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The amide linker in nonpeptide neurotensin receptor ligands plays a key role in calcium signaling at the neurotensin receptor type 2

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ABSTRACT

Compounds acting via the GPCR neurotensin receptor type 2 (NTS2) display analgesia in relevant preclinical models. The amide bond in nonpeptide NTS1 antagonists plays a central role in receptor recognition and molecular conformation. Using NTS2 FLIPR and binding assays, we found that it is also a key molecular structure for binding and calcium mobilization at NTS2. We found that reversed amides display a shift from agonist to antagonist activity and provided examples of the first competitive nonpeptide antagonists observed in the NTS2 FLIPR assay. These compounds will be valuable tools for determining the role of calcium signaling in vitro to NTS2 mediated analgesia.

The identification of novel analgesics is an ongoing challenge of discovery science. In particular, there is a need for new treatment options for alleviating chronic pain as no more than half of patients get adequate relief from currently existing medications.[1] It is thus significant that compounds acting via the GPCR neurotensin receptor type 2 (NTS2) are reported to be effective in managing both acute and chronic pain in animal models.[2–4] This analgesia is synergistic with opioid mediated analgesia and may offer compounds that either supplant opioids or that work in concert with existing opioid receptor-based drugs.[5,6] It has also been shown that the NTS2 receptor produces analgesia without the side effects of hypothermia and hypotension that are the hallmark of NTS1 interaction.[4,7,8] Collectively, these findings point to the NTS2 receptor as an attractive target to explore for treating acute and chronic pain with a lower adverse effect profile.

The NTS2 receptor is one of two GPCR's that modulate the action of the tridecapeptide neurotensin (NT, pGlu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu), which acts as both a neuromodulator and neurotransmitter in the CNS and periphery, overseeing a host of biological functions including regulation of CNS dopamine, hypothermia, hypotension and, nonopioid analgesia.[9–11] Compared with the NTS1 receptor, few selective compounds have been identified for the NTS2 receptor over the last forty years. The peptide ligands that established our current understanding of the physiological role of NTS2 include JMV-431[12,13] and NT79.[4] In addition to this, highly potent and ultra-selective peptide–peptoid hybrids with selectivity ratios reaching 12,000 and 22,000-fold have been identified recently.[14,15]

In search of NTS2 selective nonpeptide analgesics, we concluded that the delayed progress of NTS2 research compared with NTS1 might be due to the variable results obtained when the receptor is expressed in different cell lines. 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 as well as a neutral antagonist depending upon the cell expression system.[16–19] To better understand this issue, we analyzed the calcium mobilization at NTS2 with the goal of establishing a link between FLIPR assay activity and analgesia in vivo. Our initial goal was to identify a group of nonpeptide compounds that included full agonists, partial agonists and antagonists believing that this spectrum of activity would provide the tools necessary to establish the desired in vitro to in vivo correlation. We recently reported our initial efforts towards this goal that began with our establishment of a baseline of FLIPR activity for compounds commonly described in

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NTS2 receptor research. We found that compounds SR48692 (**1a**) and SR142948a (**2**) Chart 1, which are known antagonists in vivo and at NTS1, were full agonists at NTS2 in agreement with literature reports.[16,18–20] We also found that the NTS2 selective compound Levocabastine (**3**), an analgesic in vivo, was found to be a potent partial agonist in vitro.[3] NT on the other hand, which is also an analgesic in vivo, was found to be an antagonist in the FLIPR assay. Collectively, this preliminary data set aligned partial agonist and antagonist activity with analgesia in animal models.

We reported recently using the same FLIPR assay to expand the pool existing of NTS2 selective nonpeptide compounds to include NTRC-739 (**4**), NTRC-808 (**5**), and NTRC-824 (**6**).[21–23] The first two are potent partial agonists while the latter compound mimics the activity of the endogenous ligand NT in the FLIPR assay. Compounds **4** and **5** were identified during SAR studies that focused on changes to the perimeter of **1a**. In the current Letter, we examined the effect on calcium signaling resulting from changes to the central amide bond of **1a** and **1b**. This study led us to compound **7**, the first competitive (surmountable) antagonist that we have identified using the NTS2 FLIPR assay. The details of this effort are provided herein.

In the pioneering studies with **1a** that led to the first NTS1 antagonist pharmacophore model, Quéré demonstrated that the amide group forms an intramolecular H-bond with the pyrazole nitrogen in the crystal structure of **1a**.[24,25] This structure thus plays an important role in molecular conformation and overall structural rigidity. It could also act as an H-bond donor/acceptor and could therefore contribute significantly to the process of ligand/receptor recognition. Given this background, we imagined that the amide could also play a critical role in calcium signaling and receptor recognition at NTS2.

