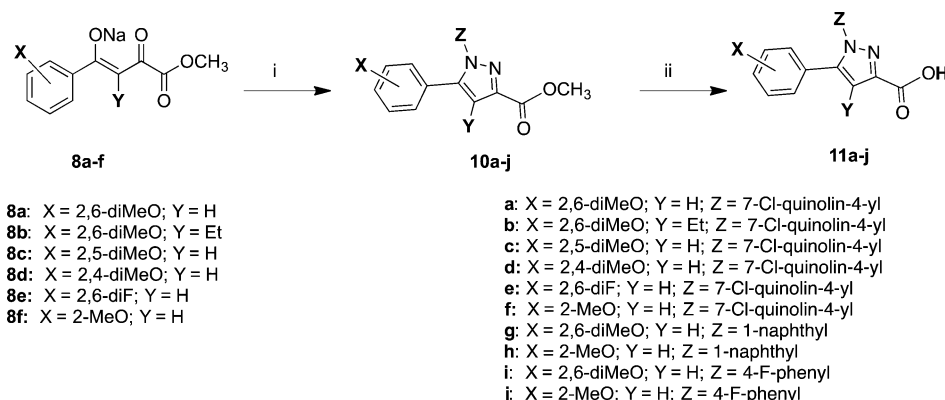
AlThough few in number, there are three prominent nonpeptide compounds that interact with NTS2 that have been used extensively in the characterization of the NT receptors. These include the two pyrazole-based compounds **5a** (SR48692) and SR142948a (**5b**) and the histamine blocker levocabastine (**6**).[37−39] Pyrazole compound **5a** prefers NTS1 to NTS2 while **5b** is nonselective, but they both antagonize the activity of NT at NTS1. Compound **6** is selective for NTS2 versus NTS1, but it is also a potent antagonist at histamine receptor 1 (H1). These compounds (**5a,b** and **6**) highlight the fact that while NTS2-selective peptides exist, selective nonpeptide compounds are rare.

The SAR of pyrazole compounds **5a** and **5b** has been described at NTS1 but, to our knowledge, no systematic survey o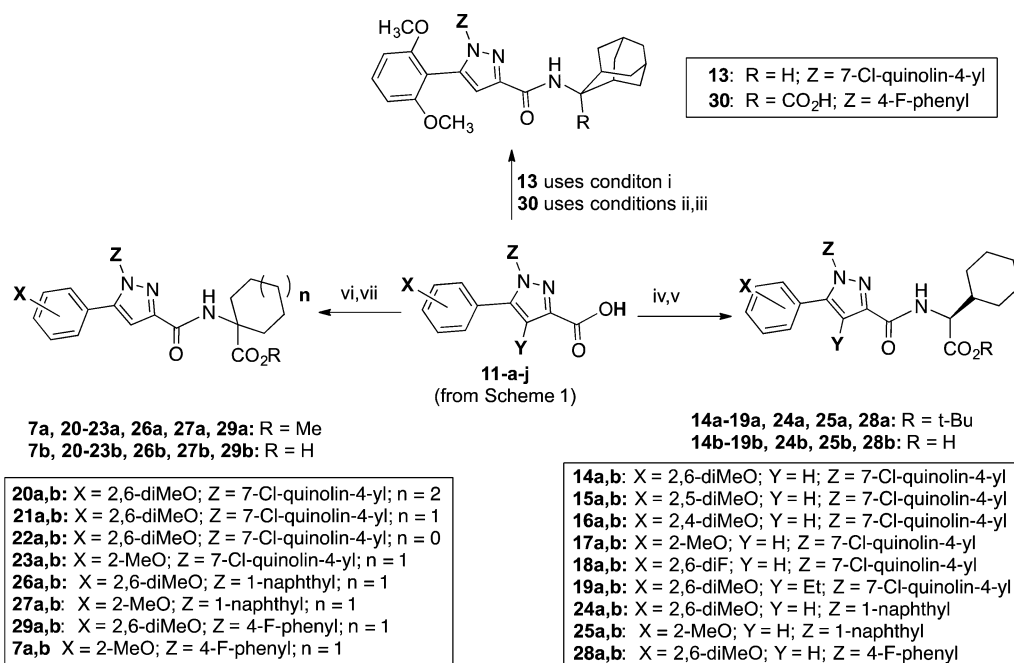f SAR has ever been described at NTS2. With the goal of discovering novel NTS2 selective ligands, we undertook this endeavor by adapting a previously described calcium mobilization assay to our high throughput FLIPR Tetra system and confirmed the functional results of active compounds using a radioligand binding assay.[40] This effort led to the identification of a nonpeptide compound that is selective for NTS2 versus NTS1, 1-({[1-(4-fluorophenyl)-5-(2-methoxyphenyl)-1H-pyrazol-3-yl]carbonyl}amino)cyclohexane carboxylic acid (**7b**), starting from the nonselective compound **5a**. The newly discovered compound (**7b**) displays neither calcium nor binding activity at NTS1, but is a potent partial agonist at NTS2 (EC₅₀ of 12 ± 6 nM and an E_{max} of 7% relative to **5b**) that gave a K_i of 153 ± 10 nM in competition with [¹²⁵I]NT. The details of this effort are presented herein.

■ CHEMISTRY

The target compounds, depicted in Tables 2 and 3, were synthesized as described in Schemes 1−2. Commercially available materials were used when available. Those not commercially available were prepared according to literature precedent. Scheme 1 illustrates the synthesis of the key intermediate pyrazole carboxylic acids (**11a−j**). These were prepared as described by Labeeuw,[41] although improved methods were recently described by Jiang et al.[42] and also Baxendale et al.[43] This employed a Knorr [3 + 2]-cyclization reaction between 4-aryl-2,4-diketoesters (**8a−f**) and arylhydrazines **9a−c** in acetic acid at reflux. The resulting esters **10a−j** were hydrolyzed using LiOH and dioxane to give **11a−j**. The 4-aryl-2,4-diketoesters were commercially available with the exception of **8b**. The 2,6-dimethoxy-butyrophenone used to synthesize compound **8b** was prepared exactly as described by Lindh.[44]

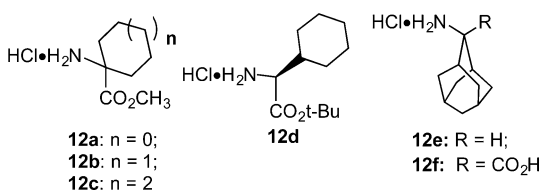**Scheme 1. Synthesis of Key Pyrazole Intermediates 11a–j[a]**

8a: X = 2,6-diMeO; Y = H
8b: X = 2,6-diMeO; Y = Et
8c: X = 2,5-diMeO; Y = H
8d: X = 2,4-diMeO; Y = H
8e: X = 2,6-diF; Y = H
8f: X = 2-MeO; Y = H

a: X = 2,6-diMeO; Y = H; Z = 7-Cl-quinolin-4-yl
b: X = 2,6-diMeO; Y = Et; Z = 7-Cl-quinolin-4-yl
c: X = 2,5-diMeO; Y = H; Z = 7-Cl-quinolin-4-yl
d: X = 2,4-diMeO; Y = H; Z = 7-Cl-quinolin-4-yl
e: X = 2,6-diF; Y = H; Z = 7-Cl-quinolin-4-yl
f: X = 2-MeO; Y = H; Z = 7-Cl-quinolin-4-yl
g: X = 2,6-diMeO; Y = H; Z = 1-naphthyl
h: X = 2-MeO; Y = H; Z = 1-naphthyl
i: X = 2,6-diMeO; Y = H; Z = 4-F-phenyl
j: X = 2-MeO; Y = H; Z = 4-F-phenyl

[a]Reagents and conditions: (i) HOAc, HCl, and **9a** (7-chloroquinolin-4-yl)hydrazine·HCl)) or **9b** (1-naphthylhydrazine·HCl) or **9c** (4-fluorophenylhydrazine·HCl), reflux 4 h; (ii) LiOH 3 equiv, dioxane, RT 16 h.

Scheme 2. Synthesis of Target Compounds 7b, 13, 14b–29b, and 30[a]

13: R = H; Z = 7-Cl-quinolin-4-yl
30: R = CO₂H; Z = 4-F-phenyl

13 uses condition i
30 uses conditions ii,iii

7a, 20-23a, 26a, 27a, 29a: R = Me
7b, 20-23b, 26b, 27b, 29b: R = H

20a,b: X = 2,6-diMeO; Z = 7-Cl-quinolin-4-yl; n = 2
21a,b: X = 2,6-diMeO; Z = 7-Cl-quinolin-4-yl; n = 1
22a,b: X = 2,6-diMeO; Z = 7-Cl-quinolin-4-yl; n = 0
23a,b: X = 2-MeO; Z = 7-Cl-quinolin-4-yl; n = 1
26a,b: X = 2,6-diMeO; Z = 1-naphthyl; n = 1
27a,b: X = 2-MeO; Z = 1-naphthyl; n = 1
29a,b: X = 2,6-diMeO; Z = 4-F-phenyl; n = 1
7a,b: X = 2-MeO; Z = 4-F-phenyl; n = 1

14a-19a, 24a, 25a, 28a: R = t-Bu
14b-19b, 24b, 25b, 28b: R = H

14a,b: X = 2,6-diMeO; Y = H; Z = 7-Cl-quinolin-4-yl
15a,b: X = 2,5-diMeO; Y = H; Z = 7-Cl-quinolin-4-yl
16a,b: X = 2,4-diMeO; Y = H; Z = 7-Cl-quinolin-4-yl
17a,b: X = 2-MeO; Y = H; Z = 7-Cl-quinolin-4-yl
18a,b: X = 2,6-diF; Y = H; Z = 7-Cl-quinolin-4-yl
19a,b: X = 2,6-diMeO; Y = Et; Z = 7-Cl-quinolin-4-yl
24a,b: X = 2,6-diMeO; Y = H; Z = 1-naphthyl
25a,b: X = 2-MeO; Y = H; Z = 1-naphthyl
28a,b: X = 2,6-diMeO; Y = H; Z = 4-F-phenyl

[a]Reagents and conditions: (i) HBTU, Et₃N, CH₂Cl₂ 2-aminoadamantane·HCl, RT, 16 h (**12e**); (ii) SOCl₂, toluene, reflux, 3 h; (iii) NaOH, THF, **12f**, RT, 16 h; (iv) HBTU, Et₃N, CH₂Cl₂ amino acid ester **12d**, RT, 16 h; (v) TFA, CH₂Cl₂, RT, 16 h; (vi) HBTU, Et₃N, CH₂Cl₂ amino acid ester **12a–c**, RT, 16 h; (vii) LiOH, dioxane, RT, 16 h.

In Scheme 2, the synthetic methods used to produce target compounds **7b**, **13**, **14b–29b**, and **30** are described. Thus, a given pyrazole carboxylic acid (**11a–j**) and amino acid ester (**12a–d**) or amine (**12e**) from Chart 2 were coupled using *O*-benzotriazol-1-yl-*N,N,N′,N′*-tetramethyluronium

hexafluorophosphate (HBTU) and triethylamine to give ester intermediates **7a**, and **14a–29a** and the descarboxy target compound **13**. The ester intermediates **7a**, and **14a–29a** were hydrolyzed to give final products using either basic (LiOH and dioxane) or acidic (trifluoroacetic acid (TFA) in CH₂Cl₂) conditions as shown. We used the *tert*-butyl rather than the methyl esters of L-cyclohexylglycine (**12d**) to avoid racemization of the chiral amino acid. We used the basic conditions for hydrolysis of achiral amino acid esters. The synthesis of target compound **30** was accomplished according to the method of Quére;[45] wherein pyrazole acid **11i** was converted to its acid chloride using SOCl₂ in toluene, followed by coupling of the acid chloride intermediate with the adamantyl amino acid **12f** under Schotten–Bauman conditions. The adamantyl amino acid **12f** was prepared as described by Nagasawa.[46]

Chart 2. Amines, Amino Acids, and Amino Acid Esters Used to Prepare Target Compounds 7b, 13, and 14b–29b

12a: n = 0;
12b: n = 1;
12c: n = 2

12d

12e: R = H;
12f: R = CO₂H

Table 1. Functional and Radioligand Binding Data Obtained for NT, 1, 5a, 5b, and 6 at the NTS1 and NTS2 Receptors

	FLIPR assay						binding[a]
	NTS1			NTS2			NTS2
compd	EC_{50}[b]	E_{max}[c]	K_e	EC_{50}	E_{max}[d]	IC_{50}	K_i
NT	0.04 ± 0.012	100 ± 3		NA[e]		18.5 ± 1.2	18.9 ± 3
1	0.01 ± 0.002	114 ± 7		NA		5.4 ± 0.6	33 ± 11
5a			4.7 ± 0.8	120 ± 20	100 ± 3		62 ± 35
5b			1.5 ± 0.6	20 ± 5	100 ± 5		6 ± 2
6	NA		NA	28 ± 4	16 ± 3		33 ± 5

[a][^{125}I]NT, [b]EC_{50}, K_e, IC_{50}, and K_i values are nM ± SEM. [c]E_{max} value is % NT. [d]E_{max} value is % 5b. [e]Not active.

■ BIOLOGICAL RESULTS

The binding affinities of the test compounds for the rNTS1 and rNTS2 receptors listed in Tables 1 through 3 were determined using previously reported competitive binding assays.[40] The NTS1 and 2 receptors were labeled using [^{125}I]NT. The cells used in the binding assay were CHO-k1 cells (American Type Culture Collection) engineered to overexpress either the rNTS1 or rNTS2 receptor. Measures of functional agonist and antagonist activity were obtained by measuring changes in intensity of a calcium-sensitive fluorescent dye as an indirect measure of changes in internal calcium concentrations. These measurements were performed using a FLIPR Tetra (NTS2) or a FlexStation II plate reader for NTS1 (Molecular Devices) and were analyzed using GraphPad Prism software. Antagonist activity was measured as inhibition of NT-induced calcium release (NTS1) or inhibition of 5b-induced calcium release (NTS2).

■ RESULTS AND DISCUSSION

In this article, we provide the details of our effort to identify nonpeptide compounds that are selective for NTS2 starting from the nonselective compound 5a. This included developing an assay capable of detecting activity at the NTS2 receptor based upon literature reports that compound 5a is an agonist at NTS2 (induced calcium mobilization at the NTS2 receptor). A summary of the key SAR elements of 5a discovered using the NTS2 calcium mobilization assay that led to the identification of the potent partial agonist 7b (NTRC-739) are provided in Table 2 and includes the NTS2 EC_{50} as well as the NTS1 K_e and the NTS2 binding affinity (K_i). Each of the appended molecular regions of 5a is discussed herein and includes: the dimethoxyphenyl ring, the amino acid side chain, the 7-chloroquinoline ring, and the 4-position of the pyrazole ring. The data obtained from the testing of NT reference compounds in this assay, including NT, 1, 5a,b and levocabastine (6), are provided in Table 1.

