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REVIEW

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Opening the amino acid toolbox for peptide-based NTS2-selective ligands as promising lead compounds for pain management

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Chronic pain is one of the most critical health issues worldwide. Despite considerable efforts to find therapeutic alternatives, opioid drugs remain the gold standard for pain management. The administration of μ-opioid receptor (MOR) agonists is associated with detrimental and limiting adverse effects. Overall, these adverse effects strongly overshadow the effectiveness of opioid therapy. In this context, the development of neurotensin (NT) ligands has shown to be a promising approach for the management of chronic and acute pain. NT exerts its opioid-independent analgesic effects through the binding of two G protein-coupled receptors (GPCRs), NTS1 and NTS2. In the last decades, modified NT analogues have been proven to provide potent analgesia in vivo. However, selective NTS1 and nonselective NTS1/NTS2 ligands cause antinociception associated with hypothermia and hypotension, whereas selective NTS2 ligands induce analgesia without altering the body temperature and blood pressure. In light of this, various structure–activity relationship (SAR) studies provided findings addressing the binding affinity of ligands towards NTS2. Herein, we comprehensively review peptide-based NTS2-selective ligands as a robust alternative for future pain management. Particular emphasis is placed on SAR studies governing the desired selectivity and associated in vivo results.

KEYWORDS
neurotensin, opioid-independent analgesic effect, pain management, peptide NTS2-selective ligands, structure–activity relationships

1 | INTRODUCTION

Despite considerable efforts made over the past decades, pain remains one of the world's major health burdens. According to the Center for Disease Control and Prevention (USA), only in the United States, approximately 50 million adults suffer from chronic pain, which consequently has a significant impact on their daily lives and work.[1] To identify the most affected populations, a new concept has been coined, namely *high-impact chronic pain* (HICP), which couples the pain duration to the disabilities it causes. To date, patients

with moderate to severe pain are commonly treated with μ-opioid receptor (MOR) agonists, such as morphine, methadone, fentanyl and oxycodone.[2] Unfortunately, opioid treatment programs (OTPs) are associated with multiple adverse effects, such as nausea, vomiting, dizziness, constipation, hormonal dysfunction and respiratory depression, among others.[3] In addition, psychological and physical dependence, as well as analgesic tolerance, seriously compromises standard treatment protocols.[4,5] As such, it is evident that OTPs are still problematic and difficult to enforce over the long term. In recent years, a plethora of independent studies has been conducted in an effort to find a valid alternative to OTPs. Among these, the development of non-opioid analgesics has proven to be a particularly promising approach.[6,7]

Neurotensin (NT) is a tridecameric neuropeptide (pyroGlu^{1}-Leu^{2}-Tyr^{3}-Glu^{4}-Asn^{5}-Lys^{6}-Pro^{7}-Arg^{8}-Arg^{9}-Pro^{10}-Tyr^{11}-Ile^{12}-Leu^{13}-OH; Figure 1), which was firstly isolated from bovine hypothalamus extracts in 1973[8] and from bovine intestinal tissue a few years later.[9] Like all neuropeptides, NT is cleaved from a biologically inactive protein precursor, namely pro-NT/NN, which also contains the NT-like hexapeptide neuromedin N (NN, H-Lys-Ile-Pro-Tyr-Ile-Leu-OH).[10] The effects of NT are mediated through the binding and activation of three receptors[11]: NTS1 and NTS2, which belong to the G protein-coupled receptor (GPCR) superfamily, and NTS3, a sortilin-like receptor with a single transmembrane domain. In attempting to define the NT pharmacophore, it has been clearly demonstrated that the *C*-terminal fragment H-Arg^{8}-Arg^{9}-Pro^{10}-Tyr^{11}-Ile^{12}-Leu^{13}-OH, also known as NT8–13, represents the minimal active sequence of NT.[12–14] For this reason, the majority of subsequently developed NT analogues bear only the NT8–13 sequence or variations thereof. With regard to metabolism, the proteolytic degradation of NT has been extensively studied and several critical cleavage sites identified, such as Tyr^{11}-Ile^{11}, Pro^{10}-Tyr^{11} and Arg^{8}-Arg^{8} peptide bonds (Figure 1). Several Zn-metallo-endopeptidases are involved in the proteolytic process[15–17]: In particular, thimet oligopeptidase (EC 3.4.24.15) cleaves the Arg^{8}-Arg^{9} bond, enkephalinase (EC 3.4.24.11) is responsible for the cleavage between Tyr^{10} and Ile^{11} and neurolysin (EC 3.4.24.16) and enkephalinase act at the Pro^{10}-Tyr^{11} bond. Considering its ubiquitous distribution, neurolysin is considered to represent the main player in NT inactivation.[18,19] Additionally, human carboxypeptidase A4 (CPA4) contributes to NT degradation, via cleavage of the *C*-terminal residue.[20] As a consequence of all the above hydrolytic activities, NT exhibits a plasma half-life ($t_{1/2}$) of less than 2 min.

NT exerts its effects in both the central nervous system (CNS) and in the periphery. In the brain, NT-producing neurons are involved in dopamine transmission (mainly through the D2 receptor), which attributes to NT a neuroleptic role in dopamine-related diseases, such as schizophrenia, Parkinson's and Huntington's diseases.[21] NT also influences hormone release from the anterior pituitary gland and hypothalamus[22] and is involved in feeding regulation,[23] gut motility,[24,25] and modulation of the cardiovascular system.[26] NT receptors have also been shown to be present on serotonergic and glutamatergic neurons.[22] Intracerebral injection of NT induces significant and prolonged hypothermia, suggesting its involvement in the thermoregulatory homeostasis.[27,28] NT-induced hypothermia, useful as neuroprotective treatment,[29] is primarily mediated by NTS1 binding and activation.[30,31] Similarly, NTS1 seems to be mainly responsible for the NT-induced hypotensive effect after central and peripheral administration.[32,33]

Of high relevance to the current review, the influence of NT in pain transmission has been reported in rodent studies[34] and confirmed by a number of other groups.[35–37] NT exerts a profound opioid-independent analgesic effect through binding with NTS1 and NTS2, as widely reported in the literature.[11,37–40] Although the antinociceptive effects are mediated by both receptors,[41,42] selective targeting of NTS2 has led to promising results in terms of analgesia with limited undesirable effects, such as NTS1-induced hypothermia and vasodilation.[43–45]

To direct the affinity of the newly developed ligands to NTS2, unnatural amino acids and peptide backbone modifications were incorporated into the NT8–13 pharmacophore. These types of chemical modifications are aimed at exploiting the differences between the NTS1 and NTS2 receptor binding sites while improving NT's half-life for therapeutic use. Herein, we describe the most important features