To carry out our investigation, we synthesized and tested target compound **12** (Scheme 1), the reduced amide variant of **1b**, and also compounds **7** and **16b**–**18b** (Scheme 2) that possessed the reversed amide group (a comparison of reversed and conventional amide structures is shown in the Table 1 chart).

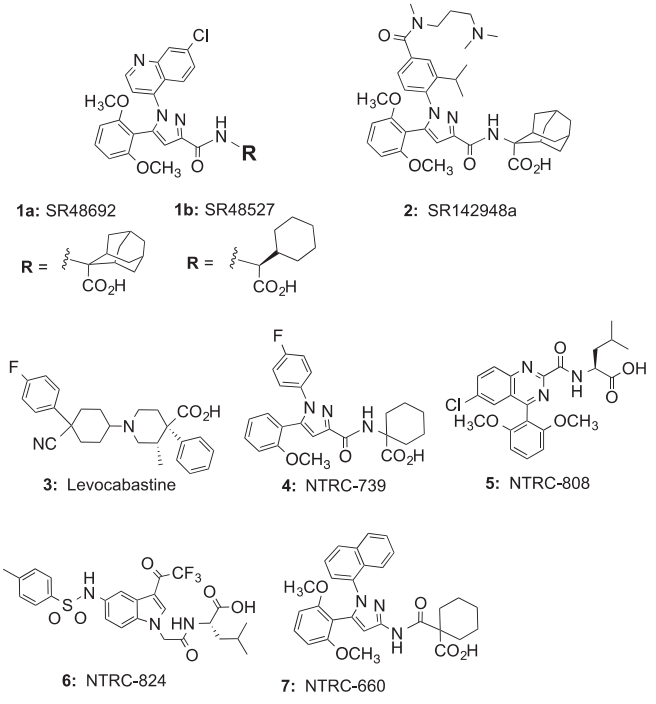
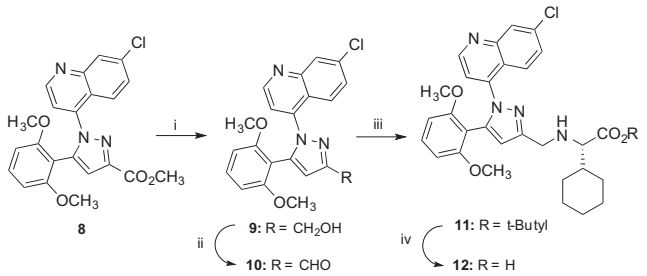


Chart 1. NTS2 FLIPR agonists (**1a**,**b**,**2**), NTS2 subtype-selective potent partial agonists levocabastine (**3**), NTRC-739 (**4**) and NTRC-808 (**5**), NTS2-selective noncompetitive antagonist NTRC-824 (**6**) and newly discovered competitive antagonist NTRC-660 (**7**).



Scheme 1. Synthesis of target compound **12**. Reagents and conditions: (i) LAH, THF, rt, 16 h; (ii) MnO_2, CH_2Cl_2, rt, 16 h; (iii) L-cyclohexylglycine *tert*-butyl ester, $NaBH(OAc)_3$, MeOH, rt, 16 h; (iv) TFA, CH_2Cl_2, rt, 12 h.

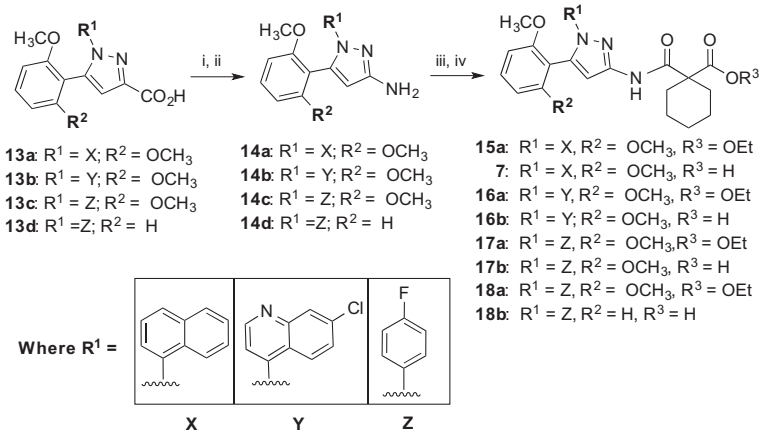
The synthesis of the reduced amide target compound **12** was accomplished as illustrated in Scheme 1. Thus, methyl 1-(7-chloroquinolin-4-yl)-5-(2,6-dimethoxyphenyl)-1*H*-pyrazole-3-carboxylate[21] (**8**) was reduced using lithium aluminum hydride (LAH) to give alcohol **9** that was subsequently oxidized using MnO_2 to give aldehyde **10**. This intermediate was then coupled reductively with L-cyclohexylglycine *tert*-butyl ester to give **11**. Target compound **12** was then available via deprotection of **11** using trifluoroacetic acid (TFA) in methylene chloride.