In attempting to develop a method of screening for NTS2 activity, we observed that the literature reports dealing with functional assays of NTS2 receptors yielded contradictory data, exhibiting cell-type expression- and species-dependent pharmacological properties with opposing patterns. Indeed, NT has been reported to be an agonist, an inverse agonist, and a neutral antagonist depending upon the cell expression system.[13,47−49] Similar findings were reported for compounds 5a, 5b, and 6 as well when tested alongside NT in these systems. Specifically, the potent NTS1 antagonists 5a and 5b were found to be agonists at NTS2 as they mobilized calcium release while levocabastine (6) was found to behave either as an agonist, antagonist, or even as a partial inverse agonist.[47−49]

While the information above illustrated that there was much inconsistency between cell lines and expression systems for

NTS2, we believed that each system would produce internally consistent (reproducible) data. We moved forward with the working hypothesis that the calcium mobilization described by these various laboratories was indeed NTS2-mediated and chose one system to study in greater detail. As we already had the CHO-k1-rNTS2 cell line available from previous work, this became the obvious choice.[40] We studied the calcium release produced by analogues of the nonpeptide compound 5a by measuring their ability to either stimulate release of calcium or to block the calcium release stimulated by 5b and found that it responded to changes in structure (SAR), Table 2. This information was compared with that obtained for (NT and 1) as well as levocabastine, which revealed that this assay was capable of identifying a range of activities from potent agonist (5a and 5b) to partial agonist (6) to antagonist (NT and 1), Table 1 below, which implied that the structure of the compound made a direct impact on the resulting mobilization of calcium. This indicated that our hypothesis was correct and that we could identify NTS2 agonists directly and NTS2 antagonists indirectly against the agonist activity of 5b.

Further support for our hypothesis was obtained by collecting the binding affinity data in competition with [^{125}I]NT for our study set of analogues of 5a,Table 2. These data revealed that compounds that modulated calcium release also competed with NT for binding at NTS2. Involvement of NTS2 was implicated further by the observation that compounds that mobilized calcium release in the CHO-k1-rNTS2 cell line did not produce calcium mobilization in the parent CHO line. Taken together, these data indicated that this assay method could be used to steer SAR studies in search of novel NTS2 selective compounds.

As described above, the baseline for our study was established using the compounds common to NT receptor research. In Table 1, we have summarized the calcium release and binding data obtained for NT, 1, 5a, and 5b, and the NTS2-selective compound levocabastine (6) in both our rNTS1 and rNTS2 cell lines. In our CHO-k1-NTS2 cell line, neither NT nor 1 showed any calcium release in our FLIPR assay as previously reported.[49] Furthermore, we did not observe constitutive activity in this expression system as has been reported by other investigators using alternate expression systems.[35,50] On the other hand, both 5a and 5b stimulated calcium release with EC_{50} values of 120 and 20 nM, respectively. Compound 5b was more potent than compound 5a in the calcium mobilization assay but equally efficacious, and was designated as our NTS2 full agonist standard (the use of the term agonist here refers to a compound that stimulates calcium release in this assay). Levocabastine (6), a compound used for years to distinguish NTS1 from NTS2, was also tested and found to be a potent partial agonist with an EC_{50} of 28 nM and

D

dx.doi.org/10.1021/jm5003843 | J. Med. Chem. XXXX, XXX, XXX−XXX

Table 2. Antagonism of NT Induced Calcium Release at NTS1 Compared to Target Compound Induced Calcium Mobilization and Binding Affinity at NTS2 for 7-Chloroquinolyl (A), Naphthyl (B), and 4-F-Phenyl (C) Substituted Pyrazole Carboxamides at the NTS1 and NTS2 Receptors

Cmpd	A B C	Y	R^1	R^2	FLIPR Assays			Binding[a]
					NTS1	NTS2		NTS2
					K_e	EC_{50}[b]	E_{max}[c]	K_i
6					NA[d]	28±4	16±3	33±5
5a	A	H			4.7±0.8	120±20	100±15	62±35
13	A	H			NA	NA		>11 μM
14b	A	H			23±6	217±19	86±3	644± 90
15b	A	H			1275±544	216±29	19±3	604±141
16b	A	H			>10 μM	382±33	12±3	1585±505
17b	A	H			1682±527	258±14	25±2	1418±468
18b	A	H			>10 μM	166±3	15±3	1001±227
19b	A	Et			>10 μM	94±17	15±1	1177±155
20b	A	H			42±6	21±5	89±5	170±71
21b	A	H			157±45	29±2	78±7	151±67
22b	A	H			222±14	20±4	76±4	102±27
23b	A	H			4549±636	62±9	35±1	533±153

Table 2. continued

Cmpd	A B C	Y	R^1	R^2	FLIPR Assays NTS1 K_e	FLIPR Assays NTS2 EC_{50}[b]	FLIPR Assays NTS2 E_{max}[c]	Binding[a] NTS2 K_i
24b	B	H	(2,6-dimethoxyphenyl; H$_3$CO, OCH$_3$)	(cyclohexyl, CO$_2$H)	58±6	159±9	45±1	536±20
25b	B	H	(2-methoxyphenyl; H$_3$CO)	(cyclohexyl, CO$_2$H)	1404±330	229±8	18±2	2687±639
26b	B	H	(2,6-dimethoxyphenyl; H$_3$CO, OCH$_3$)	(cyclohexane, CO$_2$H)	230±79	18±1.6	35±1	116±39
27b	B	H	(2-methoxyphenyl; H$_3$CO)	(cyclohexane, CO$_2$H)	7380±1130	108±8	18±2	694±178
30	C	H	(2,6-dimethoxyphenyl; H$_3$CO, OCH$_3$)	(adamantyl, CO$_2$H)	191±47	68±25	34±5	110±29
28b	C	H	(2,6-dimethoxyphenyl; H$_3$CO, OCH$_3$)	(cyclohexyl, CO$_2$H)	3344±549	271±24	22±5	622±245
29b	C	H	(2,6-dimethoxyphenyl; H$_3$CO, OCH$_3$)	(cyclohexane, CO$_2$H)	>10 µM	19±3	12±0.5	140±29
7b	C	H	(2-methoxyphenyl; H$_3$CO)	(cyclohexane, CO$_2$H)	>10 µM	12±6	7±2	153±10

[a][^{125}I]NT. [b]EC_{50}, K_e, IC_{50}, and K_i values are all nM ± SEM. [c]E_{max} value is % **5b**. [d]Not active.

an E_{max} of 16% relative to **5b**. The activity of **5b** and **6** are illustrated in Figure 1.

Neurotensin and **1** did not stimulate calcium release, but they both blocked the calcium release stimulated by compound **5b** in an insurmountable manner as they showed a rightward shift in the dose–response curve with an accompanying dose-dependent drop in the E_{max} value of **5b**.[51,52] This is illustrated for NT in Figure 2. We thus determined and report here the IC_{50} for both NT and **1** in competition with **5b** and found these to be 18.9 and 5.4 nM, respectively. The IC_{50} curve generated for NT against **5b** is depicted in Figure 3.

Corroboration of the functional data was obtained in the binding assay. We found that compound **5a** gave a K_i of 62 nM, while **5b** showed a 10-fold higher affinity with a K_i of 6 nM. As in the FLIPR assay, compound **5b** has a higher affinity than **5a**. NT gave a K_i of 18.5 nM. Compound **1** showed similar affinity to NT, while levocabastine (**6**) gave a K_i of 33 nM. The binding affinities found for NT, **1**, and levocabastine are slightly higher than those found in other laboratories, while those for **5a** and **b** are similar. We attribute this to our use of an in-plate whole cell binding method[40] as opposed to the use of membrane preparations.

Relating the functional behaviors observed in vitro for these reference compounds to their demonstrated in vivo behaviors is instructive from a drug discovery perspective. NT possesses
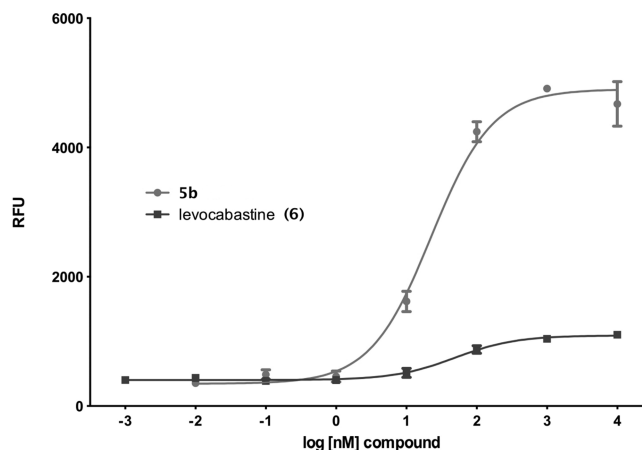

Figure 1. Dose–response curves for **5b** and **6** in CHO-k1-rNTS2 cells.

antipsychotic activity and mediates nonopioid analgesia, while both NT and levocabastine (**6**) are active in models of persistent pain.[29,34] The pyrazole compounds **5a** and **5b**, on the other hand, are not analgesics in vivo but instead block the analgesic activity of NT in animal models of pain.[28,53,54] This is also observed in animal models based on runaway mesolimbic dopamine where the neuroleptic action of NT is
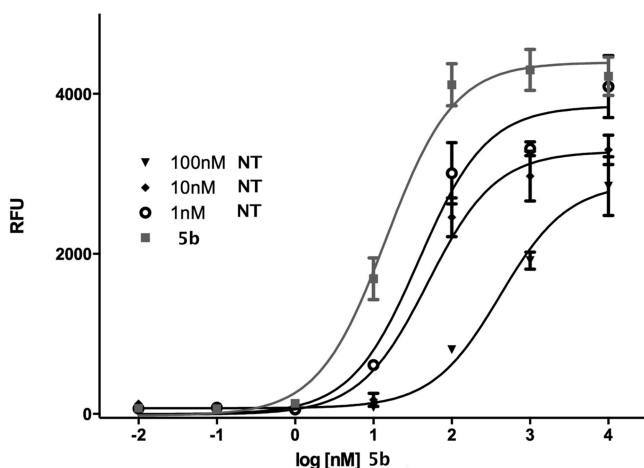
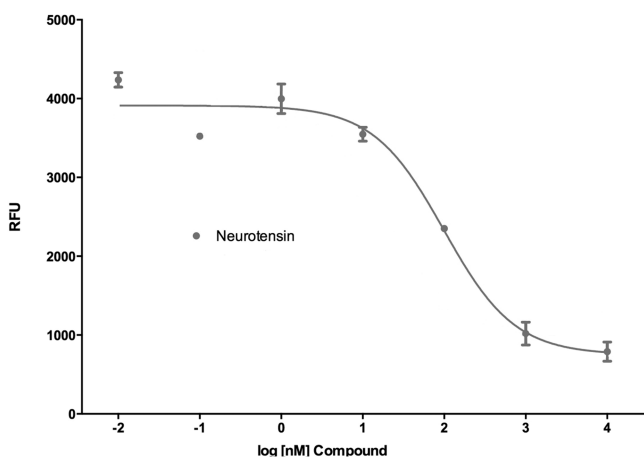**Figure 2.** NT is an insurmountable antagonist of **5b** mediated calcium mobilization.

Figure 3. IC_{50} curve for NT versus **5b**.

blocked by compounds **5a** and **5b**.[21,38,55,56] According to the functional data in Table 1, the proven analgesic and antipsychotic compounds appeared as either antagonists of calcium mobilization (NT and peptide **1**) or potent partial agonists of calcium mobilization (levocabastine, **6**), while the compounds that possess neither analgesic nor antipsychotic activity in vivo, compounds **5a** and **5b**, appeared as potent agonists. This point of reference provided a means of parsing in vitro active compounds into two categories, those that might possess desirable behaviors in animal models and those that might not. As this data set is small, we set out to buttress these relationships using our functional assay to identify additional examples of novel NTS2-selective antagonists and potent partial agonists. This article focuses attention on the latter.

The results obtained in our SAR study to find a potent partial agonist based on compound **5a** were centered on the three pyrazole scaffolds A–C, Table 2. We began by examining the effect of deleting the carboxylic acid group, compound **13**, as the carboxyl group is known to be a primary attachment point for ligands of the NT receptors.[57–59] We found that the descarboxy derivative **13** was inactive in both NTS1 and NTS2 FLIPR assays and gave a $K_i > 11 \mu M$ in the radioligand binding assay. This behavior stands in stark contrast to the activity found for **5a** and provided additional evidence that the calcium release observed for **5a** (and **5b**) results from these ligands interacting with NTS2.

We chose to use compound **14b** as a surrogate for compound **5a** because it was easier to produce compound libraries using the L-cyclohexyl glycine amino acid compared with the adamantyl amino acid. Compound **14b**, like **5a**, was previously reported to be a potent NTS1 antagonist by Quéré[60] and thus was expected to be an agonist in the NTS2. The L-isomer was chosen as it is known to be more active than the R isomer.[61] The data from **14b** indicated that it would be a suitable stand in for **5a** as it provided comparable agonist potency and efficacy for NTS2 (EC_{50} of 217 nM and E_{max} 86% of **5b**) and NTS1 antagonist activity (K_e of 23 nM). The 10-fold difference in its relative binding affinity at NTS2 illustrated to us that the structure of the amino acid side chain significantly impacts binding affinity.

A comparison of the data obtained for compounds **14b−18b** (Table 2) illustrates the SAR realized from changing the position of the 6-methoxy group (**14b**, **15b**, and **16b**), elimination of the 6-methoxy group (**17b**), and replacing the 2,6-methoxy groups with fluorine atoms (**18b**). We found that the NTS1 antagonist activity decreased across the series of positional isomers **14b−16b** with K_e values of 23 nM, 1275 nM, and >10 μM, respectively. This trend continued for **17b** and the difluoro derivative (**18b**), with K_e values of 1682 nM and >10 μM. Their NTS2 efficacy (E_{max}) also fell across this series of compounds while the functional potency (EC_{50}) was little varied. The binding affinities showed surprisingly little variation compared with the change seen going between **5a** and **14b** and reinforced the notion that the structure of the amino acid side chain has a strong impact on binding affinity and appeared to set the range of observed activity within the study group.

Taken together, we found that the potency of antagonist activity for **14b** at the NTS1 receptor relied heavily upon the 2,6-dimethoxyphenyl ring for its activity. At NTS2, however, it was the efficacy of calcium release that was most significantly affected by alteration of the 2,6-dimethoxyphenyl ring. The binding affinity data and NTS2 potency were much less affected by changes to this molecular region. This in turn suggested that an NTS2 potent partial agonist would not possess the 2,6-dimethoxyphenyl ring.

The data from compound **19b** illustrated the effect on in vitro activity produced by alkylation of the 4-position of the pyrazole ring. The changes seen here resembled the previous set of compounds as **19b** showed lower NTS1 antagonist relative to **14b** and decreased efficacy at NTS2 with less effect on NTS2 potency and binding affinity. Overall, this example illustrated that alkylation of this position provided compounds that favored NTS2 activity over NTS1 but with only modest levels of affinity at NTS2. Collectively, the data suggested that this was not a substitution that would lead in the right direction.

The amino acid side chain is known to have a strong influence on ligand behavior, presumably due to its proximity to the carboxyl group, the primary anchoring point of the ligand to the receptor. The data obtained for compounds **5a**, **14b**, and **20b−23b** in Table 2 illustrate the SAR of the large amino acid side chains. This group of compounds demonstrated that large cyclic (cycloheptyl, **20b**), large bicyclic (adamantyl, **5a**), and large pendant alicyclic (**14b**) structures are potent NTS1 antagonists with K_e values of 42, 4.7, and 23 nM, respectively. Compounds with smaller cyclic rings were somewhat less potent at NTS1, K_e = 222 nM for the five-membered cyclic ring (**22b**) and 157 nM for the six-membered cyclic ring (**21b**). According to these data then, the compounds with smaller cyclic rings (**21b**, **22b**) trended toward NTS2 selectivity.

Compounds **20b–22b** also showed increased NTS2 agonist potency (lower EC_{50}), but this was accompanied by full efficacy (high E_{max} relative to **5b**) at NTS2, exactly opposite of that found in the potent partial agonist levocabastine (**6**).