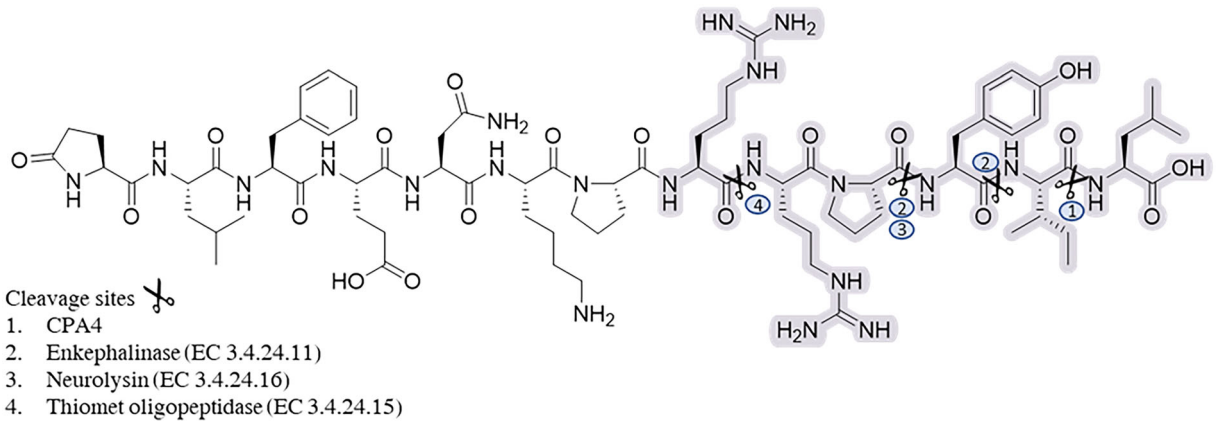


FIGURE 1 Neurotensin with the pharmacophore NT8–13 shown in grey, and iconic proteolytic enzymes acting at the cleavage sites

of NT analogues directing the selectivity and SAR studies in which NTS2-selective ligands were developed and illustrate their subsequent application in the treatment of pain.

2 | HISTORICAL MILESTONE NT LIGANDS

Between 1990 to 2010, several SAR studies yielded potent NT ligands that are still used today as reference pharmacological tools, positive controls and lead compounds for further development of novel NT derivatives. Most of them focus only on the pharmacophore moiety and differ from the native NT8–13 sequence only in the presence of unnatural amino acids and backbone modifications. In this section, we provide an overview of the key ('*milestone*') NT ligands and recapitulate their biological characterization (Figure 2). Thanks to these studies, the individual role of subtype receptors has been elucidated and different NT-mediated effects in vivo, such as hypothermic and antipsychotic-like effects, impact on blood pressure and analgesic response in different pain models have been demonstrated.

JMV449 is an important historical NT analogue, first reported by Lugrin and co-workers in 1991.[51] With respect to NT8–13, JMV449 differs only by the presence of a reduced pseudopeptide Ψ[CH_2NH] bond between the two Lys residues at positions 8 and 9 (Figure 2), which produces an additional positive charge along the peptide backbone under physiological conditions. This backbone modification was well tolerated, showing high affinity binding to hNTS2 (K_i = 0.29 nM) with a clear improvement in affinity over the pharmacophore NT8–13 (i.e., K_i = 2.29 nM).[46] Conversely, K_i at hNTS1 was maintained (JMV449 K_i = 2.02 nM vs. NT8–13 = 1.65 nM), with an NTS1/NTS2 selectivity ratio equal to 7. Importantly, the presence of the reduced bond slightly improved plasma stability, now up to 8 min, due to the bypass of thimet oligopeptidase's proteolytic activity.

Another fully characterized, reduced pseudopeptide Ψ[CH_2NH] bond-containing NT analogue is PD149163 (Figure 2), which was synthesized by Wustrow and co-workers in 1995.[52] Unlike most NT analogues, PD149163 bears a *C*-terminal ester group, which confers a prodrug profile.[53] The ethyl ester portion can be rapidly hydrolysed in the blood through the action of esterases, releasing the biologically active form. Trp and *tert*-leucine (Tle) were inserted at positions 11 and 12, respectively, and the amide bond between the pair of basic Lys residues was reduced to the pseudopeptide Ψ[CH_2NH] bond, as in JMV449. With respect to native NT, PD149163 possesses modifications at all critical cleavage sites, resulting in proteolytic resistance.[52] Biological evaluation at both NT receptors showed a clear preference towards the subtype 1 receptor, with a K_d value of 159 nM, and displaying comparable in vivo effects to NT.[47] More specifically, intrathecal administration of PD149163 led to significant antiallodynic and antihyperalgesic effects in rat models of neuropathic pain.[54] It also decreased pain responses in the formalin test.[41]

NT69L was synthesized in 2000 by Tyler-McMahon et al.[48] The chemical structure of NT69L differs from NT8–13 by the presence of *N*MeArg[8], Lys[9] and the replacement of Ile[12] and Tyr[11] with the unnatural amino acids Tle[12] and neo-Trp[11], respectively. Together, these modifications conferred to NT69L high resistance to enzymatic degradation. As shown in Figure 2, NT69L exhibited comparable nM-range binding to both hNTS1 and hNTS2. Central administration of NT69L produced antipsychotic-like effects,[55] a significant reduction in pain awareness in the formalin test[41] and attenuation of neuropathic pain