The synthesis of target compounds **7** and **16b**–**18b** was accomplished using a Curtius rearrangement and the appropriate pyrazole-3-carboxylic acid (**13a**–**13d**) as illustrated in Scheme 2.[21] Thus, acids **13a**–**13d** were heated with diphenylphosphoryl azide (DPPA) in ethanol and 1,4-dioxane and triethylamine under reflux overnight and the resulting carbamates were subsequently hydrolyzed using 10% NaOH in ethanol to give intermediates **14a**–**14d**. From this point the target compounds were obtained by coupling **14a**–**14d** with 1-(ethoxycarbonyl)cyclohexanecarboxylic acid (**21**, Scheme 3) using *O*-benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU) and triethylamine to give ester intermediates **15a**–**18a**. Hydrolysis of these ethyl esters using 1N LiOH in dioxane provided target compounds **7**, **16b**, **17b** and **18b**.

The synthesis of 1-(ethoxycarbonyl)cyclohexanecarboxylic acid (**21**) used to prepare target compounds **7**, **16b**–**18b** was accomplished as illustrated in Scheme 3.[26] Thus, the alkylation/cyclization of *tert*-butyl ethyl malonate (**19**) and 1,5-dibromopentane with NaH gave the cyclic mixed diester **20**.[27] Subsequent deprotection using trifluoroacetic acid (TFA) then gave **21**. We initially tried to hydrolyze the ethyl ester first leaving the tertiary butyl ester in place, but found that this product was difficult to isolate from a basic work up. Doing the TFA deprotection first, on the other hand, led to **21** that could be used in crude form following removal of solvents.

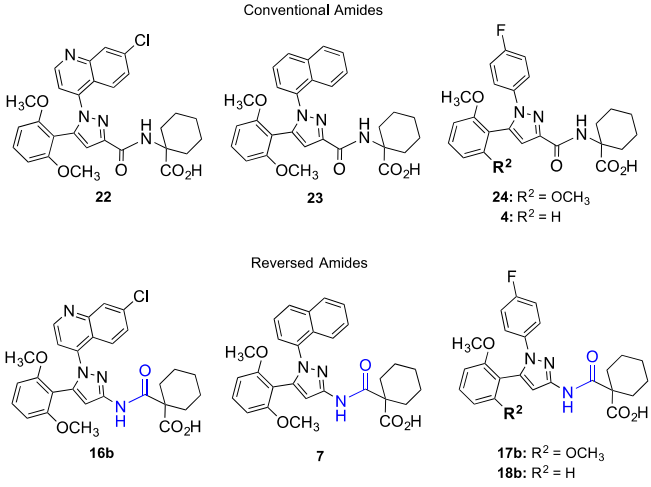
In Table 1, we present the data obtained from the FLIPR assays and binding assays for our test compounds. This includes the data for the reduced amide **12** followed by the data for the reversed amides **7** and **16b**–**18b**. Accompanying the data for **12**, we provided the data (obtained previously) for the unreduced amide **1b** (Chart 1). The data for the reversed amides are presented along with the data previously obtained for their conventional amide compounds (**4**, **22**–**24**). This is displayed above the data of their reversed amide counterparts so that the impact of the structural change is more readily appreciated.

We found that compound **12**, with a reduced amide bond, showed no activity at either NT receptor in the FLIPR assays or the binding assays. Since compound **1b** is quite active, we find that this functional group, intact, is necessary for activity at NT receptors. There are several explanations for this observation. It is



Scheme 2. Synthesis of target compounds **7** and **16b**–**18b**. Reagents and conditions: (i) DPPA, Et_3N, 1,4-dioxane, refl., 14 h; (ii) 10% NaOH, refl., 5 h; (iii) (ethoxycarbonyl) cyclohexanecarboxylic acid (**21**, Scheme 3), HBTU, Et_3N; (iv) LiOH, 1,4-dioxane.