In their favor, however, these same compounds (**20b–22b**) showed improved binding affinity at NTS2 versus **14b** (K_i = 170, 151, and 102 nM versus 644 nM for **14b**), more in line with **5a** (K_i = 62 nM). For these compounds, the binding affinity and NTS2 potency improvement worked in concert with the lower NTS1 activity to provide enhanced NTS2 affinity and potency while maintaining low efficacy and lower NTS1 activity. This comparison of NTS2 agonist potency to the NTS1 antagonist potency for compounds **21b** and **23b** was an important clue to achieving selectivity in this series of compounds as this data reinforced the data from the previous series, which showed that the NTS1 receptor relied much more heavily upon the 2,6-dimethoxyphenyl ring for its activity compared with NTS2 and that the amino acid side chain could work in concert with the methoxyphenyl ring to retain these desired properties. This trend was further advanced in compounds that did not possess the chloroquinoline group.

Two key substitutions for the 7-chloroquinoline group (**A**) were the naphthyl (**B**) and 4-fluorophenyl (**C**) groups, Table 2. These substitutions were included in our compound libraries because the naphthyl compounds were known to yield NTS1 antagonists[45] and the 4-fluorophenyl group was found in levocabastine (**6**). Like their 7-chloroquinolyl counterparts, the naphthyl-substituted 2,6-dimethoxyphenyl derivatives **24b** and **26b** possess potent NTS1 antagonist activity and also NTS2 agonist activity; however, distinct differences were observed that led us in the right direction.

Most apparent was that these compounds were inherently less potent at NTS1 and less efficacious at NTS2. The K_e values for NTS1 antagonist activity and the NTS2 E_{max} values were roughly half of that found for similarly substituted chloroquinoline-based compounds. This was observed for both the L-cyclohexyl glycine (**24b**) and the 1-aminocyclohexancarboxylic acid side chains (**26b**). Comparing the naphthyl (**24b**) and the 7-chloroquinolyl (**14b**) compounds, we found K_e values of 58 versus 23 nM and NTS2 E_{max} values of 45 and 86% of **5b**, respectively. Comparing compounds **26b** and **21b**, we found K_e values of 230 versus 157 nM and NTS2 E_{max} values of 35 and 78%, respectively.

As seen in the 7-chloroquinolyl series (**A**), the naphthyl-substituted 2-methoxyphenyl derivatives **25b** and **27b** were less potent NTS1 antagonists than their 2,6-dimethoxyphenyl substituted counterparts. More surprising was the observation that the NTS2 efficacies for these compounds were nearly half again as low as that found in similarly substituted 7-chloroquinolyl compounds **17b** and **23b**. This is readily revealed in a comparison of the NTS2 efficacy data obtained for compound **21b**, **23b**, and the 2-methoxy naphthyl substituted compound **27b**, with NTS2 E_{max} values of 78, 35, and 18%, respectively.

The naphthyl-substituted compounds with the 2,6-dimethoxyphenyl ring were more potent at NTS2 than their chloroquinoline-based counterparts (**14b**, **17b**, **21b**, and **23b**), but naphthyl compounds bearing the 2-methoxyphenyl ring were equally potent. This was observed for compounds with the L-cyclohexyl glycine as well as those with 1-aminocyclohexancarboxylic acid as in **26b** and **27b**.

The NTS2 binding data observed for the naphthyl-substituted compounds **24b–27b** was found to be in line with that seen for the similarly substituted 7-chloroquinolyl-substituted compounds, with the 2-methoxy derivatives showing lower affinity (higher K_i values) than the 2,6-dimethoxy counterparts. As before though, the compounds bearing the L-cyclohexyl glycine side chain (**24b,25b**) were less potent than those with the 1-aminocyclohexancarboxylic side chain (**26b,27b**).

Toward obtaining an NTS2 selective potent partial agonist, the calcium data profile for compound **26b** looked promising as it moved closer to that of levocabastine (**6**). Compound **26b** showed 2-fold greater NTS2 potency and comparable efficacy. The binding data was also similar, but in this case, compound **6** was more potent, showing a K_i 3.5-fold greater than that for **26b**. The similarity between these two compounds ended, however, when comparing receptor selectivity, as compound **26b** showed substantial NTS1 antagonist activity while levocabastine (**6**) displayed no activity at NTS1. The 2-methoxy naphthyl-substituted analogue **27b** was not able to compensate for this deficit, for while its NTS1 antagonist activity was lowered by 20-fold, the NTS2 potency and binding affinity also suffered substantial losses.

Fortunately, the pyrazole scaffold (**C**) bearing the 4-fluorophenyl-substituent seen in compounds **30**, **28b**, **29b**, and **7b** overcame the deficiencies found in scaffold **B** to provide selective potent partial agonists. Comparison of the adamantyl-substituted compounds **5a** and **30** highlights the contribution of the 4-fluorophenyl ring. These data show that the NTS1 antagonist activity was diminished by 40-fold for **30** versus **5a**, with K_e values of 191 and 4.7 nM, respectively. The NTS2 potency, on the other hand, was doubled (EC_{50} values of 120 versus 68 nM) while the efficacy was less than half relative to **5a** (E_{max} values of 100 versus 34% of **5b**). This phenomenon was also observed in the data for compounds **28b**, **29b**, and **7b**. These compounds are all far less active at NTS1 compared with their corresponding 7-chloroquinolyl (**14b**, **21b**, and **23b**) or naphthyl (**24b**, **26b**, and **27b**) analogues and thereby attain enhanced NTS2 selectivity with respect to calcium release. The 4-fluorophenyl-substituted compounds (**29b** and **7b**), bearing the 1-aminocyclohexancarboxylic acid substituent, showed the most levocabastine-like profiles whether they had a 2,6-dimethoxyphenyl ring (**29b**) or a 2-methoxyphenyl ring (**7b**). In keeping with previous observations, the transition to the 2-methoxyphenyl ring (**7b**) from the 2,6-dimethoxyphenyl ring (**29b**) led to a nearly 50% reduction in NTS2 efficacy, with NTS2 E_{max} values of 15 and 8% of **5b**, respectively.

The NTS2 binding affinity observed for **29b** and **7b** was consistent with similarly substituted 7-chloroquinolyl (**21b**, **23b**) or naphthyl-substituted (**26b**, **27b**) analogues bearing the 1-aminocyclohexanecarboxylic acid, with one important exception; for **7b**, the binding affinity did not decrease in going from the 2,6-dimethoxyphenyl (**29b**) to the 2-methoxyphenyl ring substitution (**7b**) as seen in the K_i values of 140 and 153 nM, respectively. We imagine that this change in SAR for **29b** and **7b** could result from a change in binding mode at NTS2 that is permitted for 4-fluorophenyl but not 7-chloroquinolyl or naphthyl-substituted analogues. In the end, the 4-fluorophenyl-substituted pyrazole scaffold **C** was able to overcome the deficits found in scaffolds **A** and **B** to provide pyrazole-based compounds (**29b** and **7b**) with enhanced NTS2 potency and binding affinity with significantly lower efficacy compared to **5a** at the NTS2 receptor.

From the standpoint of calcium mobilization at NTS1 and NTS2, compounds (**29b** and **7b**) appear to be selective for the

NTS2 receptor. However, this was not a fair comparison, as one assay measures calcium mobilization (NTS2) while the other blockade of calcium mobilization (NTS1). We therefore acquired the radioligand binding data for both **29b** and **7b** at NTS1 in order to compare like measurements. As seen in Table 3, comparison of the relative binding affinities at NTS1

Table 3. Selectivity Ratios for 29b and 7b at the NTS1 and NTS2 Receptors Determined Using [^{125}I]NT Radioligand Binding

compd	NTS1 K_i nM ± SEM	NTS2 K_i nM ± SEM	NTS2/NTS1
29b	3210 ± 879	140 ± 29	23
7b	>25 μM	153 ± 10	161

and NTS2 showed that compound **7b** is 161-fold selective for NTS2 versus NTS1 while the dimethoxy analogue **29b** shows only a 23-fold preference for NTS2 over NTS1. These data point out that while the calcium assay described herein is useful for identifying compounds that are active at NTS2, conclusions about selectivity require the use of the binding assay.

The identification of **7b** as an NTS2 selective compound demonstrated that this calcium assay was useful for driving SAR studies in the pyrazole carboxamide series of compounds. However, the low efficacy of **7b**, less than half of that found for levocabastine, prompted us to revisit mobilization of calcium in the parent CHO cell line as a negative control. We thus carried out a final study and examined all of the compounds reported herein that showed NTS2 E_{max} values less than 15% of **5b**. This was done collectively by comparing all of the low efficacy compounds together in the same assay, under identical conditions, in our CHO-k1-NTS2 cells and simultaneously in the parent CHO line. The results of these experiments confirmed that the calcium mobilization observed was only found in the CHO cells stably expressing the NTS2 receptor.

■ CONCLUSIONS

In summary, we tested the compounds common to neurotensin research and have identified their associated calcium mobilization patterns at NTS1 and 2 using a FLIPR tetra using NT as our agonist standard for NTS1 and **5b** as our agonist standard for NTS2. We found that compounds known to possess analgesic and antipsychotic activity in vivo appear as antagonists (NT and **1**)) or potent partial agonists (levocabastine (**6**)) in this NTS2 in vitro assay. Using the assays described above in combination with binding data, we identified structural changes to compound **5a** that led to the discovery of the NTS2 selective compound **7b**, a pyrazole-based compound with in vitro properties similar to levocabastine in this assay, a compound known to be active against chronic pain. We are now working to identify the relevance of the functional data produced using this method by investigating compound **7b** in recognized models of neuropathic pain in an attempt to establish an in vitro/in vivo correlation.

■ EXPERIMENTAL SECTION

Reactions were conducted under a nitrogen atmosphere using oven-dried glassware as required. All solvents and chemicals used were reagent grade. Anhydrous tetrahydrofuran (THF), dichloromethane (DCM), and *N,N*-dimethylformamide (DMF) were purchased from VWR and used without further purification. (7-Chloroquinolin-4-yl)-hydrazine·HCl (**9a**) was purchased from Aldrich Chemical Corp. 1-Naphthylhydrazine·HCl (**9b**) was purchased from TCI America. 4-Fluorophenylhydrazine·HCl (**9c**) was purchased from Alfa Aesar.

Most other reagents were obtained from either Aldrich Chemical Corp. or Fischer Scientific and used without further purification. Flash column chromatography was carried out using a Teledyne ISCO Combiflash Rf system and Redisep Rf gold prepacked HP silica columns. Evaporation of solvents was accomplished using rotary evaporators. The purity of target compounds was determined to be ≥95% by combustion analysis. Characterization of compounds was accomplished by a combination of TLC, combustion analysis, mass spectrometry (MS) as well as ^{1}H and ^{13}C NMR. NMR spectra were recorded on a Bruker Avance DPX-300 (300 MHz). Chemical shifts are reported in ppm relative to the tetramethylsilane, and coupling constant (*J*) values are reported in hertz (Hz). Low-resolution mass spectra were obtained using a Waters Alliance HT/Micromass ZQ system (ESI) in both positive and negative mode. Optical rotations were measured on an Auto Pol III polarimeter at the sodium D line. Thin layer chromatography (TLC) was performed on EMD precoated silica gel 60 F$_{254}$ plates, and spots were visualized with UV light and I$_2$ or phosphomolybdic acid stain. CHO-k1 cells were from the American Type Culture Collection, ^{125}I−neurotensin was from PerkinElmer, Calcium 5 dye was from Molecular Devices, and cell culture reagents were from Life Technologies. The graphs provided in Figures 1−3 were obtained using Graphpad Prism Software.

General Methods. *General Method for Preparation of Pyrazole Carboxylic Acid Esters (10a−j).* A magnetically stirred solution of a 4-aryl-2,4-diketoester sodium salt (**8a−f**, 5 mmol) and arylhydrazine hydrochloride (**9a−c**, 5 mmol) in glacial acetic acid (35 mL) and concd HCl (1.5 mL) was heated under reflux for 5 h and then cooled to rt. This was then poured into 300 mL of water and extracted five times with CH$_2$Cl$_2$. The extracts were washed carefully with satd NaHCO$_3$ and then water and brine then dried over Na$_2$SO$_4$ and concentrated to give a crude material. This material was purified using flash chromatograph with a gradient of 0−100% EtOAc in hexanes over 30 min collecting peaks only. Flow rates are automatically set based on column size chosen for separation. Combination of desired fractions and evaporation provided methyl esters **10a−j** as foamy solids.

General Method for Preparation of Pyrazole Carboxylic Acids (11a−j). Pyrazole carboxylic acids **11a−j** were prepared from the esters (**10a−j**) using the methyl ester hydrolysis method described below.

General Amide Coupling. To a magnetically stirred suspension of the appropriate pyrazole carboxylic acid (**11a−j**, 0.122 mmol) in anhydrous CH$_2$Cl$_2$ (20 mL) were added successively triethylamine (0.366 mmol), HBTU (0.146 mmol), and the appropriate amino acid ester hydrochloride salt (**12a−e**, 0.122 mmol). After stirring for 16 h, the resulting mixture was concentrated and the residue purified by flash chromatography using a gradient of 0−100% EtOAc in hexanes over 30 min collecting peaks only. Flow rates are automatically set based on column size chosen for separation. This gave an intermediate ester that was taken directly to the hydrolysis step.

General Methyl Ester Hydrolysis. To a magnetically stirred solution of methyl ester (**7a,20−23a, 26a, 27a,** and **29a**) from the coupling reaction (0.1 mmol) in 1,4-dioxane (5 mL) was added 1N LiOH (2 mL), followed by stirring at room temperature overnight. The mixture was then concentrated, and the residue was taken up in water (2 mL) and extracted with ethyl acetate (15 mL). After this, 2 N HCl was added to the aqueous layer to precipitate the carboxylic acid final product. This was extracted twice with CH$_2$Cl$_2$, and the combined extracts were dried over Na$_2$SO$_4$ and concentrated to give solid final products.

General tert-Butyl Ester Hydrolysis. To a magnetically stirred solution of the *tert*-butyl ester intermediate (**14−19a,24a,25a,** and **28a**) obtained from the coupling reaction (0.08 mmol) in CH$_2$Cl$_2$ (10 mL) was added excess TFA (10 mL) at room temp. After stirring for 16 h, the mixture was concentrated and then triturated with ethyl ether to give a solid product that was isolated by vacuum filtration, washed with ether, and then dried under high vacuum overnight.

Methyl 1-(1-(4-Fluorophenyl)-5-(2-methoxyphenyl)-1H-pyrazole-3-carboxamido)cyclohexanecarboxylate (7a). Following the general amide coupling procedure, **7a** was prepared from **11j** and **12b** as an amorphous off-white solid (55% yield). ^{1}H NMR (CDCl$_3$) δ 7.36 (t, *J* = 7.8 Hz, 1H) 7.23−7.31 (m, 3H), 7.18 (s, 1H), 6.94−7.04 (m, 3H),

6.80 (d, J = 8.5 Hz, 1H), 3.75 (s, 3H), 3.48 (s, 3H), 2.15−2.25 (m, 2H), 1.88−2.02 (m, 2H), 1.46−1.56 (m, 6H).