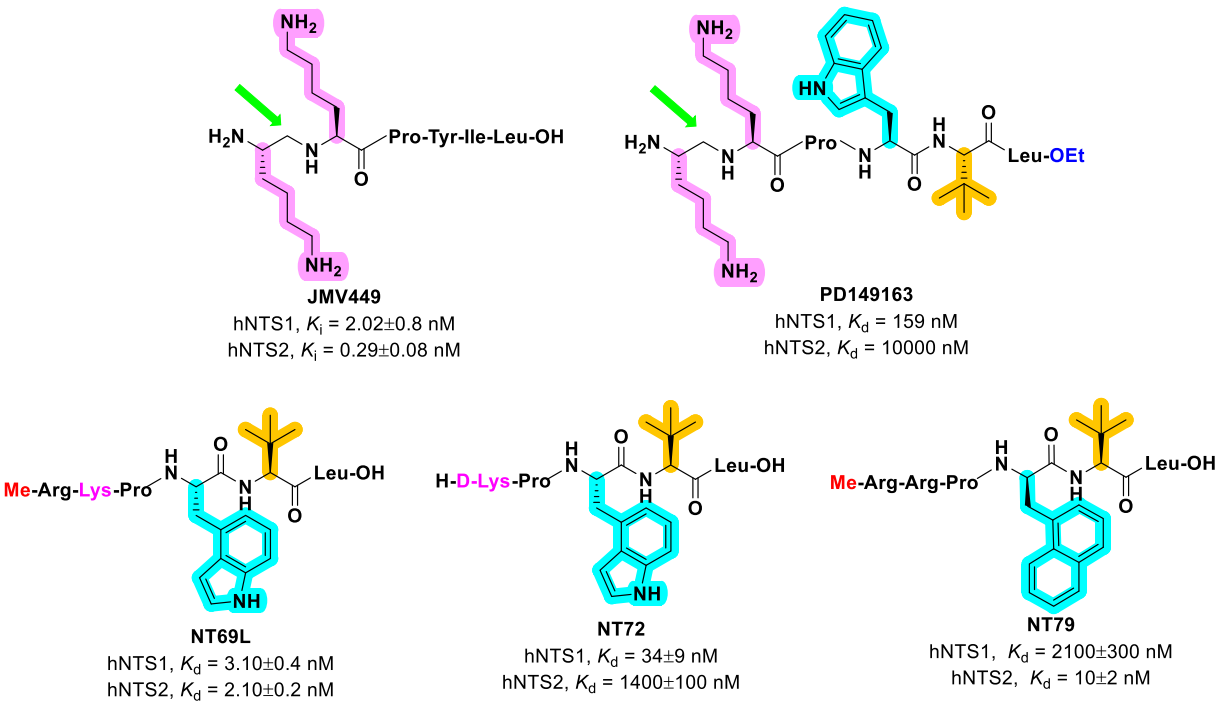


FIGURE 2 Chemical structures of milestone NT ligands and their relative binding affinity towards hNTS1 and hNTS2. K_i's for NTS1 and NTS2 are values reported in the original articles: JMV449,[46] PD149163,[47] NT69L,[48] NT72[49] and NT79.[50]

in rats.[54] Intraperitoneal administration of NT69L produced potent and persistent analgesia in hot plate test[48] and limited acetic acid-induced writhing.[42] Along with this, a significant reduction in body temperature was recorded, which could be useful in asphyxia cardiac arrest, as reported by Katz et al.[56] Nonetheless, a rapid onset of tolerance to NT69L effects, including analgesia, was observed.[57]

NT72 was originally reported by Richelson and co-workers.[49] As shown in Figure 2, it contains only five amino acids with the basic residue at position 8 being absent. Additionally, D-Lys was introduced at position 9 along with neo-Trp and Tle at positions 11 and 12, respectively. Interestingly, the introduction of D-Lys at position 9 seems to be a key feature for NTS1 selectivity, leading to a K_d value in the low nanomolar range at hNTS1, whereas a loss of binding to NTS2 was recorded, with a 41-fold selectivity of NTS1 over NTS2. In vivo evaluation of NT72 resulted in potent analgesic effects in the hot plate test and a significant reduction of acetic acid-induced writhing and pain responses.[42] Additionally, this modified pentapeptide exhibited a comparable analgesic effect in NTS2 knock-out mice, indicating that its effect is NTS1-mediated.[42]

As another milestone ligand, NT79 can be considered one of the first reported NTS2-selective ligands.[50] Compared with NT8–13, NT79 contains Tle^{12}, D-1-naphtylalanine (D-1-Nal) at position 11 and NMeArg^8. These modifications led to K_d values in the low nanomolar range for NTS2 (Figure 2), associated with moderate selectivity (NTS1/NTS2 = 210). During in vivo evaluation, NT79 showed a potent analgesic effect in a visceral pain model (writhing test, ED_{50} = 0.14 μg/kg), whereas the analgesic effect was not observed in a thermal pain model (hot plate test). Considering the weak binding to NTS1, NT79 caused a limited decrease in body temperature (−1.5°C that returned to baseline after 1 h). By analogy, blood pressure was also not influenced upon the administration of this compound.[50]

As described above, the NT ligands reported here paved the way for the development of NT ligands. The replacement of Arg^8-Arg^9 portion with Lys^8-Lys^9 has become one of the most common modifications, as has the introduction of Tle instead of Ile at position 12. Observations made through the development of NT79 clarified the key role played by the residue at position 11 in binding to NTS2.

Regarding the plasma stability, methylation of Lys^8, reduction of the pseudopeptide Ψ[CH_2NH] bond between Lys^8-Lys^9, introduction of unnatural amino acids at position 11 and Tle^{12} proved to improve the half-life of the NT analogues.

Despite the promising results obtained, historical 'milestone' NT ligands lack selectivity towards NTS2: In fact, only NT79 showed an appreciable preference towards NTS2 (SI = 210), along with a slight decrease in binding affinity compared with NT8–13.

In addition, because of the inability to cross the BBB, all NT ligands require intracerebroventricular, intraperitoneal or intrathecal administration. Since NT receptors involved in analgesia are distributed in the CNS, a good candidate for pain management should cross the BBB, in order to reach appropriate concentrations in cerebrospinal fluid.

Building further on the milestone ligands depicted in Figure 2, NTS2-selective ligands were developed, which will be described in the following sections.

3 | TOWARDS PEPTIDE-BASED NTS2 LIGANDS

3.1 | Peptide-peptoid hybrids

In 2011, Einsiedel et al. reported a SAR study in which backbone modifications were inserted at position 11.[58] However, as a first step, a large set of previously reported NT analogues was prepared to re-evaluate their affinity for hNTS1 and hNTS2 because only binding to NTS1 was originally reported. The Ala-scan series confirmed the key role played by Tyr^{11} and Leu^{13} in hNTS1 binding: Analogue **1** (Table 1), bearing Ala^{11}, showed a double-digit nanomolar affinity towards hNTS2 (K_i = 83 nM), whereas high binding was also eliminated for analogue **2**, containing Ala^{13} (K_i = 1300 nM).[59] Subsequently, compounds incorporating D-amino acids were revisited[60,61]: With the exception of (D-Arg^8)NT8–13, which showed a K_i equal to 0.61 and 5.4 nM towards NTS1 and NTS2, respectively, all analogues exhibited lower affinity towards both NT receptors when compared with NT8–13, and no appreciable selectivity was recorded. Similarly, with the exception of the β^3-$homolle^{12}$ derivative **3**, which showed a K_i value towards hNTS2 comparable with that of native NT (K_i = 5.4 nM) and a selectivity index (SI, equal to K_i NTS1/K_i NTS2) of 46 over hNTS1, the β^3-homo-amino acid-bearing ligands were unselective. Finally, peptoid derivative **4**, which bears the common Arg^8-Arg^9 to Lys^8-Lys^9 substitution, exhibited a slight preference for NTS2 (SI = 30), despite a significant loss of affinity.