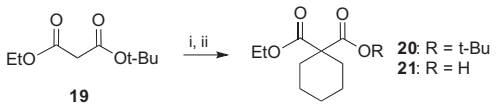
Table 1
FLIPR and binding assay data for reference compound **1b**, reduced amide **12**, reverse amides **7**, **16b**, **17b** and **18b**, and conventional amides **4**, **22**–**24** at rNTS1 and rNTS2 receptors



	FLIPR Assays				Binding Assays [a]	
	NTS1	NTS2			NTS1	NTS2
#	K_e [b]	EC_{50}	E_{max} [c]	K_e	K_i	K_i
1b[d]	23 ± 6	217 ± 19	86 ± 3		ND [e]	644 ± 90
12	NA [f]	NA		NA	>10 μM	>10 μM
22[d]	157 ± 45	29 ± 2	78 ± 7		177 ± 73	151 ± 67
16b	4388 ± 539	NA		3046 ± 992	7181 ± 1512	140 ± 18
23[d]	230 ± 79	18 ± 1.6	35 ± 1		1210 ± 225	116 ± 39
7	4812 ± 253	NA		181 ± 38	10162 ± 1487	128 ± 13
24[d]	>10 μM	19 ± 3	12 ± 0.5		3210 ± 879	140 ± 29
17b	NA	55 ± 17	10 ± 2		>25 μM	271 ± 53
4[d]	NA	12 ± 6	7 ± 2		>25 μM	153 ± 20
18b	NA	951 ± 268	13 ± 2		>25 μM	6339 ± 755

[a] [^{125}I]NT.
[b] K_e, EC_{50}, and K_i values are nM ± SEM.
[c] E_{max} value is % of **2**.
[d] Data, except for NTS1 binding, is from Ref. 22.
[e] Not determined.
[f] Not active.

believed that this functional group acts as an H-bond donor/acceptor and thus helps stabilize the ligand to receptor interaction. From this perspective, the data suggests that this H-bond donor/acceptor is a key feature of the ligand recognition architecture. An alternative explanation exits however. Rather than resulting from the loss of an H-bond interaction, it could be that the fundamental shift in character of the amide nitrogen from neutral to basic may be the cause of the loss of activity. This would enable zwitterion



Scheme 3. Synthesis of (ethoxycarbonyl)cyclohexane carboxylic acid (**21**). Reagents and conditions: (i) *tert*-butyl ethyl malonate, NaH, DMF, 1,5-dibromopentane, rt, 16 h; (ii) CH_2Cl_2, TFA, rt, 3 h.

formation and in turn would change not only the shape of the ligand but also the ionic character of the resulting molecule. This could, in turn, render it unrecognizable to the associated binding domains of the NT receptors and thereby eliminate binding and thereby signaling.

Unlike **12**, the reversed amide derivatives (**7** and **16b**–**18b**) were active at both NTS1 and NTS2 and also demonstrated SAR. Considering the NTS1 receptor first, we found that all of the reversed amide compounds lacked agonist activity (data not shown). This is inline with the parent compounds (**4**, **22**–**24**) that that were either inactive or antagonists at NTS1. Thus, reversing the amide does not convert antagonists into agonists at this receptor. However, the antagonist activity (K_e data) found for the reverse amides **16b** and **7** was much weaker than that found for the conventional amide compounds (**22** and **23**). This degradation in activity suggests that the amide group is a key component for antagonist recognition and blockade of NT mediated calcium signaling at NTS1.

The 4-fluorophenyl substituted reversed amide derivatives **17b** and **18b** did not show antagonist activity in the NTS1 FLIPR assay but neither did their conventional amide analogues (**24** and **4**). Thus, reversing the amide does not over ride the NTS2 selectivity promoted by the 4-fluorophenyl substituent. In the binding assay at NTS1, this trend continued with **17b** showing less affinity for NTS1 compared with the conventional amide compound **24**.

In the NTS2 FLIPR assay, we found that the reverse amide **16b** showed a significant change in behavior relative to **22**. Here, the potent full agonist activity of **22** was supplanted with weak antagonist activity in the reversed amide **16b**. While this activity was weak, it was remarkable since its antagonist behavior was different from that reported previously for NT or **6**. In this case, **16b** showed competitive (surmountable) antagonist activity whereas both NT and **6** demonstrated insurmountable antagonism of the calcium release mediated by compound **2**.[21,22] In fact, **16b** was the first competitive antagonist observed in our studies of nonpeptide NTS2 compounds. The napthyl substituted compound **7** showed substantially improved antagonist activity compared with **16b** (K_e of 181 vs 3046 nM, respectively). As before, the potent partial agonist activity of the conventional amide (**23**) gave way to competitive antagonist activity in the reversed amide **7**.