1-({[1-(4-Fluorophenyl)-5-(2-methoxyphenyl)-1H-pyrazol-3-yl]-carbonyl}amino)cyclohexanecarboxylic Acid (7b). Following the general methyl ester hydrolysis procedure, **7b** was obtained from **7a** as a white solid (85%). ^{1}H NMR (300 MHz, CDCl$_3$) δ 7.33−7.42 (m, 1H), 7.18−7.31 (m, 4H), 6.94−7.05 (m, 4H), 6.81 (d, J = 8.2 Hz, 1H), 3.44 (s, 3H), 2.28 (d, J = 13.9 Hz, 2H), 1.94−2.08 (m, 2H), 1.33−1.83 (m, 6H). ^{19}F NMR (282 MHz, CDCl$_3$) δ −113.42. ^{13}C NMR (CDCl$_3$) δ 175.00, 163.76, 163.45, 160.16, 156.36, 145.66, 142.48, 136.54, 136.50, 131.24, 131.16, 125.81, 125.70, 120.87, 118.66, 115.71, 115.41, 111.24, 109.45, 60.25, 54.94, 32.20, 25.13, 21.30. MS (ESI) m/z: 436.7 (M − H)$^-$. Anal. (C$_{24}$H$_{24}$FN$_3$O$_4$) C, H, N.

Methyl 1-(7-Chloroquinolin-4-yl)-5-(2,6-dimethoxyphenyl)-1H-pyrazole-3-carboxylate (10a).[45] Ester **10a** was prepared via the general procedure starting from methyl 4-(2,6-dimethoxyphenyl)-2,4-dioxobutanoate sodium salt (**8a**) and 7-chloro-4-hydrazinylquinoline hydrochloride (**9a**) to give **10a** as a yellow foam (68%). ^{1}H NMR (CDCl$_3$) δ 8.98 (d, J = 5.2 Hz, 1H), 8.88 (d, J = 1.8 Hz, 1H), 8.37 (d, J = 9.2 Hz, 1H), 7.82 (dd, J = 1.8, 9.2 Hz, 1H), 7.45 (d, J = 5.2 Hz, 1H), 7.34 (t, J = 8.4 Hz, 1H), 7.15−7.24 (m, 1H), 6.50 (d, J = 8.4 Hz, 2H), 4.01 (s, 3H), 3.48 (s, 6H).

Methyl 1-(7-Chloroquinolin-4-yl)-5-(2,6-dimethoxyphenyl)-4-ethyl-1H-pyrazole-3-carboxylate (10b). Ester **10b** was prepared from methyl 3-[(2,6-dimethoxyphenyl)carbonyl]-2-oxopentanoate, sodium salt (**8b**), and **9a** according to the general method (pale-yellow solid, 45%). ^{1}H NMR (CDCl$_3$) δ 8.87 (d, J = 4.8 Hz, 1H), 8.18 (d, J = 1.9 Hz, 1H), 7.95 (d, J = 9.1 Hz, 1H), 7.50 (d, J = 2.0 Hz, 1H), 7.12−7.27 (m, 2H), 6.42 (d, J = 8.5 Hz, 2H), 3.76 (s, 3H), 3.50 (s, 6H), 2.69 (q, J = 7.4 Hz, 2H), 1.17 (t, J = 7.4 Hz, 3H).

Methyl 1-(7-Chloroquinolin-4-yl)-5-(2,5-dimethoxyphenyl)-1H-pyrazole-3-carboxylate (10c). Ester **10c** was prepared from methyl 4-(2,5-dimethoxyphenyl)-2,4-dioxobutanoate sodium salt (**8c**) and **9a** according to the general method (pale-yellow solid, 57%). ^{1}H NMR (CDCl$_3$) δ 8.77 (d, J = 4.5 Hz, 1H), 8.14 (d, J = 2.1 Hz, 1H), 7.91 (d, J = 9.0 Hz, 1H), 7.54 (dd, J = 2.1, 8.95 Hz, 1H), 7.14 (s, 1H), 7.01 (d, J = 4.5 Hz, 1H), 6.78−6.91 (m, 2H), 6.57 (d, J = 8.9 Hz, 1H), 3.99 (s, 3H), 3.73 (s, 3H), 2.91 (s, 3H).

Methyl 1-(7-Chloroquinolin-4-yl)-5-(2,4-dimethoxyphenyl)-1H-pyrazole-3-carboxylate (10d). Ester **10d** was prepared from methyl 4-(2,4-dimethoxyphenyl)-2,4-dioxobutanoate sodium salt (**8d**) and 7-chloro-4-hydrazinylquinoline hydrochloride (**9a**) according to the general method (yellow amorphous solid, 45%). ^{1}H NMR (CDCl$_3$) δ 8.78 (d, J = 4.6 Hz, 1H), 8.14 (d, J = 1.8 Hz, 1H), 7.87 (d, J = 9.0 Hz, 1H), 7.52 (dd, J = 2.0, 9.0 Hz, 1H), 7.20 (d, J = 8.4 Hz, 1H), 7.09 (s, 1H), 7.02 (d, J = 4.6 Hz, 1H), 6.47 (dd, J = 2.2, 8.43 Hz, 1H), 6.19 (d, J = 2.1 Hz, 1H), 3.98 (s, 3H), 3.77 (s, 3H), 2.99 (s, 3H).

Methyl 1-(7-Chloroquinolin-4-yl)-5-(2,6-difluorophenyl)-1H-pyrazole-3-carboxylate (10e). Ester **10e** was prepared from methyl 4-(2,6-difluorophenyl)-2,4-dioxobutanoate sodium salt (**8e**) and **9a** according to the general method (yellow amorphous solid, 52%). ^{1}H NMR (CDCl$_3$) δ 8.87 (d, J = 4.5 Hz, 1H), 8.14 (d, J = 1.9 Hz, 1H), 7.61−7.75 (m, 1H), 7.51 (dd, J = 1.9, 9.0 Hz, 1H), 7.41 (dd, J = 0.9, 8.7 Hz, 1H), 7.25−7.35 (m, 1H), 7.22 (d, J = 4.5 Hz, 1H), 6.77−6.89 (m, 2H), 4.01 (s, 3H).

Methyl 1-(7-Chloroquinolin-4-yl)-5-(2-methoxyphenyl)-1H-pyrazole-3-carboxylate (10f). Ester **10f** was prepared from methyl 4-(2-methoxyphenyl)-2,4-dioxo-butanoate sodium salt (**8f**) and **9a** according to the general method (yellow solid, 43%). ^{1}H NMR (CDCl$_3$) δ 8.78 (d, J = 4.7 Hz, 1H), 8.15 (s, 1H), 7.91 (d, J = 9.0 Hz, 1H), 7.50 (td, J = 1.0, 9.0 Hz, 1H), 7.27−7.32 (m, 2H), 6.96−7.01 (m, 2H), 6.65 (d, J = 8.5 Hz, 2H), 4.0 (s, 3H), 4.0 (s, 3H).

Methyl 5-(2,6-Dimethoxyphenyl)-1-naphthalen-1-yl-1H-pyrazole-3-carboxylate (10g).[62] Ester **10g** was prepared from **8a** and 1-naphthylhydrazine hydrochloride (**9b**) according to the general method (colorless foam, 55%). ^{1}H NMR (CDCl$_3$) δ 7.67−7.83 (m, 3H), 7.38−7.49 (m, 2H), 7.22−7.33 (m, 2H), 7.05−7.17 (m, 2H), 6.34 (d, J = 8.3 Hz, 2H), 3.96 (s, 3H), 3.41 (br s, 6H).

Methyl 5-(2-Methoxyphenyl)-1-naphthalen-1-yl-1H-pyrazole-3-carboxylate (10h).[62] Ester **10h** was prepared from methyl 4-(2-methoxyphenyl)-2,4-dioxobutanoate sodium salt (**8f**) and **9b** according

to the general method (off-white solid, 40%). ^{1}H NMR (CDCl$_3$) δ 7.78−7.88 (m, 2H), 7.62−7.71 (m, 1H), 7.44−7.53 (m, 2H), 7.29−7.36 (m, 1H), 7.24−7.29 (m, 1H), 7.10−7.24 (m, 3H), 6.81 (dt, J = 0.8, 7.5 Hz, 1H), 6.64 (d, J = 8.2 Hz, 1H), 3.97 (s, 3H), 3.13 (s, 3H).

Methyl 5-(2,6-Dimethoxyphenyl)-1-(4-fluorophenyl)-1H-pyrazole-3-carboxylate (10i). Ester **10i** was prepared from **8a** and 4-fluorophenyl hydrazine hydrochloride (**9c**) according to the general method (white amorphous powder, 55%). ^{1}H NMR (CDCl$_3$) δ 7.22−7.33 (m, 3H), 6.89−7.00 (m, 3H), 6.51 (d, J = 8.3 Hz, 2H), 3.96 (s, 3H), 3.59 (s, 3H).

Methyl 1-(4-Fluorophenyl)-5-(2-methoxyphenyl)-1H-pyrazole-3-carboxylate (10j). Ester **10j** was prepared from **8f** and **9c** according to the general method (off-white powder, 45%). ^{1}H NMR (CDCl$_3$) δ 7.32−7.43 (m, 1H), 7.23−7.32 (m, 3H), 6.92−7.04 (m, 4H), 6.81 (d, J = 8.5 Hz, 1H), 3.97 (s, 3H), 3.44 (s, 3H).

1-(7-Chloroquinolin-4-yl)-5-(2,6-dimethoxyphenyl)-1H-pyrazole-3-carboxylic Acid (11a).[45] Pyrazole acid **11a** was prepared from ester **10a** according to the general methyl ester hydrolysis method (pale-yellow powder, 78%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.90 (d, J = 4.7 Hz, 1H), 8.17 (s, 1H), 7.73 (s, 2H), 7.26 (t, J = 8.4 Hz, 1H), 7.20 (d, J = 4.5 Hz, 1H), 6.99 (s, 1H), 6.54 (d, J = 8.5 Hz, 2H), 3.39 (s, 6H).

1-(7-Chloroquinolin-4-yl)-5-(2,6-dimethoxyphenyl)-4-ethyl-1H-pyrazole-3-carboxylic Acid (11b). Pyrazole acid **11b** was prepared from ester **10b** according to the general methyl ester hydrolysis method (pale-yellow solid, 80%). ^{1}H NMR (CDCl$_3$) δ 10.36−11.51 (m, 2H), 8.87 (d, J = 4.8 Hz, 1H), 8.19 (d, J = 2.0 Hz, 1H), 7.94 (d, J = 9.0 Hz, 1H), 7.49 (dd, J = 2.0, 9.0 Hz, 1H), 7.19−7.32 (m, 1H), 7.17 (d, J = 4.7 Hz, 1H), 6.42 (d, J = 8.4 Hz, 2H), 3.50 (s, 6H), 2.68 (q, J = 7.4 Hz, 2H), 1.16 (t, J = 7.4 Hz, 3H). MS (ESI) m/z: 436.5 (M − H)$^-$.

1-(7-Chloroquinolin-4-yl)-5-(2,5-dimethoxyphenyl)-1H-pyrazole-3-carboxylic Acid (11c). Pyrazole acid **11c** was prepared from ester **10c** according to the general methyl ester hydrolysis method (amorphous, pale-yellow solid, 94%). ^{1}H NMR (CDCl$_3$) δ 8.80 (d, J = 4.7 Hz, 1H), 8.18 (d, J = 1.9 Hz, 1H), 7.91 (d, J = 9.2 Hz, 1H), 7.56 (dd, J = 1.9, 9.0 Hz, 1H), 7.19 (s, 1H), 7.02 (d, J = 4.7 Hz, 1H), 6.91−6.82 (m, 2H), 6.57 (d, J = 9.0 Hz, 1H), 3.75 (s, 3H), 2.92 (m, 3H).

1-(7-Chloroquinolin-4-yl)-5-(2,4-dimethoxyphenyl)-1H-pyrazole-3-carboxylic Acid (11d). Pyrazole acid **11d** was prepared from ester **10d** according to the general methyl ester hydrolysis method (pale-yellow solid, 78%). ^{1}H NMR (DMSO-d_6) δ 8.91 (d, J = 4.7 Hz, 1H), 8.22 (d, J = 1.7 Hz, 1H), 7.78 (s, 1H), 7.76 (d, J = 2.0 Hz, 1H), 7.32 (d, J = 8.4 Hz, 1H), 7.24 (d, J = 4.7 Hz, 1H), 7.04 (s, 1H), 6.56 (dd, J = 2.2, 8.4 Hz, 1H), 6.34 (d, J = 2.2 Hz, 1H), 3.72 (s, 3H), 2.92 (s, 3H).

1-(7-Chloroquinolin-4-yl)-5-(2,6-difluorophenyl)-1H-pyrazole-3-carboxylic Acid (11e). Pyrazole acid **11e** was prepared from ester **10e** according to the general methyl ester hydrolysis method (pale-yellow solid, 94%). ^{1}H NMR (CD$_3$OD) δ 8.90 (d, J = 4.7 Hz, 1H), 8.10 (s, 1H), 7.80 (d, J = 9.0 Hz, 1H), 7.62 (d, J = 9.0 Hz, 1H), 7.23−7.45 (m, 2H), 7.04 (s, 1H), 6.54 (d, J = 8.5 Hz, 2H).

1-(7-Chloroquinolin-4-yl)-5-(2-methoxyphenyl)-1H-pyrazole-3-carboxylic Acid (11f).[63] Pyrazole acid **11f** was prepared from ester **10f** according to the general methyl ester hydrolysis method (pale-yellow solid, 92%). ^{1}H NMR (DMSO-d_6) δ 8.80 (d, J = 4.7 Hz, 1H), 8.15 (d, J = 2.1 Hz, 1H), 7.98 (d, J = 9.0 Hz, 1H), 7.69 (dd, J = 2.1, 9.0 Hz, 1H), 7.25−7.39 (m, 2H), 7.04 (d, J = 4.7 Hz, 1H), 6.97 (t, J = 7.4 Hz, 1H), 6.79 (d, J = 8.1 Hz, 1H), 6.70 (s, 1H), 2.91 (s, 3H).

5-(2,6-Dimethoxyphenyl)-1-naphthalen-1-yl-1H-pyrazole-3-carboxylic Acid (11g).[62] Pyrazole acid **11g** was prepared from ester **10g** according to the general methyl ester hydrolysis method (off-white solid, 70%). ^{1}H NMR (CDCl$_3$) δ 7.78−7.86 (m, 2H), 7.72 (dd, J = 3.5, 6.3 Hz, 1H), 7.44−7.52 (m, 2H), 7.22−7.35 (m, 2H), 7.10−7.19 (m, 2H), 6.35 (d, J = 8.3 Hz, 2H), 3.41 (s, 6H).

5-(2-Methoxyphenyl)-1-naphthalen-1-yl-1H-pyrazole-3-carboxylic Acid (11h).[62] Pyrazole acid **11h** was prepared from ester **11h** according to the general methyl ester hydrolysis method (off-white powder, 92%). ^{1}H NMR (CDCl$_3$) δ 7.81−7.91 (m, 2H), 7.68 (dd, J = 3.4, 6.2 Hz, 1H), 7.46−7.56 (m, 2H), 7.28−7.37 (m, 1H), 7.14−7.24 (m, 3H), 6.83 (t, J = 7.4 Hz, 1H), 6.65 (d, J = 8.3 Hz, 1H), 3.12 (s, 3H). MS (ESI) m/z: 343.2 (M − H)$^-$.

5-(2,6-Dimethoxyphenyl)-1-(4-fluorophenyl)-1H-pyrazole-3-carboxylic Acid (11i). Pyrazole acid **11i** was prepared from ester **10i** according to the general methyl ester hydrolysis method (off-white powder, 93%). ^{1}H NMR (CDCl$_3$) δ 7.23–7.36 (m, 3H), 6.91–7.03 (m, 3H), 6.52 (d, J = 8.5 Hz, 2H), 3.60 (s, 6H).