On the basis of these findings, a new series of peptide-peptoid hybrids was developed (Table 2), in which the N-$homoTyr^{11}$ (N-$hTyr^{11}$) peptoid residue was inserted at position 11, and the impact of Lys and Arg at positions 8 and 9 was assessed.[58] Among the

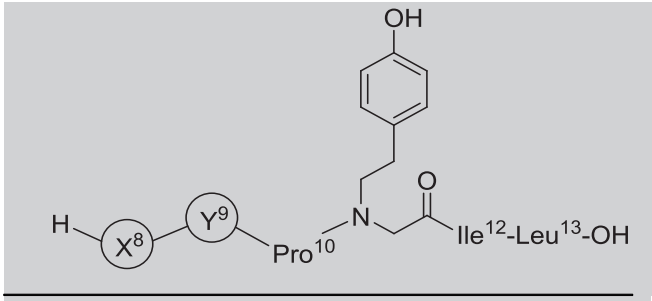
TABLE 1 Binding affinity of the most promising Ala, β^3-h and peptoid derivatives

Compounds	Modifications	K_i, nM		
		hNTS1	hNTS2	SI NTS1/NTS2
NT8–13	-	0.59	4.9	0.12
1	[Ala^{11}]NT8-13	1300	83	16
2	[Ala^{13}]NT8-13	1100	1300	0.85
3	[β^3-$hIle^{12}$]NT8-13	250	5.4	46
4	[Lys^8-Lys^9-$NTyr^{11}$]NT8-13	30,000	1000	30

Note: Adapted from research article published by Einsiedel and co-workers.[58]

synthesized analogues, the best ligands **5–8** showed comparable and single-digit nanomolar K_i values towards hNTS2, whereas moderate differences were observed in terms of selectivity. In particular, introduction of Lys at position 9 led to the most NTS2-selective analogue **6** (SI = 12,000), whereas the same substitution at position 8 gave analogue **7** with an almost sixfold lower SI value than **6**. No significant difference in binding affinity was observed between analogues **5** and **8**, which bear the same residues (either Arg or Lys) at positions 8 and 9. Additionally, derivatives of ligand **5** (not shown), in which the phenolic hydroxyl was removed or replaced by a methoxy group, showed detrimental results compared with the parent compound **5**. Similarly, substitution of the phenol moiety with a 2-pyridyl ring was poorly tolerated. The hexapeptide H-Arg-Arg-Pro-hTyr-Ile-Leu-OH (not shown) exhibited almost comparable binding affinity towards both receptors (NTS1, K_i = 210 and NTS2, K_i = 17, SI = 12), supporting the key role played by the *N*-hTyr[11] peptoid.

TABLE 2 NT-peptoid/peptide analogues with *N*-hTyr at position 11 and their biological evaluation



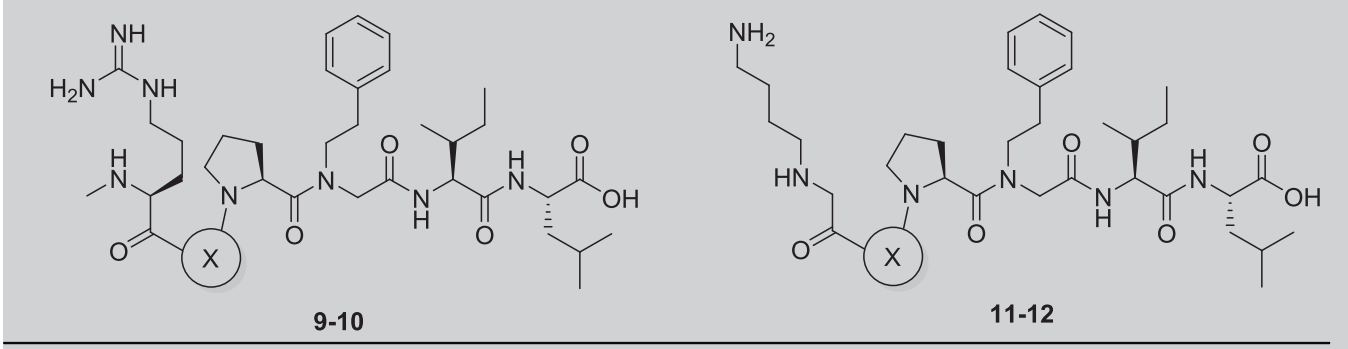
			K_i (nM)		
Cmp	**X⁸**	**Y⁹**	**hNTS1**	**hNTS2**	**SI NTS1/NTS2**
5[58]	Arg	Arg	28,000	8.8	3200
6	Arg	Lys	80,000	6.7	12,000
7	Lys	Arg	8300	4.4	1900
8	Lys	Lys	32,000	4.3	7400

Note: Adapted from research article published by Einsiedel and co-workers.[58]

As mentioned above, the introduction of *N*-hTyr at position 11 led to selective analogues for NTS2.[58] Based on these findings, Held and co-workers developed metabolically stable NTS2 ligands via the peptide-peptoid strategy with a peptoid residue at position 11 and modifications at positions 8 and 9 (Table 3).[62] Indeed, the terminal amino group was methylated and the *N*-(4-aminobutyl)glycine residue (denoted as *N*lys) was inserted at position 8. Additionally, Arg and Lys in position 9 were alternatively inserted. Apart from compound **11**, all the modified NT analogues showed higher SI values, compared with lead compound **5**. Methylation (compounds **9** and **10**) and *N*lys insertion (compounds **11** and **12**) at position 8 were well-tolerated by NTS2, providing single-digit nanomolar K_d values, whereas low NTS1 binding was recorded. Compounds **10** and **12** bearing Lys at position 9 showed poor binding towards the receptor subtype 1, with the highest selectivity displayed for NTS2. Of note, analogue **10** exhibited an impressive proteolytic stability in serum degradation assays, with a plasma half-life of over 32 h. The single introduction of *N*-hTyr residue in lead compound **5** slightly improved the half-life value (ca. 1 h), whereas a single *N*-methylation at position 8 (as in H-*N*Me-Arg-Lys-Pro-Tyr-Ile-Leu-OH) led to a plasma half-life of approximately 12 h.[62] These data confirmed that the amide bond between two basic amino acids could be considered the primary cleavage site.