In Figure 1, the antagonist behaviors of compounds **6** and **7** versus the agonist **2** are illustrated. Compound **7** shows competitive (surmountable) antagonist activity versus the agonist **2** as it shifts the agonist response curve to the right. On other hand, compound **6** demonstrated insurmountable antagonist activity versus compound **2** in the FLIPR assay as it shifts the curve of **2** to the right but also lowers the maximal response of **2** as its concentration is increased. NT demonstrates behavior similar to **6** (not shown in Fig. 1).[22]

In the binding assay, compound **7** revealed that it possessed good affinity for NTS2 but quite low affinity for NTS1 and while it does not show the >100-fold separation characteristic of a selective compound, it possesses a 79-fold preference for NTS2 versus NTS1. Together with its competitive antagonist nature in the FLIPR assay, its affinity and preference for NTS2 makes

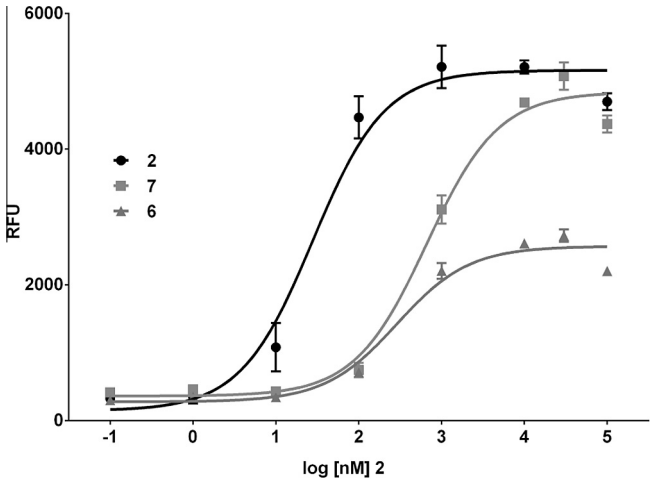


Figure 1. K_e assays were run against dose–response curves of the control agonist **2** in CHO-k1-rNTS2 cells. Compound **7** displays competitive (surmountable) antagonist activity versus the agonist **2** as seen in the rightward shift of the dose–response curve of **2**. Compound **6** illustrates insurmountable antagonist activity as the curve of **2** is shifted to the right but its maximal response is damped.

analogue **7** a useful addition to the toolkit needed to probe the relevance of calcium signaling at NTS2 to analgesia in vivo.

Unlike the naphthyl substituted compound **7**, the 4-fluorophenyl substituted reverse amide compounds **17b** and **18b** did not display a shift from agonist to antagonist at NTS2, Table 1. Instead, **17b** possessed a profile of activity similar to that of the conventional amide **24** but with roughly half of the activity across the various assays. The reverse amide **18b**, on the other hand, was found to be virtually inactive at both NT receptors. Though limited in scope, the data for **17b** is in line with earlier results that suggested that compounds with the 4-fluorophenyl substitution do not behave like those with the naphthyl substitution, perhaps as a consequence of binding to the receptor in an alternate manner. Viewed from this perspective then, the lack of a shift from agonist to antagonist activity for **17b** suggests that the amide group is not situated in a manner conducive to this transformation providing additional evidence that these two types of compounds bind NTS2 differently.

Overall, the information provided by this study, together with the work of Quéré, demonstrates that the amide bond in compounds derived from **1a** plays a critical role in mobilization of calcium and receptor recognition at both GPCR NT receptors. At the NTS2 receptor, we found that reversing the amide can trigger antagonist activity that is of a competitive nature. This feature differentiates compound **7** from the insurmountable antagonists described in previous work.[22] As such, compound **7** provides an additional tool to further our understating of the relevance of calcium signaling at NTS2 to analgesia in relevant animal models. These studies are currently in progress and will be reported in the near future.

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Supplementary data

Supplementary data (experimental details for compound synthesis and biological testing as well as catalog numbers of the

compounds identified for testing in our database mining) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2015.03.083. These data include MOL files and InChiKeys of the most important compounds described in this article.

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