1-(4-Fluorophenyl)-5-(2-methoxyphenyl)-1H-pyrazole-3-carboxylic Acid (11j). Pyrazole acid **11j** was prepared from ester **10j** according to the general methyl ester hydrolysis method (off-white powder, 91%). ^{1}H NMR (CDCl$_3$) δ 7.34–7.44 (m, 1H), 7.22–7.33 (m, 3H), 6.95–7.07 (m, 4H), 6.82 (d, J = 8.3 Hz, 1H), 3.44 (s, 3H).

1-(7-Chloroquinolin-4-yl)-5-(2,6-dimethoxyphenyl)-N-tricyclo-[3.3.1.13,7]dec-2-yl-1H-pyrazole-3-carboxamide (13). Compound **13** was prepared from **11a** and 2-aminoadamantane hydrochloride (**12e**) following the general amide coupling method (off-white powder, 65%). ^{1}H NMR δ (CDCl$_3$) 8.77 (d, J = 4.7 Hz, 1H), 8.16 (d, J = 1.9 Hz, 1H), 7.96 (d, J = 9.0 Hz, 1H), 7.55 (dd, J = 1.9, 9.0 Hz, 1H), 7.24–7.37 (m, 2H), 7.13 (s, 1H), 6.92–7.02 (m, 2H), 6.64 (d, J = 8.3 Hz, 1H), 4.28 (d, J = 8.1 Hz, 1H), 2.98 (s, 6H), 2.02–2.11 (m, 2H), 1.80–1.95 (m, 9H), 1.70–1.79 (m, 2H), 1.59–1.70 (m, 2H). MS (ESI) m/z: 544.4 (M+H)$^+$. Anal. (C$_{31}$H$_{31}$ClN$_4$O$_3$) C, H, N.

tert-Butyl (2S)-({[1-(7-Chloroquinolin-4-yl)-5-(2,6-dimethoxyphenyl)-1H-pyrazol-3-yl]carbonyl}amino)(cyclohexyl)ethanoate (14a). Following the general amide coupling procedures, **14a** was obtained from **11a** and tert-butyl (2S)-amino(cyclohexyl)ethanoate hydrochloride (**12d**). The material was purified by flash chromatography (0–100% EtOAc/hexanes) to afford **14a** (84%) as a pale-yellow film. ^{1}H NMR (CDCl$_3$) δ 8.78 (d, J = 4.7 Hz, 1H), 8.12 (d, J = 2.0 Hz, 1H), 7.97 (d, J = 9.0 Hz, 1H), 7.50 (dd, J = 2.1, 9.0 Hz, 1H), 7.42 (d, J = 9.0 Hz, 1H), 7.21 (t, J = 8.4 Hz, 1H), 7.01–7.12 (m, 2H), 6.39 (d, J = 8.2 Hz, 2H), 4.66 (dd, J = 5.1, 9.1 Hz, 1H), 3.40 (br s, 6H), 1.54–1.99 (m, 6H), 1.44–1.53 (m, 9H), 1.01–1.32 (m, 5H).

(2S)-({[1-(7-Chloroquinolin-4-yl)-5-(2,6-dimethoxyphenyl)-1H-pyrazol-3-yl]carbonyl}amino)(cyclohexyl)ethanoic Acid (14b).[58] Following the general tert-butyl ester hydrolysis procedure, **14b** was obtained as a pale-yellow solid in 91% yield from **14a**. ^{1}H NMR (CDCl$_3$) δ 9.10 (br s, 1H), 8.74 (br s, 1H), 8.31 (d, J = 8.9 Hz, 1H), 7.78 (d, J = 8.9 Hz, 1H), 7.60–7.41 (m, 2H), 7.39–7.25 (m, 1H), 6.48 (d, J = 7.5 Hz, 2H), 4.80 (dd, J = 4.7, 7.9 Hz, 1H), 3.47 (br s, 6H), 2.10–1.92 (m, 1H), 1.90–1.60 (m, 5H), 1.32–1.09 (m, 5H). MS (ESI) m/z: 548.4 (M − H)$^-$. $[α]^{25}_D$ −18.1° (c 1.94, MeOH). Anal. (C$_{29}$H$_{29}$ClN$_4$O$_5$) C, H, N.

tert-Butyl (2S)-({[1-(7-Chloroquinolin-4-yl)-5-(2,5-dimethoxyphenyl)-1H-pyrazol-3-yl]carbonyl}amino)(cyclohexyl)ethanoate (15a). Following the general amide coupling procedure, **15a** was obtained from **11c** and **12d** as a pale-yellow film (70%). ^{1}H NMR (CDCl$_3$) δ 8.78 (d, J = 4.7 Hz, 1H), 8.16 (d, J = 1.9 Hz, 1H), 8.01 (d, J = 9.2 Hz, 1H), 7.55 (dd, J = 2.3, 9.0 Hz, 1H), 7.40 (d, J = 9.0 Hz, 1H), 7.14–7.11 (m, 1H), 7.00 (d, J = 4.7 Hz, 1H), 6.90 (d, J = 3.0 Hz, 1H), 6.83 (dd, J = 3.2, 9.0 Hz, 1H), 6.55 (d, J = 9.0 Hz, 1H), 4.69–4.62 (m, 1H), 3.72 (s, 3H), 2.89 (m, 3H), 2.05–1.60 (m, 6H), 1.50 (s, 9H), 1.39–1.11 (m, 5H).

(2S)-({[1-(7-Chloroquinolin-4-yl)-5-(2,5-dimethoxyphenyl)-1H-pyrazol-3-yl]carbonyl}amino)(cyclohexyl)ethanoic Acid (15b). Following the general tert-butyl ester hydrolysis procedure, **15b** was obtained (92%) as a white foam from **15a**. ^{1}H NMR (CDCl$_3$) δ 9.00 (d, J = 5.3 Hz, 1H), 8.39 (d, J = 1.8 Hz, 1H), 8.17 (d, J = 9.2 Hz, 1H), 7.7 (dd, J = 1.9, 9.0 Hz, 1H), 7.30 (d, J = 9.0 Hz, 1H), 7.27–7.16 (m, 2H), 6.90 (d, J = 3.0 Hz, 1H), 6.83 (dd, J = 3.2, 9.0 Hz, 1H), 6.58 (d, J = 9.0 Hz, 1H), 4.81–4.72 (m, 1H), 3.78 (s, 3H), 2.90 (m, 3H), 2.05–1.60 (m, 6H), 1.37–1.03 (m, 5H). MS (ESI) m/z: 547.8 (M − H)$^-$. $[α]^{25}_D$ −2.6° (c 0.45, MeOH). Anal. (C$_{29}$H$_{29}$ClN$_4$O$_5$) C, H, N.

tert-Butyl (2S)-({[1-(7-Chloroquinolin-4-yl)-5-(2,4-dimethoxyphenyl)-1H-pyrazol-3-yl]carbonyl}amino)(cyclohexyl)ethanoate (16a). Following the general amide coupling procedure, **16a** was obtained as a pale-yellow film (86%) from **11d** and **12d**. ^{1}H NMR (CDCl$_3$) δ 8.79 (d, J = 4.7 Hz, 1H), 8.16 (d, J = 2.0 Hz, 1H), 7.96 (d, J = 9.0 Hz, 1H), 7.54 (dd, J = 2.1, 9.0 Hz, 1H), 7.40 (d, J = 9.0 Hz, 1H), 7.21 (d, J = 8.4 Hz, 1H), 7.07 (s, 1H), 7.01 (d, J = 4.6 Hz, 1H), 6.47 (dd, J = 2.3, 8.4 Hz, 1H), 6.18 (d, J = 2.3 Hz, 1H), 4.66 (dd, J = 5.1, 9.0 Hz, 1H), 3.77 (s, 3H), 2.96 (s, 3H), 1.56–1.81 (m, 6H), 1.48 (d, J = 4.5 Hz, 9H), 1.00–1.34 (m, 5H).

(2S)-({[1-(7-Chloroquinolin-4-yl)-5-(2,4-dimethoxyphenyl)-1H-pyrazol-3-yl]carbonyl}amino)(cyclohexyl)ethanoic Acid (16b). Following the general tert-butyl ester hydrolysis procedure, **16b** was obtained as a yellow solid in 86% yield from **16a**. ^{1}H NMR (CDCl$_3$) δ 9.11 (br s, 1H), 8.48 (s, 1H), 8.27 (d, J = 9.1 Hz, 1H), 7.80 (d, J = 8.9 Hz, 1H), 7.46 (d, J = 8.4 Hz, 1H), 7.42–7.35 (m, 1H), 7.31 (d, J = 8.4 Hz, 1H), 7.12 (s, 1H), 6.59 (d, J = 8.4 Hz, 1H), 6.23 (d, J = 1.7 Hz, 1H), 4.73 (dd, J = 5.4, 8.4 Hz, 1H), 3.81 (s, 3H), 3.02 (s, 3H), 1.87–1.61 (m, 5H), 1.37–1.04 (m, 6H). MS (ESI) m/z: 548.5 (M − H)$^-$. $[α]^{25}_D$ +3.1° (c 0.45, MeOH). Anal. (C$_{29}$H$_{29}$ClN$_4$O$_5$) C, H, N.

tert-Butyl (2S)-({[1-(7-Chloroquinolin-4-yl)-5-(2-methoxyphenyl)-1H-pyrazol-3-yl]carbonyl}amino)(cyclohexyl)ethanoate (17a). Following the general amide coupling procedure, **17a** was obtained from **11f** and **12d** in 94% yield as a pale-yellow solid. ^{1}H NMR (CDCl$_3$) δ 8.77 (d, J = 4.7 Hz, 1H), 8.16 (d, J = 1.9 Hz, 1H), 7.98 (d, J = 9.0 Hz, 1H), 7.55 (dd, J = 2.0, 9.1 Hz, 1H), 7.41 (d, J = 9.1 Hz, 1H), 7.23–7.35 (m, 2H), 7.09–7.16 (m, 1H), 6.89–7.03 (m, 2H), 6.64 (d, J = 8.1 Hz, 1H), 4.66 (dd, J = 5.0, 9.1 Hz, 1H), 2.98 (s, 3H), 1.53–1.99 (m, 7H), 1.49 (s, 9H), 1.01–1.35 (m, 6H).

(2S)-({[1-(7-Chloroquinolin-4-yl)-5-(2-methoxyphenyl)-1H-pyrazol-3-yl]carbonyl}amino)(cyclohexyl)ethanoic Acid (17b). Following the general tert-butyl ester hydrolysis procedure, **17b** was obtained as a pale-yellow film in 74% yield from **17a**. ^{1}H NMR (CDCl$_3$) δ 9.09 (br s, 1H), 8.76 (br s, 1H), 8.33 (d, J = 8.9 Hz, 1H), 7.82 (d, J = 8.9 Hz, 1H), 7.57–7.32 (m, 4H), 7.22 (s, 1H), 7.10 (t, J = 6.9 Hz, 1H), 6.70 (d, J = 7.7 Hz, 1H), 4.78 (dd, J = 4.8, 8.2 Hz, 1H), 3.04 (br s, 3H), 2.10–1.90 (m, 1H), 1.89–1.60 (m, 5H), 1.25–1.07 (m, 5H). MS (ESI) m/z: 518.0 (M − H)$^-$. $[α]^{25}_D$ −24.3° (c 2.04, MeOH). Anal. (C$_{28}$H$_{27}$ClN$_4$O$_4$) C, H, N.

tert-Butyl (2S)-({[1-(7-Chloroquinolin-4-yl)-5-(2,6-difluorophenyl)-1H-pyrazol-3-yl]carbonyl}-amino)(cyclohexyl)ethanoate (18a). Following the general amide coupling procedure, **18a** was obtained from **11e** and **12d** as a pale-yellow foam in 53% yield. ^{1}H NMR (CDCl$_3$) δ 8.89 (d, J = 4.7 Hz, 1H), 8.15 (d, J = 4.7 Hz, 1H), 7.75 (d, J = 9.0 Hz, 1H), 7.75 (d, J = 9.0 Hz, 1H), 7.52 (dd, J = 1.9, 9.0 Hz, 1H), 7.4 (d, J = 9.0 Hz, 1H), 7.32–7.25 (m, 3H), 7.2 (d, J = 9.0 Hz, 1H), 6.80 (t, J = 7.6 Hz, 1H), 4.69–4.63 (m, 1H), 1.90–1.85 (m, 1H), 1.84–1.60 (m, 5H), 1.50 (s, 9H), 1.32–1.06 (m, 5H).

(2S)-({[1-(7-Chloroquinolin-4-yl)-5-(2,6-difluorophenyl)-1H-pyrazol-3-yl]carbonyl}amino)(cyclohexyl)ethanoic Acid (18b). Following the general tert-butyl ester hydrolysis procedure, **18b** was obtained as a white foam (90%) from **18a**. ^{1}H NMR (CDCl$_3$) δ 8.91 (d, J = 4.7 Hz, 1H), 8.18 (s, 1H), 8.17 (d, J = 9.2 Hz, 1H), 7.74 (d, J = 9.0 Hz, 1H), 7.53 (dd, J = 1.9, J = 9.0 Hz, 1H), 7.40 (d, J = 9.0 Hz, 1H), 7.20–7.05 (m, 4H), 6.80 (t, J = 7.6 Hz, 1H), 4.82–4.74 (m, 1H), 2.08–1.94 (m, 1H), 1.89–1.60 (m, 5H), 1.37–1.05 (m, 5H). MS (ESI) m/z: 523.6 (M − H)$^-$. $[α]^{25}_D$ −13.3° (c 0.44, MeOH). Anal. (C$_{27}$H$_{23}$ClF$_2$N$_4$O$_3$) C, H, N.

tert-Butyl (2S)-({[1-(7-Chloroquinolin-4-yl)-5-(2,6-dimethoxyphenyl)-4-ethyl-1H-pyrazol-3-yl]carbonyl}amino)(cyclohexyl)-ethanoate (19a). Following the general amide coupling procedures, **19a** was obtained as a pale-yellow film (86%) **11b** and **12d**. ^{1}H NMR (CDCl$_3$) δ 8.76 (d, J = 4.6 Hz, 1H), 8.09 (d, J = 2.1 Hz, 1H), 7.98 (d, J = 9.1 Hz, 1H), 7.48 (td, J = 2.2, 9.1 Hz, 2H), 7.21 (t, J = 8.4 Hz, 1H), 7.09 (d, J = 4.6 Hz, 1H), 6.40 (dd, J = 8.4, 15.6 Hz, 2H), 4.64 (dd, J = 4.9, 9.0 Hz, 1H), 3.53 (s, 3H), 3.42 (s, 3H), 2.54–2.82 (m, 2H), 1.61–1.95 (m, 6H), 1.49 (s, 9H), 1.17–1.36 (m, 5H), 1.06–1.15 (m, 3H). MS (ESI) m/z: 633.7 (M + H)$^+$.