In 2013, Held carried out a SAR study in which modifications at position 10 were assessed, whereas the *N*-hTyr was maintained at position 11.[63] Starting with lead compound **5**, several (substituted) proline surrogates were introduced at position 10, in order to evaluate their impact on affinity and selectivity towards NTS2 (Table 4).

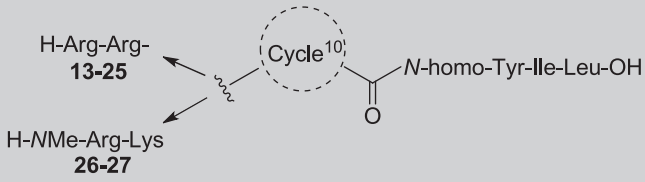
TABLE 3 Peptide-peptoid derivatives with modifications at positions 8 and 9 and their activity towards both NTS1 and NTS2



Cmp	**X⁹**	**NTS1, K_i (nM)**	**NTS2, K_i (nM)**	**SI NTS1/NTS2**
5[62]	-	31,000	8.0	3900
9	Arg	44,000	7.2	6100
10	Lys	61,000	2.8	22,000
11	Arg	47,000	110	430
12	Lys	55,000	5.2	11,000

Note: Adapted from research article published by Held and co-workers.[62]

TABLE 4 Biological evaluation of NT analogues with proline residue differently substituted



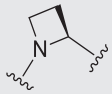
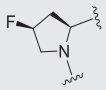
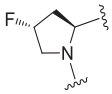
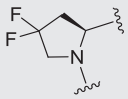
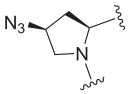
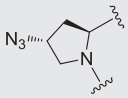
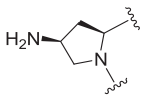
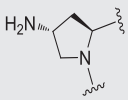
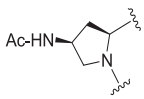
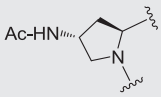
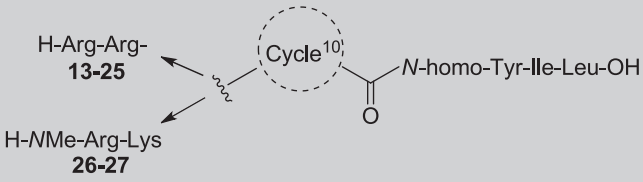
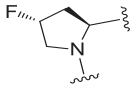
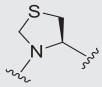
Cmp	Cycle	K_i (nM) NTS1	NTS2	SI NTS1/NTS2
5		31,000	8.0	3900
13		59,000	91	650
14		23,000	24	930
15		60,000	23	2600
16		66,000	10	6600
17		25,000	340	67
18		44,000	74	590
19		67,000	30	2200
20		59,000	190	310
21		57,000	83	690
22		>100,000	1800	55
23		59,000	880	67
24		20,000	200	100
25		55,000	52	1100

TABLE 4 (Continued)



Cmp	Cycle	K_i (nM) NTS1	NTS2	SI NTS1/NTS2
26		70,000	8.1	8600
27		39,000	16	2400

Note: Adapted from research article published by Held and co-workers.[63]

Reduction and expansion of the five-membered pyrrolidine ring of Pro (analogues **13** and **14**, respectively) led to a slight decrease in terms of affinity for NTS2. The introduction of a double bond to give the pyrroline ring (**15**) also seemed to be well tolerated. The best result was obtained with thia-analogue **16**, which possessed a K_i value at NTS2 comparable to **5**, coupled with increased selectivity over NTS1. In addition, the introduction of a fluorine atom at position 4 of Pro resulted in a loss of affinity and selectivity in the *cis* (**17**) and *trans* (**18**) configuration, whereas better results were obtained for the 4,4-difluorinated analogue **19**. Additionally, azido (**20** and **21**), amino (**22** and **23**) and amino-acetyl (**24** and **25**) groups were inserted in the *cis* and *trans* configuration at the position 4. All analogues with *trans* substituents showed better results compared to the corresponding *cis* derivatives, suggesting that NTS2 prefers the *exo*-puckered conformation of the pyrrolidine ring. Lastly, the 4-F-*trans* and thia-derivatives (**18** and **16**, respectively) were further modified through the insertion of Lys and *N*MeArg at positions 8 and 9, respectively, affording derivatives **26** and **27**. Analogue **27** showed slightly reduced binding affinity towards NTS2 compared with the parent peptide **16**, whereas compound **26** exhibited modest improvements in affinity and selectivity towards NTS2 (for **16**: K_i NTS2 = 10 nM and SI = 6600 vs. for **26**: K_i NTS2 = 8.1 nM and SI = 8600).

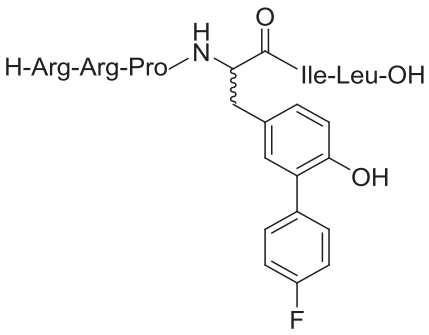
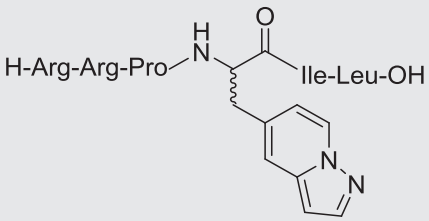
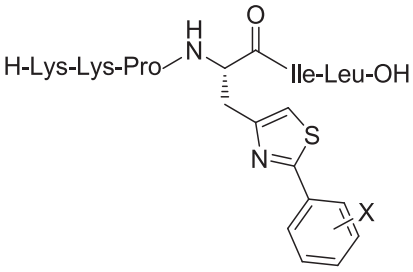
3.2 | Introduction of extended aromatic amino acids at position 11