(2S)-({[1-(7-Chloroquinolin-4-yl)-5-(2,6-dimethoxyphenyl)-4-ethyl-1H-pyrazol-3-yl]carbonyl}amino)(cyclohexyl)ethanoic Acid (19b). Following the general tert-butyl ester hydrolysis procedure, **19b** was obtained as a pale-yellow solid in 96% yield from **19a**. ^{1}H NMR (CDCl$_3$) δ 9.12 (d, J = 5.7 Hz, 1H), 8.43 (s, 1H), 8.38 (d, J = 9.2 Hz, 1H), 7.77 (dd, J = 1.3, 9.3 Hz, 1H), 7.56 (d, J = 8.7 Hz, 1H), 7.48 (d, J = 5.7 Hz, 1H), 7.34 (t, J = 8.4 Hz, 1H), 6.51 (dd, J = 8.5, 15.5 Hz, 2H), 4.77 (dd, J = 5.2, 8.7 Hz, 1H), 3.67–3.45 (m, 6H), 2.66 (dd, J = 3.2, 7.3 Hz, 2H), 2.12–1.91 (m, 1H), 1.90–1.60 (m, 6H), 1.38–1.16 (m, 4H), 1.15–1.01 (m, 3H). MS (ESI) m/z: 575.8 (M − H)$^-$. $[α]^{25}_D$ +2.3° (c 0.98, MeOH). Anal. (C$_{31}$H$_{33}$ClN$_4$O$_5$) C, H, N.

Methyl 1-({[1-(7-Chloroquinolin-4-yl)-5-(2,6-dimethoxyphenyl)-1H-pyrazol-3-yl]carbonyl}amino)cycloheptanecarboxylate (20a). Following the general amide coupling procedure, **20a** was obtained from **11a** and methyl 1-aminocycloheptanecarboxylate hydrochloride (**12c**) as an off-white powder in 65% yield. ^{1}H NMR (CDCl$_3$) δ 8.77 (d, J = 4.7 Hz, 1H), 8.16 (d, J = 1.9 Hz, 1H), 7.96 (d, J = 9.0 Hz, 1H), 7.55 (dd, J = 1.9, 9.0 Hz, 1H), 7.24–7.37 (m, 2H), 7.13 (s, 1H), 6.92–7.02 (m, 2H), 6.64 (d, J = 8.3 Hz, 1H), 4.28 (d, J = 8.1 Hz, 1H), 2.98 (s, 6H), 2.02–2.11 (m, 2H), 1.80–1.95 (m, 9H), 1.70–1.79 (m, 2H), 1.59–1.70 (m, 2H).

1-({[1-(7-Chloroquinolin-4-yl)-5-(2,6-dimethoxyphenyl)-1H-pyrazol-3-yl]carbonyl}amino)cycloheptanecarboxylic Acid (20b). Following the general methyl ester hydrolysis procedure, **20b** was obtained from **20a** as a white powder in 86% yield. ^{1}H NMR (CDCl$_3$) δ 8.80 (d, J = 4.7 Hz, 1H), 8.14 (d, J = 2.1 Hz, 1H), 7.90 (d, J = 9.0 Hz, 1H), 7.50 (dd, J = 2.1, 9.0 Hz, 1H), 7.24–7.17 (m, 1H), 7.11 (s, 1H), 7.07 (d, J = 4.7 Hz, 1H) 6.40 (d, J = 8.7 Hz, 2H), 3.42 (s, 6H), 2.42–2.28 (m, 2H), 2.24–2.13 (m, 2H), 1.74–1.53 (m, 10H). MS (ESI) m/z: 519.5 (M − H)$^-$. Anal. (C$_{29}$H$_{29}$ClN$_4$O$_5$) C, H, N.

Methyl 1-({[1-(7-Chloroquinolin-4-yl)-5-(2,6-dimethoxyphenyl)-1H-pyrazol-3-yl]carbonyl}amino)cyclohexanecarboxylate (21a). Following the general amide coupling procedures, **21a** was obtained from **11a** and ethyl 1-aminocyclohexanecarboxylate hydrochloride (**12b**) as a white solid in 87% yield. ^{1}H NMR (CDCl$_3$) δ 8.74–8.84 (m, 1H), 8.14 (d, J = 2.1 Hz, 1H), 7.96 (d, J = 9.0 Hz, 1H), 7.52 (dd, J = 2.1, 9.0 Hz, 1H), 7.15–7.25 (m, 1H), 7.04–7.15 (m, 2H), 6.39 (d, J = 8.5 Hz, 2H), 4.25 (q, J = 7.2 Hz, 1H), 3.41 (s, 6H), 2.18 (d, J = 13.6 Hz, 2H), 1.86–2.02 (m, 2H), 1.43–1.74 (m, 6H), 1.23–1.34 (m, 3H).

1-({[1-(7-Chloroquinolin-4-yl)-5-(2,6-dimethoxyphenyl)-1H-pyrazol-3-yl]carbonyl}amino)cyclohexanecarboxylic Acid (21b). Following the general methyl ester hydrolysis procedure, **21b** was obtained from **21a** in 87% yield as a white solid. ^{1}H NMR (DMSO-d_6) δ 8.92 (d, J = 4.7 Hz, 1H), 8.15 (d, J = 2.1 Hz, 1H), 7.86–7.75 (m, 2H), 7.69 (dd, J = 2.1, 9.0 Hz, 1H), 7.30–7.21 (m, 2H), 6.95 (s, 1H), 6.53 (d, J = 8.7 Hz, 1H), 3.43 (s, 6H), 2.17–2.06 (m, 2H), 1.85–1.72 (m, 2H), 1.62–1.25 (m, 6H). MS (ESI) m/z: 533.8 (M − H)$^-$. Anal. (C$_{28}$H$_{27}$ClN$_4$O$_5$) C, H, N.

Methyl 1-({[1-(7-Chloroquinolin-4-yl)-5-(2,6-dimethoxyphenyl)-1H-pyrazol-3-yl]carbonyl}amino)cyclopentanecarboxylate (22a). Following the general amide coupling procedures, **22a** was obtained from **11a** and methyl 1-aminocyclopentanecarboxylate hydrochloride (**12a**) as a white powder in 85% yield. ^{1}H NMR (CDCl$_3$) δ 8.78 (d, J = 4.7 Hz, 1H), 8.12 (d, J = 2.1 Hz, 1H), 7.94 (d, J = 9.0 Hz, 1H), 7.52 (dd, J = 2.1, 9.0 Hz, 1H), 7.29–7.22 (m, 1H), 7.21–7.16 (m, 1H), 7.11–7.05 (m, 1H), 6.39 (d, J = 8.5 Hz, 2H), 3.78 (s, 3H), 3.41 (s, 6H), 2.42–2.28 (m, 2H), 2.17–2.05 (m, 2H), 1.90–1.80 (m, 4H).

1-({[1-(7-Chloroquinolin-4-yl)-5-(2,6-dimethoxyphenyl)-1H-pyrazol-3-yl]carbonyl}amino)cyclopentanecarboxylic Acid (22b). Following the general methyl ester hydrolysis procedure, **22b** was obtained from **22a** as a white powder in 72% yield. ^{1}H NMR (CDCl$_3$) δ 8.80 (d, J = 4.7 Hz, 1H), 8.14 (d, J = 2.1 Hz, 1H), 7.90 (d, J = 9.0 Hz, 1H), 7.53 (dd, J = 2.1, J = 9.0 Hz, 1H), 7.30–7.18 (m, 1H), 7.11 (s, 1H), 7.07 (d, J = 4.7 Hz, 1H) 6.40 (d, J = 8.7 Hz, 2H), 3.42 (s, 6H), 2.54–2.40 (m, 2H), 2.20–2.07 (m, 2H), 1.90–1.77 (m, 4H). MS (ESI) m/z: 547.8 (M − H)$^-$. Anal. (C$_{28}$H$_{27}$ClN$_4$O$_5$) C, H, N.

Methyl 1-({[1-(7-Chloroquinolin-4-yl)-5-(2-methoxyphenyl)-1H-pyrazol-3-yl]carbonyl}amino)cyclohexanecarboxylate (23a). Following the general amide coupling procedure, **23a** was obtained from **11f** and ethyl 1-aminocyclohexanecarboxylate hydrochloride (**12b**) as a white solid in 63% yield. ^{1}H NMR (CDCl$_3$) δ 8.78 (d, J = 4.7 Hz, 1H), 8.17 (d, J = 1.9 Hz, 1H), 7.96 (d, J = 9.0 Hz, 1H), 7.56 (dd, J = 2.2, 9.1 Hz, 1H), 7.24–7.34 (m, 2H), 7.10 (s, 1H), 6.90–7.01 (m, 1H), 6.64 (d, J = 8.7 Hz, 2H), 3.78 (s, 3H), 2.99 (s, 3H), 2.18 (d, J = 13.8 Hz, 2H), 1.88–2.01 (m, 2H), 1.63–1.74 (m, 6H).

1-({[1-(7-Chloroquinolin-4-yl)-5-(2-methoxyphenyl)-1H-pyrazol-3-yl]carbonyl}amino)cyclohexanecarboxylic Acid (23b). Following the general methyl ester hydrolysis procedure, **23b** was obtained from **23a** as a white solid in 77% yield. ^{1}H NMR (CDCl$_3$) δ 8.80 (d, J = 4.7 Hz, 1H), 8.17 (d, J = 2.1 Hz, 1H), 7.90 (d, J = 9.0 Hz, 1H), 7.5 (dd, J = 2.1, J = 9.0 Hz, 1H), 7.35–7.21 (m, 1H), 7.20–7.11 (m, 2H), 6.63

(d, J = 8.7 Hz, 1H), 2.98 (s, 3H), 2.30–2.16 (m, 2H), 2.10–1.96 (m, 2H), 1.76–1.53 (m, 6H). MS (ESI) m/z: 503.6 (M − H)$^-$. Anal. (C$_{28}$H$_{27}$ClN$_4$O$_4$) C, H, N.

tert-Butyl (2S)-Cyclohexyl({[5-(2,6-dimethoxyphenyl)-1-naphthalen-1-yl-1H-pyrazol-3-yl]carbonyl}amino)ethanoate (24a). Following the general amide coupling procedures, **24a** was obtained from **11g** and **12d** as a white solid in 53% yield. ^{1}H NMR (CDCl$_3$) δ 7.88–7.72 (m, 3H), 7.51–7.38 (m, 3H), 7.36–7.21 (m, 2H), 7.11 (t, J = 8.4 Hz, 1H), 7.07–7.03 (m, 1H), 6.33 (d, J = 5.9 Hz, 2H), 4.66 (dd, J = 5.4, 9.1 Hz, 1H), 3.40 (br s, 6H), 1.96–1.52 (m, 8H), 1.47 (s, 9H), 1.33–1.02 (m, 6H).

(2S)-Cyclohexyl({[5-(2,6-dimethoxyphenyl)-1-naphthalen-1-yl-1H-pyrazol-3-yl]carbonyl}amino)ethanoic Acid (24b).[45] Following the general *tert*-butyl ester hydrolysis procedure **24b** was obtained as a slightly yellow solid in 53% yield from **24a**. ^{1}H NMR (CDCl$_3$) δ 7.82 (d, J = 6.8 Hz, 2 H), 7.73 (d, J = 6.4 Hz, 2 H), 7.48 (dd, J = 3.2, 6.2 Hz, 2 H), 7.38–7.21 (m, 2 H), 7.19–7.01 (m, 2 H), 6.33 (d, J = 8.1 Hz, 2 H), 4.73 (dd, J = 5.8, 8.3 Hz, 1 H), 3.40 (br s, 6 H), 1.93 (br s, 1 H), 1.83–1.55 (m, 5 H), 1.23–0.99 (m, 5 H). MS (ESI) m/z: 512.4 (M − H)$^-$. $[\alpha]^{25}_D$ −26.7° (c 0.24, MeOH). Anal. (C$_{30}$H$_{31}$N$_3$O$_5$) C, H, N.

tert-Butyl (2S)-Cyclohexyl({[5-(2-methoxyphenyl)-1-naphthalen-1-yl-1H-pyrazol-3-yl]carbonyl}amino)ethanoate (25a). Following the general amide coupling procedure, **25a** was obtained from **11h** and **12d** in 81% yield as a colorless film. ^{1}H NMR (CDCl$_3$) δ 7.84 (s, 1H), 7.81 (s, 1H), 7.75 (d, J = 3.5 Hz, 1H), 7.55–7.46 (m, 2H), 7.43 (d, J = 9.1 Hz, 1H), 7.36–7.28 (m, 1H), 7.23–7.14 (m, 3H), 7.12 (s, 1H), 6.85–6.75 (m, 1H), 6.61 (d, J = 8.6 Hz, 1H), 4.66 (dd, J = 5.4, 9.1 Hz, 1H), 3.07 (s, 3H), 1.96–1.57 (m, 7H), 1.47 (s, 9H), 1.32–1.05 (m, 6H).

(2S)-Cyclohexyl({[5-(2-methoxyphenyl)-1-naphthalen-1-yl-1H-pyrazol-3-yl]carbonyl}amino)ethanoic Acid (25b). Following the general *tert*-butyl ester hydrolysis procedure, **25b** was obtained from **25a** as a white foam (43%). ^{1}H NMR (CDCl$_3$) δ 7.91–7.81 (m, 2 H), 7.73–7.61 (m, 2 H), 7.56–7.47 (m, 2 H), 7.39–7.29 (m, 1 H), 7.25–7.18 (m, 2 H), 7.15 (s, 2 H), 6.80 (t, J = 7.4 Hz, 1 H), 6.63 (d, J = 8.3 Hz, 1 H), 4.74 (dd, J = 5.7, 8.8 Hz, 1 H), 3.12 (s, 3 H), 1.93 (br s, 1 H), 1.83–1.53 (m, 5 H), 1.24–1.02 (m, 5 H). MS (ESI) m/z: 482.3 (M − H)$^-$. $[\alpha]^{25}_D$ −32.7° (c 0.56, MeOH). Anal. (C$_{29}$H$_{29}$N$_3$O$_4$) C, H, N.

Methyl 1-({[5-(2,6-Dimethoxyphenyl)-1-naphthalen-1-yl-1H-pyrazol-3-yl]carbonyl}amino)cyclohexanecarboxylate (26a). Following the general amide coupling procedure, **26a** was obtained from **11g** and **12b** as a white solid in 98% yield. ^{1}H NMR (CDCl$_3$) δ 7.73–7.89 (m, 3H), 7.45–7.52 (m, 2H), 7.27–7.37 (m, 2H), 7.06–7.16 (m, 2H), 7.02 (s, 1H), 6.32 (d, J = 8.3 Hz, 2H), 3.78 (s, 3H), 3.23–3.63 (m, 6H), 2.08–2.32 (m, 4H), 1.56 (br s, 8H).

1-({[5-(2,6-Dimethoxyphenyl)-1-naphthalen-1-yl-1H-pyrazol-3-yl]carbonyl}amino)cyclohexanecarboxylic Acid (26b).[45] Following the general ethyl ester hydrolysis procedure, **26b** was obtained from **26a** as an off-white powder in 35% yield. ^{1}H NMR (CDCl$_3$) δ 7.88–7.78 (m, 2 H), 7.77–7.67 (m, 1 H), 7.54–7.45 (m, 2 H), 7.37–7.29 (m, 1 H), 7.28–7.22 (m, 4 H), 7.15 (t, J = 8.4 Hz, 1 H), 7.08 (s, 1 H), 6.34 (d, J = 8.5 Hz, 2 H), 3.41 (br s, 6 H), 2.24 (d, J = 14.1 Hz, 2 H), 2.10–1.94 (m, 2 H), 1.79–1.48 (m, 6 H). MS (ESI) m/z: 498.3 (M − H)$^-$. Anal. (C$_{30}$H$_{31}$N$_3$O$_5$) C, H, N.

Methyl 1-({[5-(2-Methoxyphenyl)-1-naphthalen-1-yl-1H-pyrazol-3-yl]carbonyl}amino)cyclohexanecarboxylate (27a). Following the general amide coupling procedures, **27a** was obtained from **11h** and **12b** as a colorless film in 73% yield. ^{1}H NMR (CDCl$_3$) δ 7.93–7.80 (m, 2H), 7.80–7.71 (m, 1H), 7.59–7.46 (m, 2H), 7.40–7.29 (m, 1H), 7.25–7.12 (m, 4H), 7.10 (s, 1H), 6.87–6.74 (m, 1H), 6.62 (d, J = 8.2 Hz, 1H), 3.77 (s, 3H), 3.09 (s, 3H), 2.13 (br s, 2H), 1.96 (br s, 3H), 1.71–1.30 (m, 10H).

1-({[5-(2-Methoxyphenyl)-1-naphthalen-1-yl-1H-pyrazol-3-yl]carbonyl}amino)cyclohexanecarboxylic Acid (27b). Following the general ethyl ester hydrolysis procedure, **27b** was obtained from **27a** as a white foam (66%). ^{1}H NMR (CDCl$_3$) δ 7.92–7.82 (m, 2 H), 7.68 (d, J = 3.4 Hz, 1 H), 7.53 (dd, J = 3.3, 6.3 Hz, 2 H), 7.38–7.30 (m, 1 H), 7.25–7.09 (m, 5 H), 6.82 (t, J = 7.4 Hz, 1 H), 6.63 (d, J = 8.2 Hz, 1 H), 3.10 (s, 3 H), 2.22 (d, J = 13.9 Hz, 2 H), 2.01 (t, J = 11.2 Hz,

2 H), 1.77−1.19 (m, 8 H), 0.97 (br s, 1 H). MS (ESI) m/z: 503.6 (M − H)⁻. Anal. ($C_{28}H_{27}N_3O_4$) C, H, N.

tert-Butyl (2S)-Cyclohexyl({[5-(2,6-dimethoxyphenyl)-1-(4-fluoro-phenyl)-1H-pyrazol-3-yl]carbonyl}amino)ethanoate (28a). Following the general amide coupling procedure, **28a** was obtained from **11i** and **12d** as an off-white powder in 94% yield. ^{1}H NMR (CDCl₃) δ 7.42−7.54 (m, 1H), 7.21−7.35 (m, 3H), 6.90−7.03 (m, 3H), 6.50 (dd, *J* = 8.5, 13.9 Hz, 2H), 4.67 (dd, *J* = 5.09, 9.14 Hz, 1H), 3.62 (s, 3H), 3.52 (s, 3H), 1.60−1.92 (m, 7H), 1.48−1.52 (m, 9H), 1.16−1.33 (m, 5H). MS (ESI) m/z: 536.5 (M − H)⁻.

(2S)-Cyclohexyl({[5-(2,6-Dimethoxyphenyl)-1-(4-fluorophenyl)-1H-pyrazol-3-yl]carbonyl}amino)ethanoic Acid (28b). Following the general *tert*-butyl ester hydrolysis procedure, **28b** was obtained as a white foam (83%) from **28a**. ^{1}H NMR (CD₃OD) δ 7.23−7.42 (m, 3H), 6.99−7.15 (m, 2H), 6.76−6.86 (m, 1H), 6.62 (d, *J* = 8.29 Hz, 2H), 4.57 (d, *J* = 6.03 Hz, 1H), 3.60 (s, 6H), 1.74−2.10 (m, 7H), 1.21−1.44 (m, 4H). MS (ESI) m/z: 480.6 (M − H)⁻. $[\alpha]^{25}_D$ −19.7° (*c* 0.33, MeOH). Anal. ($C_{26}H_{26}FN_3O_5$) C, H, N.

Methyl 1-({[5-(2,6-Dimethoxyphenyl)-1-(4-fluorophenyl)-1H-pyr-azol-3-yl]carbonyl}amino)cyclohexanecarboxylate (29a). Following the general amide coupling procedure, **29a** was prepared from **11i** and **12b** as a colorless solid in 50% yield. ^{1}H NMR (CDCl₃) δ 7.33−7.25 (m, 4H), 7.00−6.90 (m, 2H), 6.50 (d, *J* = 8.5 Hz, 2H), 3.75 (s, 3H), 3.58 (s, 6H), 2.25−2.13 (m, 2H), 2.01−1.89 (m, 2H), 1.74−1.52 (m, 6H).

1-({[5-(2,6-Dimethoxyphenyl)-1-(4-fluorophenyl)-1H-pyrazol-3-yl]carbonyl}amino)cyclohexanecarboxylic Acid (29b). Following the general methyl ester hydrolysis procedure, **29b** was obtained from **29a** as a white solid (89%). ^{1}H NMR (CDCl₃) δ 7.36−7.21 (m, 4H), 7.03−6.95 (m, 2H), 6.50 (d, *J* = 8.5 Hz, 2H), 3.60 (s, 6H), 2.34−2.20 (m, 2H), 2.10−1.96 (m, 2H), 1.79−1.47 (m, 6H). MS (ESI) m/z: 466.4 (M − H)⁻. Anal. ($C_{25}H_{26}FN_3O_5$) C, H, N.

2-({[5-(2,6-Dimethoxyphenyl)-1-(4-fluorophenyl)-1H-pyrazol-3-yl]carbonyl}amino)tricycle-[3.3.1.13,7]decane-2-carboxylic Acid (30). Following the method of Quéré,[45] **30** was obtained from **11i** and 9-aminobicyclo[3.3.1]nonane-9-carboxylic acid (**12f**). Briefly, **11i** was heated under reflux in SOCl₂ for 2 h, followed by cooling and concentration on a rotavap. The residue was then dissolved in toluene and evaporated 3× to remove HCl. The residue was then taken up in dry THF and added to a well agitated mixture of 5:1 THF:water (15 mL/g of acid chloride) containing 1.1 equiv of 2-amino-adamantane-carboxylic acid and 2.2 equiv of NaOH at 5 °C. Following the addition, the stirring was continued for 18 h at ambient temp. After this time, the mixture is made acidic with 1 N HCl and extracted 3× with CH₂Cl₂, dried over sodium sulfate, and evaporated. The residue was chromatographed using a 0−15% CH₂Cl₂:MeOH gradient. Evaporation provided the desired compound as an off-white solid (71%). ^{1}H NMR (CDCl₃) δ 7.20−7.36 (m, 4H), 6.90−7.04 (m, 3H), 6.51 (d, *J* = 8.5 Hz, 2H), 3.54−3.63 (m, 6H), 2.71−2.81 (m, 2H), 2.23 (d, *J* = 12.8 Hz, 2H), 2.07 (d, *J* = 12.2 Hz, 2H), 1.68−1.95 (m, 8H). MS (ESI) m/z: 518.9 (M − H)⁻. Anal. ($C_{29}H_{30}FN_3O_5$) C, H, N.

Pharmacological Methods. *Calcium Mobilization Assay for NTS1 Receptor.* CHO-k1-rNTS1 cells were maintained in DMEM/F12 medium supplemented with 10% fetal bovine serum (Gibco), 100 U/mL penicillin/100 μg/mL streptomycin, 100 μg/mL normocin (Invivogen), and 250 μg/mL geneticin. For calcium mobilization assays, cells were plated at 30000 cells/well in black, clear-bottom 96-well plates the day of the assay and incubated at 37 °C, 5% CO₂. Prior to the assay, Calcium 5 dye (Molecular Devices) was reconstituted according to manufacturer instructions. The reconstituted dye was diluted 1:40 in assay buffer (1× HBSS, 20 mM HEPES, and 2.5 mM Probenicid (Sigma), pH 7.4). Growth media was removed, and 200 μL of this diluted dye was added to each well. Plates were incubated for 45 min at 37 °C, 5% CO₂ after dye addition. For agonist assays, cells were pretreated with 1:10 addition (20 μL) of 10% DMSO in assay buffer, and the plates were returned to 37 °C, 5% CO₂ for 15 min. Full-log serial dilutions of the test compounds were made at 10× the desired final concentration in 1% DMSO assay buffer and warmed to 37 °C. After the pretreatment incubation, fluorescence

intensity was measured on a FlexStation II fluorometric imaging plate reader (Molecular Devices). Relative fluorescence units (RFU) were measured before (20 readings) and after (40 readings) the agonist compound addition for a total 60 s read time (excitation = 485 nm, emission = 525 nm, cutoff = 515 nm). For antagonist assays, cells were treated with Calcium 5 dye for 45 min at 37 °C, 5% CO₂ as with the agonist assays. Then 1/10th volume of 10× final concentration of the test antagonist compounds were added in 1% DMSO in assay buffer, and the plate was incubated for 15 min at 37 °C, 5% CO₂. For K_e assays, the cells were pretreated with a single concentration of test compound and the FlexStation II added serial dilutions of the control agonist NT. For IC₅₀ assays, cells were pretreated with serial dilutions of test compound and the FlexStation II added 1 nM NT.

Calcium Mobilization Assay for NTS2 Receptor. CHO-k1-rNTS2 cells were maintained in DMEM/F12 supplemented with 10% FBS, pen/strep, 100 μg/mL normocin, and 400 μg/mL geneticin. For calcium mobilization assays, cells were plated at 25000 cells/well in black, clear-bottom 96-well plates the day before the assay and incubated at 37 °C, 5% CO₂. Then 100 μL of reconstituted Calcium 5 dye (Molecular Devices, diluted 1:20 in assay buffer (1× HBSS, 20 mM HEPES, 2.5 mM Probenicid, pH 7.4)) was added per well and plates were incubated for 45 min at 37 °C, 5% CO₂. For agonist assays, cells were pretreated with 1:10 addition of 10% DMSO and the plates were returned to 37 °C, 5% CO₂. Full-log serial dilutions of the test compounds were made at 10× the desired final concentration in 1% DMSO assay buffer and warmed to 37 °C. After the pretreatment incubation, fluorescence intensity was measured on a FLIPR Tetra fluorometric imaging plate reader (Molecular Devices). Relative fluorescence units (RFUs) were measured every second for 100 s (14 readings before compound addition to establish baseline fluorescence, 85 after), exposure time 0.53 s, gain = 2000, excitation intensity = 30%, gate open = 10%. For antagonist assays, 1/10 volume of 10× concentration of the test compound dilutions in 10% DMSO in assay buffer was added to cells in lieu of the 10% DMSO only in assay buffer for the pretreatment. K_e assays were run against dose−response curves of the control agonist **5b**, and IC₅₀ assays were run against the EC₈₀ of **5b** (73 nM final concentration).

Competitive Binding Assays. Relative binding affinity was evaluated using ^{125}I labeled neurotensin and CHO-k1 cell lines overexpressing either the rNTS1 or rNTS2 receptor essentially as described by Gendron.[40] Briefly, cells were plated at 100000 cells/well in 24-well plates in complete DMEM/F12 medium supplemented with 10% fetal bovine serum, 100 units of penicillin/mL, 100 mg of streptomycin/mL, 0.1 mg/mL of normocin, and 250 mg/mL geneticin. Cells were incubated at 37 °C with 5% CO₂ and 95% humidity for 48 h. Cells were then equilibrated for 10 min in Earle's buffer (130 mM NaCl, 5 mM KCl, 1.8 mM CaCl₂, 0.8 mM MgCl₂, and 20 mM HEPES, pH 7.4) supplemented with 0.1% BSA and 0.1% glucose and then incubated with or without 10 nM test compound in 0.1 nM [^{125}I]NT at 37 °C for 30 min. Cells were washed twice in Earle's buffer, extracted in 1 mL of 0.1N NaOH and counted in a Packard Cobra II gamma counter for 1 min. Total binding was determined in the absence of test compound, and nonspecific binding was determined in the presence of 1.0 μM nonradiolabeled neurotensin.

Data Analysis. To determine EC₅₀, IC₅₀, and K_e values, data were fit to a three-parameter logistic equation using GraphPad Prism software. K_i values for radioligand binding assays were determined from IC₅₀ values using the equation of Cheng and Prusoff. All data are from at least three independent experiments run in duplicate wells.

■ ASSOCIATED CONTENT

ⓈSupporting Information

Elemental analysis results for test compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: (919)-541-6375. Fax: (919) 541-6499. E-mail: jbthomas@rti.org.

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS USED

NT, neurotensin; NTS1, neurotensin receptor 1; NTS2, neurotensin receptor 2; CNS, central nervous system 5; H1, histamine receptor 1; FLIPR, fluorometric imaging plate reader; CHO, Chinese hamster ovary

■ REFERENCES

(1) Caraway, R.; Leeman, S. E. The isolation of a new hypotensive peptide, neurotensin, from bovine hypothalami. *J. Biol. Chem.* **1973**, *248*, 6854−6861.

(2) Boules, M.; Li, Z.; Smith, K.; Fredrickson, P.; Richelson, E. Diverse roles of neurotensin agonists in the central nervous system. *Front. Endocrinol.* **2013**, *4*, 1−16.

(3) Hokfelt, T.; Everitt, B. J.; Theodorsson-Norheim, E.; Goldstein, M. Occurrence of neurotensinlike immunoreactivity in subpopulations of hypothalamic, mesencephalic, and medullary catecholamine neurons. *J. Comp. Neurol.* **1984**, *222*, 543−559.

(4) Kalivas, P. W.; Miller, J. S. Neurotensin neurons in the ventral tegmental area project to the medial nucleus accumbens. *Brain. Res.* **1984**, *300*, 157−160.

(5) Kalivas, P. W.; Nemeroff, C. B.; Prange, A. J., Jr. Neuroanatomical site specific modulation of spontaneous motor activity by neurotensin. *Eur. J. Pharmacol.* **1982**, *78*, 471−474.

(6) Ford, A. P.; Marsden, C. A. In vivo neurochemical and behavioural effects of intracerebrally administered neurotensin and D-Trp11-neurotensin on mesolimbic and nigrostriatal dopaminergic function in the rat. *Brain Res.* **1990**, *534*, 243−250.

(7) Nemeroff, C. B.; Hernandez, D. E.; Luttinger, D.; Kalivas, P. W.; Prange, A. J., Jr. Interactions of neurotensin with brain dopamine systems. *Ann. N. Y. Acad. Sci.* **1982**, *400*, 330−344.

(8) Clineschmidt, B. V.; McGuffin, J. C. Neurotensin administered intracisternally inhibits responsiveness of mice to noxious stimuli. *Eur. J. Pharmacol.* **1977**, *46*, 395−396.

(9) Nemeroff, C. B.; Osbahr, A. J., III; Manberg, P. J.; Ervin, G. N.; Prange, A. J., Jr. Alterations in nociception and body temperature after intracisternal administration of neurotensin, beta-endorphin, other endogenous peptides, and morphine. *Proc. Natl. Acad. Sci. U. S. A.* **1979**, *76*, 5368−5371.

(10) Tanaka, K.; Masu, M.; Nakanishi, S. Structure and functional expression of the cloned rat neurotensin receptor. *Neuron* **1990**, *4*, 847−854.

(11) Vita, N.; Laurent, P.; Lefort, S.; Chalon, P.; Dumont, X.; Kaghad, M.; Gully, D.; Le Fur, G.; Ferrara, P.; Caput, D. Cloning and expression of a complementary DNA encoding a high affinity human neurotensin receptor. *FEBS Lett.* **1993**, *317*, 139−142.

(12) Chalon, P.; Vita, N.; Kaghad, M.; Guillemot, M.; Bonnin, J.; Delpech, B.; Le Fur, G.; Ferrara, P.; Caput, D. Molecular cloning of a levocabastine-sensitive neurotensin binding site. *FEBS Lett.* **1996**, *386*, 91−94.