Since the first SAR studies on NT, the tyrosine at position 11 has been considered crucial for binding to NTS1. Interestingly, [D-Tyr11]NT showed a 10-fold higher relative potency than native NT to induce hypothermia and similar results were obtained for [D-Phe11]NT.[64] In 2008, Richelson et al. demonstrated that the introduction of D-amino acids at position 11, along with the extension of the aromatic region, was well-tolerated by NTS2, unlike NTS1.[49] As position 11 was found to play a crucial role in terms of efficient binding to NT receptors, Pratsch explored the impact of a more bulky aromatic amino acid at position 11.[65] To further validate the role played by aromatic D-amino acids at position 11, the same unnatural Tyr derivative was inserted in both D- and L-configuration (Table 5). Specifically, the Tyr side chain was functionalized with a 4-F-phenyl ring in the 3′-position. The introduction of the two enantiomeric 4-fluorophenylated-Tyr derivatives at position 11 provided the analogues **28**(11*S*) and **29**(11*R*). As expected, hexapeptide **29**(11*R*) showed a K_d value in the low nanomolar range towards NTS2 (K_d = 63 nM), with a SI of 290. On the contrary, comparable binding values were recorded for the corresponding **28**(11*S*) analogue towards both receptors. Compared with the analogue NT50 containing D-1-naphthylalanine (Nal) at position 11 in NT8–13 (K_i NTS1 = 1800 nM, K_i NTS2 = 17 nM, SI = 104), identified by Richelson as one of the first NTS2-selective ligands,[49] compound **29**(11*R*) showed comparable affinity towards NTS2 with enhanced selectivity.

In another attempt to extend the aromatic region at position 11, the introduction of L-azaindolylalanine led to the analogue **30** (11*S*), which demonstrated single-digit nanomolar binding towards NTS2, coupled with a selectivity index of 27 over NTS1 (Table 5).[66] As expected, the corresponding analogue **31**(11*R*) containing D-azaindolylalanine, exhibited a higher SI towards NTS2, although a substantial loss in affinity was observed.

More recently, a small set of molecules with modifications at this key position was also developed by Hapău and co-workers.[67] Accordingly, Tyr[11] was replaced by L-(β-arylthiazol-4-yl)alanine residues, with differently decorated phenyl rings at C2 of the 1,3-thiazole core. The novel arylthiazole derivatives also encompassed either the Arg[8]-Arg[9] or Lys[8]-Lys[9] dipeptide motif. Compared with NT8–13, the presence of arylthiazoles at position 11 decreased binding towards both receptors (Table 5). Only **34** showed an IC_{50} value at NTS1 comparable with that of NT8–13, whereas binding at NTS2 was found to be 40-fold lower. Despite the clear loss of affinity, **32** and **33** still showed a

TABLE 5 Sequences and biological evaluation of NT ligands with extended aromatic region at position 11

Cmps	Sequences	X	IC_{50}, nM hNTS1	hNTS2	SI NTS1/NTS2
28(11*S*)		-	39	11	3.5
29(11*R*)		-	18,000	63	290
30(11*S*)		-	130	4.8	27
31(11*R*)		-	52,000	83	630
32		4-Me	1377	86.9	15.8
33		3-Me	1285	80.0	16.1
34		-	3.46	139	0.02

Note: Adapted from research articles published by Pratsch et al.,[65] Schaab et al.[66] and Hapău et al.[67]

modest selectivity towards NTS2, with a SI of approximatively 16 in both cases. Considering the comparable nanomolar binding affinity for NTS2 displayed by **32** and **33**, the methyl group in these analogues clearly played an important role in preferential binding to NTS2, as the unsubstituted phenyl analogue **34** showed a reversed preference for NTS1. Regarding the plasma half-life, the introduction of arylthiazole residues at position 11 led to a negligible improvement in proteolytic stability (i.e., **34** = half-life: 2.7 min vs. NT8–13 half-life: 0.78 min), indicating that the mere presence of these unnatural amino acids does not significantly prevent enzymatic degradation.

3.3 | Introduction of beta- and beta-homo-amino acids at position 11

In 2014, Schaab et al. developed a series of NT sequences in which β^2-homo-amino acids, as Tyr bioisosteres, were inserted at position 11.[66] As mentioned above, the introduction of D-residues led to an increase in selectivity. For this reason, all analogues were developed with the residue at position 11 in both *S*- and *R*-configuration. The two derivatives bearing a β^2-homo-amino acid **35**(11*R*)-**36**(11*S*) exhibited a huge loss of affinity at both receptor subtypes compared with NT8–13 (Table 6). Additional homologation of the Tyr side chain, as in **37**(11*R*) and **38**(11*S*), did not afford further improvement.

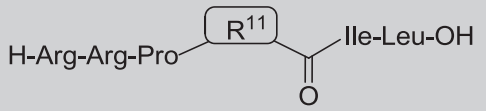
3.4 | Conformationally constrained Tyr surrogates

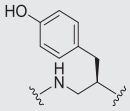
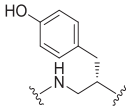
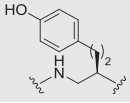
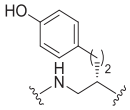
The introduction of conformationally constrained amino acids has been considered a valid strategy for the development of receptor (sub)type-selective ligands.[68,69] Using this approach, Simeth et al. reported a small library of tetrahydrofuran-containing NT analogues, in which spirocyclic amino acids were inserted at position 11 (Table 7).[70] Structurally, the substituted tetrahydrofuran amino acid (TAA) can serve as a mimic of Tyr^{11} by retaining aromaticity and inclusion of the hydroxyl group while possessing a fixed χ^1 angle of the Tyr side chain. With this modification, it was possible to extend the half-life of the NT analogues because of the presence of this type of unnatural amino acid. Among all the NT analogues synthesized, the analogue **39**R_{trans} exhibited a SI over 1200, with a K_i value of 29 nM towards NTS2 (Table 7). The stereochemistry of the side chain stereocenter played a critical role in the binding affinity towards NTS2. Indeed, when the α-carbon possesses the (*S*)-configuration and the additional chiral carbon on the side chain is positioned in *trans* to it

(**40**S_{trans}), a significantly higher K_i value was recorded with respect to the **39**R_{trans} analogue. In addition to the unnatural TAA amino acids in position 10, in both the **39**R_{trans} and **40**S_{trans} ligands, Ile^{12} was replaced by Gly and this modification appears to be critical for selectivity. In fact, the analogue **41**, which contains the racemic TAA residue and differs from the NTS2 selective ligand **39**S_{trans} only by the replacement of Gly^{12} with Ile^{12}, exhibited promising K_i values in the nanomolar range towards NTS1 and NTS2 but devoid of selectivity. Interesting results were observed when the hydroxy group of TAA was replaced by bromine: derivate **42** showed comparable binding affinity to **41** towards NTS2 (K_i NTS2 = 12 nM for both analogues), but the K_i at NTS1 became significantly higher (**42**, K_i NTS1 = 870 nM vs. **41**, K_i NTS1 = 7 nM), implying good selectivity. Therefore, unlike NTS1, the presence of bromine was well tolerated in the NTS2 binding pocket. Interestingly, replacement of the Arg^8-Arg^9 sequence with Lys^8-Lys^9 (**45**), Lys^8-Arg^9 (**44**) and Arg^8-Lys^9 (**43**) was not productive and led to analogues with higher K_i values towards NTS2, compared with **42**. Finally, the introduction of (*S*)-Phe(4-Br) (**46**) and its enantiomer (*R*)-Phe(4-Br) (**47**) at position 10 led to unselective analogues, indicating that the constraint and side chain topology induced by the TAA is essential for the purpose of selectivity towards NTS2. Based on these findings, it was apparent that the bulkiness of the main chain and side chain impact of TAA residues fit more easily into the NTS2 binding region, compared with the subtype 1 receptor.