(13) Mazella, J.; Botto, J. M.; Guillemare, E.; Coppola, T.; Sarret, P.; Vincent, J. P. Structure, functional expression, and cerebral localization of the levocabastine-sensitive neurotensin/neuromedin N receptor from mouse brain. *J. Neurosci.* **1996**, *16*, 5613−5620.

(14) Mazella, J.; Zsurger, N.; Navarro, V.; Chabry, J.; Kaghad, M.; Caput, D.; Ferrara, P.; Vita, N.; Gully, D.; Maffrand, J. P.; Vincent, J. P. The 100-kDa neurotensin receptor is gp95/sortilin, a non-G-protein-coupled receptor. *J. Biol. Chem.* **1998**, *273*, 26273−26276.

(15) Nemeroff, C. B. Neurotensin: perchance an endogenous neuroleptic? *Biol. Psychiatry* **1980**, *15*, 283−302.

(16) Kitabgi, P. Targeting neurotensin receptors with agonists and antagonists for therapeutic purposes. *Curr. Opin. Drug Discovery Dev.* **2002**, *5*, 764−776.

(17) Boules, M.; Fredrickson, P.; Richelson, E. Neurotensin agonists as an alternative to antipsychotics. *Expert Opin. Invest. Drugs* **2005**, *14*, 359−369.

(18) Tatetsu, S.; Takaki, M.; Miyagawa, T.; Kozuma, Y.; Kozuma, Y. A study on the experimental production in the brain of histopathological changes resembling schizophrenia by chronic methamphetamine administration. *Seishin Shinkeigaku Zasshi* **1967**, *69*, 1363−1370.

(19) Machiyama, Y. Chronic methamphetamine intoxication model of schizophrenia in animals. *Schizophrenia Bull.* **1992**, *18*, 107−113.

(20) Frankel, P. S.; Hoonakker, A. J.; Alburges, M. E.; McDougall, J. W.; McFadden, L. M.; Fleckenstein, A. E.; Hanson, G. R. Effect of methamphetamine self-administration on neurotensin systems of the basal ganglia. *J. Pharmacol. Exp. Ther.* **2011**, *336*, 809−815.

(21) Hanson, G. R.; Hoonakker, A. J.; Robson, C. M.; McFadden, L. M.; Frankel, P. S.; Alburges, M. E. Response of neurotensin basal ganglia systems during extinction of methamphetamine self-administration in rat. *J. Pharmacol. Exp. Ther.* **2013**, *346*, 173−181.

(22) Dobner, P. R. Neurotensin and pain modulation. *Peptides* **2006**, *27*, 2405−2414.

(23) Boules, M.; Johnston, H.; Tozy, J.; Smith, K.; Li, Z.; Richelson, E. Analgesic synergy of neurotensin receptor subtype 2 agonist NT79 and morphine. *Behav. Pharmacol.* **2011**, *22*, 573−581.

(24) Bredeloux, P.; Cavelier, F.; Dubuc, I.; Vivet, B.; Costentin, J.; Martinez, J. Synthesis and biological effects of c(Lys-Lys-Pro-Tyr-Ile-Leu-Lys-Lys-Pro-Tyr-Ile-Leu) (JMV2012), a new analogue of neurotensin that crosses the blood−brain barrier. *J. Med. Chem.* **2008**, *51*, 1610−1616.

(25) Bredeloux, P.; Costentin, J.; Dubuc, I. Interactions between NTS2 neurotensin and opioid receptors on two nociceptive responses assessed on the hot plate test in mice. *Behav. Brain Res.* **2006**, *175*, 399−407.

(26) Smith, K. E.; Boules, M.; Williams, K.; Richelson, E. NTS1 and NTS2 mediate analgesia following neurotensin analog treatment in a mouse model for visceral pain. *Behav. Brain Res.* **2012**, *232*, 93−97.

(27) Hughes, F. M.; Shaner, B. E.; May, L. A.; Zotian, L.; Brower, J. O.; Woods, R. J.; Cash, M.; Morrow, D.; Massa, F.; Mazella, J.; Dix, T. A. Identification and functional characterization of a stable, centrally active derivative of the neurotensin (8−13) fragment as a potential first-in-class analgesic. *J. Med. Chem.* **2010**, *53*, 4623−4632.

(28) Sarret, P.; Esdaile, M. J.; Perron, A.; Martinez, J.; Stroh, T.; Beaudet, A. Potent spinal analgesia elicited through stimulation of NTS2 neurotensin receptors. *J. Neurosci.* **2005**, *25*, 8188−8196.

(29) Tetreault, P.; Beaudet, N.; Perron, A.; Belleville, K.; Rene, A.; Cavelier, F.; Martinez, J.; Stroh, T.; Jacobi, A. M.; Rose, S. D.; Behlke, M. A.; Sarret, P. Spinal NTS2 receptor activation reverses signs of neuropathic pain. *FASEB J.* **2013**, *27*.

(30) Finnerup, N. B.; Sindrup, S. H.; Jensen, T. S. The evidence for pharmacological treatment of neuropathic pain. *Pain* **2010**, *150*, 573−581.

(31) Carraway, R. L., S, E. Structural requirements for the biological activity of neurotensin, a new vasoactive peptide. In *Peptides: Chemistry, Structure and Biology*; Walter, R., Meienhofer, J., Eds.; Ann Arbor Science: Ann Arbor, MI, 1975; pp 679−685.

(32) Dubuc, I.; Sarret, P.; Labbe-Jullie, C.; Botto, J. M.; Honore, E.; Bourdel, E.; Martinez, J.; Costentin, J.; Vincent, J. P.; Kitabgi, P.;

Mazella, J. Identification of the receptor subtype involved in the analgesic effect of neurotensin. *J. Neurosci.* **1999**, *19*, 503−510.

(33) Doulut, S.; Rodriguez, M.; Lugrin, D.; Vecchini, F.; Kitabgi, P.; Aumelas, A.; Martinez, J. Reduced peptide bond pseudopeptide analogues of neurotensin. *Pept. Res.* **1992**, *5*, 30−38.

(34) Boules, M.; Liang, Y.; Briody, S.; Miura, T.; Fauq, I.; Oliveros, A.; Wilson, M.; Khaniyev, S.; Williams, K.; Li, Z.; Qi, Y.; Katovich, M.; Richelson, E. NT79: A novel neurotensin analog with selective behavioral effects. *Brain Res.* **2010**, *1308*, 35−46.

(35) Einsiedel, J.; Held, C.; Hervet, M.; Plomer, M.; Tschammer, N.; Hubner, H.; Gmeiner, P. Discovery of highly potent and neurotensin receptor 2 selective neurotensin mimetics. *J. Med. Chem.* **2011**, *54*, 2915−2923.

(36) Held, C.; Plomer, M.; Hubner, H.; Meltretter, J.; Pischetsrieder, M.; Gmeiner, P. Development of a metabolically stable neurotensin receptor 2 (NTS2) ligand. *ChemMedChem* **2013**, *8*, 75−81.

(37) Gully, D.; Canton, M.; Boigegrain, R.; Jeanjean, F.; Molimard, J. C.; Poncelet, M.; Gueudet, C.; Heaulme, M.; Leyris, R.; Brouard, A. Biochemical and pharmacological profile of a potent and selective nonpeptide antagonist of the neurotensin receptor. *Proc. Natl. Acad. Sci. U. S. A.* **1993**, *90*, 65−69.

(38) Gully, D.; Labeeuw, B.; Boigegrain, R.; Oury-Donat, F.; Bachy, A.; Poncelet, M.; Steinberg, R.; Suaud-Chagny, M. F.; Santucci, V.; Vita, N.; Pecceu, F.; Labbe-Jullie, C.; Kitabgi, P.; Soubrie, P.; Le Fur, G.; Maffrand, J. P. Biochemical and pharmacological activities of SR 142948A, a new potent neurotensin receptor antagonist. *J. Pharmacol. Exp. Ther.* **1997**, *280*, 802−812.

(39) Dahl, R.; Pedersen, B.; Larsen, B. Intranasal levocabastine for the treatment of seasonal allergic rhinitis: a multicentre, double-blind, placebo-controlled trial. *Rhinology* **1995**, *33*, 121−125.

(40) Gendron, L.; Perron, A.; Payet, M. D.; Gallo-Payet, N.; Sarret, P.; Beaudet, A. Low-affinity neurotensin receptor (NTS2) signaling: internalization-dependent activation of extracellular signal-regulated kinases 1/2. *Mol. Pharmacol.* **2004**, *66*, 1421−1430.

(41) Labeeuw, B.; Gully, D.; Jeanjean, F.; Molimard, J.; Boigegrain, R. Substituted 1-Phenyl-3-pyrazolecarboxamides Active on the Neurotensin Receptors Their Preparation and Pharmaceutical Compositions Containing Them. U.S. Patent 5,723,483, 1998.

(42) Jiang, J.; Huang, W.; Zhai, J.; Liu, H.; Cai, Q.; Xu, L.; Wang, W.; Ji, Y. 'One-pot' synthesis of 4-substituted 1,5-diaryl-1*H*-pyrazole-3-carboxylates via lithium *tert*-butoxide-mediated sterically hindered Claisen condensation and Knorr reaction. *Tetrahedron* **2013**, *69*, 627−635.

(43) Baxendale, I. R.; Cheung, S.; Kitching, M. O.; Ley, S. V.; Shearman, J. W. The synthesis of neurotensin antagonist SR 48692 for prostate cancer research. *Biorg. Med. Chem.* **2013**, *21*, 4378−4387.

(44) Lindh, J.; Sojberg, P. J. R.; M, L. Synthesis of aryl ketones by palladium(II)-catalyzed decarboxylative addition of benzoic acids to nitriles. *Angew. Chem. Int. Ed.* **2010**, *49*, 7733−7737.

(45) Quéré, L. Etude de l'interaction ligand-récepteur neurotensinergique. Ph.D. Thesis. University of Namur, Namur, Belgium, 1995.

(46) Nagasawa, H. T.; Eberling, J. A.; Shirota, F. N. 2-Amino-adamantane-2-carboxylic acid, a rigid, achiral, tricyclic alpha-amino acid with transport inhibitory properties. *J. Med. Chem.* **1973**, *16*, 823−826.

(47) Yamada, M.; Yamada, M.; Lombet, A.; Forgez, P.; Rostene, W. Distinct functional characteristics of levocabastine sensitive rat neurotensin NT2 receptor expressed in Chinese hamster ovary cells. *Life Sci.* **1998**, *62*, 375−380.

(48) Richard, F.; Barroso, S.; Martinez, J.; Labbe-Jullie, C.; Kitabgi, P. Agonism, inverse agonism, and neutral antagonism at the constitutively active human neurotensin receptor 2. *Mol. Pharmacol.* **2001**, *60*, 1392−1398.

(49) Vita, N.; Oury-Donat, F.; Chalon, P.; Guillemot, M.; Kaghad, M.; Bachy, A.; Thurneyssen, O.; Garcia, S.; Poinot-Chazel, C.; Casellas, P.; Keane, P.; Le Fur, G.; Maffrand, J. P.; Soubrie, P.; Caput, D.; Ferrara, P. Neurotensin is an antagonist of the human neurotensin NT2 receptor expressed in Chinese hamster ovary cells. *Eur. J. Pharmacol.* **1998**, *360*, 265−272.

(50) Holst, B.; Holliday, N. D.; Bach, A.; Elling, C. E.; Cox, H. M.; Schwartz, T. W. Common structural basis for constitutive activity of the ghrelin receptor family. *J. Biol. Chem.* **2004**, *279*, 53806−53817.

(51) Gaddum, J. H. Theories of drug antagonism. *Pharmacol. Rev.* **1957**, *9*, 211−218.

(52) Kenakin, T.; Jenkinson, S.; Watson, C. Determining the potency and molecular mechanism of action of insurmountable antagonists. *J. Pharmacol. Exp. Ther.* **2006**, *319*, 710−723.

(53) Roussy, G.; Dansereau, M. A.; Baudisson, S.; Ezzoubaa, F.; Belleville, K.; Beaudet, N.; Martinez, J.; Richelson, E.; Sarret, P. Evidence for a role of NTS2 receptors in the modulation of tonic pain sensitivity. *Mol. Pain* **2009**, *5*, 38.

(54) Smith, D. J.; Hawranko, A. A.; Monroe, P. J.; Gully, D.; Urban, M. O.; Craig, C. R.; Smith, J. P.; Smith, D. L. Dose-dependent pain-facilitatory and -inhibitory actions of neurotensin are revealed by SR 48692, a nonpeptide neurotensin antagonist: influence on the antinociceptive effect of morphine. *J. Pharmacol. Exp. Ther.* **1997**, *282*, 899−908.

(55) Costa, F. G.; Frussa-Filho, R.; Felicio, L. F. The neurotensin receptor antagonist, SR48692, attenuates the expression of amphetamine-induced behavioural sensitisation in mice. *Eur. J. Pharmacol.* **2001**, *428*, 97−103.

(56) Gully, D.; Lespy, L.; Canton, M.; Rostene, W.; Kitabgi, P.; Le Fur, G.; Maffrand, J. P. Effect of the neurotensin receptor antagonist SR48692 on rat blood pressure modulation by neurotensin. *Life Sci.* **1996**, *58*, 665−674.

(57) Labbe-Jullie, C.; Barroso, S.; Nicolas-Eteve, D.; Reversat, J. L.; Botto, J. M.; Mazella, J.; Bernassau, J. M.; Kitabgi, P. Mutagenesis and modeling of the neurotensin receptor NTR1. Identification of residues that are critical for binding SR 48692, a nonpeptide neurotensin antagonist. *J. Biol. Chem.* **1998**, *273*, 16351−16357.

(58) Labbe-Jullie, C.; Botto, J. M.; Mas, M. V.; Chabry, J.; Mazella, J.; Vincent, J. P.; Gully, D.; Maffrand, J. P.; Kitabgi, P. [3H]SR 48692, the first nonpeptide neurotensin antagonist radioligand: characterization of binding properties and evidence for distinct agonist and antagonist binding domains on the rat neurotensin receptor. *Mol. Pharmacol.* **1995**, *47*, 1050−1056.

(59) Henry, J. A.; Horwell, D. C.; Meecham, K. G.; Rees, D. C. A structure−affinity study of the amino acid side-chains in neurotensin: N and C terminal deletions and Ala-scan. *Bioorg. Med. Chem. Lett.* **1993**, *3*, 949−952.

(60) Quéré, L.; Boigegrain, R.; Jeanjean, F.; Gully, D.; Evrard, G.; Durant, F. Structural requirements of non-peptide neurotensin receptor antagonists. *J. Chem. Soc., Perkin Trans.* **1996**, *2*, 2639−2646.

(61) Labbe-Jullie, C.; Deschaintres, S.; Gully, D.; Le Fur, G.; Kitabgi, P. Effect of the nonpeptide neurotensin antagonist, SR 48692, and two enantiomeric analogs, SR 48527 and SR 49711, on neurotensin binding and contractile responses in guinea pig ileum and colon. *J. Pharmacol. Exp. Ther.* **1994**, *271*, 267−276.

(62) Labeeuw, B.; Gully, D.; Jeanjean, F.; Molimard, J.; Boigegrain, R. Substituted 1-naphthyl-3-pyrazolecarboxamides which are active on neurotensin, their preparation and pharmaceutical compositions containing them. US005502059, 1996.

(63) Thomas, J. B.; Navarro, H.; Warner, K. R.; Gilmour, B. The identification of nonpeptide neurotensin receptor partial agonists from the potent antagonist SR48692 using a calcium mobilization assay. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 1438−1441.