Computational studies on the most promising analogue. **39**R_{trans} revealed that it adopted different conformations upon binding to the two receptors, which differed from the binding position of NT8–13. In NTS2, Tyr^{11} of NT8–13 is stabilized both by an H-bond to the extracellular loop 1 (ECL1) and by an extensive network of van der Waals interactions. For **39**R_{trans}, the H-bond between the OH of and the ECL1 residues is lacking, but the van der Waals interactions are likely strong enough to promote effective binding. Furthermore, a transient H-bond between the phenol group of TAA residue and Thr195 of ECL2 was observed. With respect to NTS1 binding, key interactions

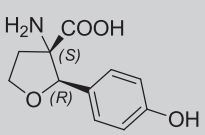
TABLE 6 Binding affinity of modified NT analogues and resulting selectivity indexes



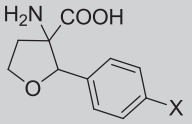
Cmp	R^{11}	K_i (nM) hNTS1	K_i (nM) hNTS2	SI NTS1/NTS2
NT8–13	-	0.24	1.2	0.20
35(11*R*)		79,000	5600	14
36(11*S*)		19,000	5400	3.5
37(11*R*)		1700	2500	0.68
38(11*S*)		2500	3600	0.69

Note: Adapted from research articles published by Schaab and co-workers.[66]

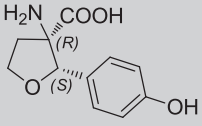
TABLE 7 Sequences of NT derivatives, binding affinity, and selectivity index of TAA-containing NT analogues



(S_{trans})TAA(OH)



TAA (*R*,*S* and *S*,*R*)
X = -Br, -OH



(R_{trans})TAA(OH)

Cmps	Sequences	K_i (nM) hNTS1	K_i (nM) hNTS2	SI NTS1/NTS2
39R_{trans}	H-Arg-Arg-Pro-(R_{trans})TAA (OH)-Gly-Leu-OH	~35,000	29	~1207
40S_{trans}	H-Arg-Arg-Pro-(S_{trans})TAA (OH)-Gly-Leu-OH	~20,000	700	~29
41	H-Arg-Arg-Pro-TAA (OH)-Ile-Leu-OH	7	12	0.58
42	H-Arg-Arg-Pro-TAA (Br)-Ile-Leu-OH	870	12	72.5
43	H-Arg-Lys-Pro-TAA (Br)-Ile-Leu-OH	~10,000	147	68
44	H-Lys-Arg-Pro-TAA (Br)-Ile-Leu-OH	5000	110	45
45	H-Lys-Lys-Pro-TAA (Br)-Ile-Leu-OH	~13,000	227	57
46	H-Lys-Lys-Pro-(*S*)-Phe(4-Br)-Ile-Leu-OH	93	69	1.3
47	H-Lys-Lys-Pro-(*R*)-Phe(4-Br)-Ile-Leu-OH	2170	873	2.5

Note: Adapted from research articles published by Simeth and co-workers.[70]

involving Pro[10], Tyr[11] and Ile[12] appear to be impaired, as well as binding of the *C*-terminal moiety. The distance between the *N*-terminal region of **39**R_{trans} and ECL3 appears to be higher compared with NT8–13, resulting in a weaker interaction between Pro[10] and Trp344. It should be noted that the presence of Gly at position 12 causes a conformational change in TAA, which results in deeper binding, negatively influencing binding to NTS1.

Recently, Eiselt and co-workers, based on preliminary data,[71] reported a small library of modified NT analogues in which sterically hindered, modified or cyclic Tyr surrogates were incorporated at position 11.[72] In an effort to mimic Tyr[11] in a conformationally constrained manner, 6- and 7-hydroxyl-substituted tetrahydroisoquinoline (Tic) residues were inserted (Table 8). In addition to HO-Tic residues, the uncommon amino acids 2,6-dimethyl-Tyr (Dmt) and *meta*-Tyr (*m*-Tyr) were also introduced at position 11. In this series, Ile[12] was systematically replaced by the unnatural amino acid Tle, which had previously led to beneficial affinity, selectivity and enzymatic stability.[73] Indeed, as shown in Table 8, the NT8–13 analogue carrying only Tle (**48**) possessed a sub-nanomolar K_i value for NTS2 ($K_i = 0.46$ nM), similar to that of NT8–13, with a slight decrease of affinity towards NTS1 ($K_i = 3.6$ nM) and an SI value of 8. In addition, aiming to avoid proteolytic cleavage between the two basic residues at positions 8 and 9, the authors introduced the modified amino acid β^3-hLys at position 8. The incorporation of β^3-hLys, Dmt and Tle at positions 8, 11 and 12, respectively, collectively led to the modified analogue **49**, which showed the best K_i value for NTS2 in this series, while also displaying a moderate selectivity (SI = 89). Slightly better selectivity was obtained following the introduction of *meta*-Tyr into the two analogues **50** and **51**, which carry the Lys[8]-Lys[9] segment and β^3-hLys at position 8, respectively. Incorporation of (6-OH)Tic led to the most interesting hexapeptides **52** and **53**. The latter, **53**, with β^3-hLys at position 8, showed a single-digit nanomolar K_i value for NTS2 and a SI of 1324, whereas the analogue **52**, harbouring the Lys[8]-Lys[9] fragment, exhibited a K_i value of 21.2 nM at NTS2 and no affinity for NTS1 up to 10 μM. The replacement of (6-OH)Tic with the isomer of structure (7-OH)Tic (not shown) was not tolerated, suggesting a critical OH orientation for NTS2 affinity and selectivity. The most promising NT analogues displayed large differences in plasma stability. Indeed, the β^3-hLys-containing hexapeptide **53** showed a half-life value of more than 24 h, whereas the analogue **52** with Lys at position 8 exhibited plasma degradation similar to that of NT8–13 (half-life = 4.4 min). LC–MS analyses identified the NT9–13 as the first metabolite of ligand **52**, further highlighting the importance of backbone modifications between the two Lys residues. In the formalin model of persistent pain, intrathecal administration of **53** induced a relevant analgesic effect; at 62 nmol, the formalin-induced behaviours, such as paw lifting and shaking, were fully inhibited for a period of 60 min, allowing the calculation of an ED_{50} value of 1.4 nmol (i.e., 3.5 μg/kg). Morphine was used as a positive control, and its analgesic effect was found to be less potent compared with that of **53** at equimolar doses (2 and 7 nmol). On the contrary, an equimolar dose of analogue **52** induced shorter lasting analgesia, corresponding to the beginning of the inflammatory phase, an observation in accordance with its low stability. The most potent NTS2 ligand, compound **49**, showed complete inhibition of nociceptive behaviour at 4.5 nmol. This clearly outperformed compound **53**, likely because of its greater affinity for both NT receptors. At a dose of 62 nmol, compound **49** caused classic NTS1-induced adverse effects, including hypotension and hypothermia. Intravenous administration of **49** (0.01 mg/kg) resulted in a robust hypotensive effect, manifested by a triphasic drop in blood pressure. The same dose of NTS2-selective ligand **53**, however, did not affect blood pressure values. Regarding hypothermia, intrathecal injection of **53** at its ED_{50} value (i.e., 3.5 μg/kg) also resulted in a non-significant temperature drop (1.5°C), comparable with vehicle. In contrast, i.t. administration of **49** caused robust and persistent hypothermia (i.e., >3°C after 1 h), which can be ascribed to its NTS1 affinity.

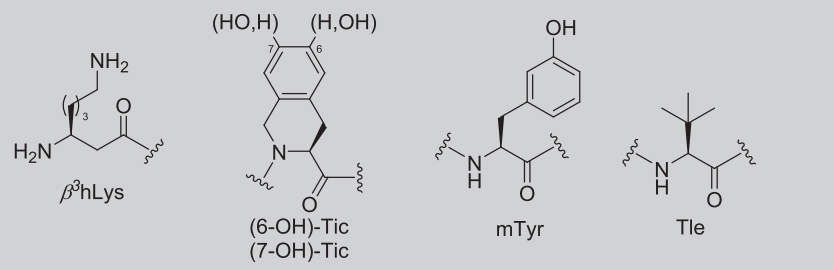


TABLE 8 Sequences of hexapeptides NT8–13 and **48**–**53** with their biological evaluation towards NT receptors

		K_i (nM)		
Cmp	**Sequence**	**hNTS1**	**hNTS2**	**SI NTS1/NTS2**
NT8–13	H-Arg-Arg-Pro-Tyr-Ile-Leu-OH	0.9	0.55	1.6
48	H-Arg-Arg-Pro-Tyr-**Tle**-Leu-OH	3.6	0.46	8
49	H-**β^3-hLys**-Lys-Pro-**Dmt**-**Tle**-Leu-OH	13.4	0.15	89
50	H-Lys-Lys-Pro-**mTyr**-**Tle**-Leu-OH	345	2.7	128
51	H-**β^3-hLys**-Lys-Pro-**mTyr**-**Tle**-Leu-OH	107	0.55	195
52	H-Lys-Lys-Pro-**(6-OH)Tic**-**Tle**-Leu-OH	>10,000	21.2	>470
53	H-**β^3-hLys**-Lys-Pro-**(6-OH)Tic**-**Tle**-Leu-OH	3786	2.9	1324

Note: Adapted from research articles published by Eiselt and co-workers.[72]

3.5 | Electrostatic interactions at position 11 for NTS2 selectivity

In 2017, based on a molecular modelling approach, a set of original NT analogues was developed.[74] Molecular dynamics simulations showed some discrepancies at the interfaces between the ligands and the two NT receptors (Figure 3). Among these differences, the most important was the identity of key residues in the NT receptor binding sites, which face the ligand's Tyr^{11} (i.e., Arg212 and Glu179 in hNTS1 and hNTS2, respectively).

Based on this observation, a small panel of acidic and basic amino acids was inserted at position 11, as well as a bis-lysine motif at the *N*-terminus of the sequence (Table 9). Taken together, the presence of Lys^{11} instead of Tyr^{11} led to the most selective analogue for NTS2 (**54**, with SI = 21.8), although a moderate loss of affinity was observed. Shortening the side chain proved to be poorly tolerated (**55**), whereas introduction of His (**56**) afforded similar mid-nanomolar K_i values for both receptors. In the latter case (**56**), the aromaticity of the His side chain partially restored the binding affinity for hNTS1–affinity being compared with the positively charged aliphatic side chains of Lys and Orn—highlighting two features for NTS2 selectivity: the positive charge and an aliphatic side chain at position 11. In contrast, insertion of acidic residues, such as Asp (**57**) and Glu (**58**), led to a significant loss of NTS2 affinity, because of the presence of Glu179 in the binding site. However, a complete loss of binding affinity for hNTS1 was also unexpectedly observed, again suggesting a key role of aromaticity at position 11 for efficient binding to hNTS1. Alternatively, it was postulated that the presence of the negative charge could drastically alter the biologically active conformation, resulting in a complete loss of binding affinity. To further validate the hypothesis of beneficial electrostatic interactions, the modified NT analogues were tested in mutated hNTS1, in which Arg212 was replaced with Glu (hNTS1-R212E). As expected, the presence of an acidic amino acid at position 212 resulted in a gain in binding affinity for analogue **54** that carries Lys at position 11.

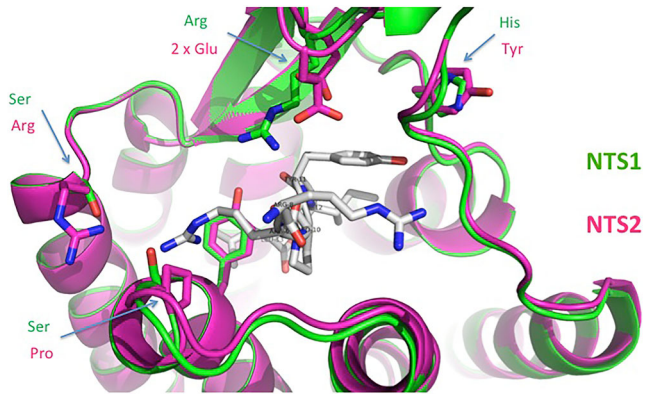


FIGURE 3 Superimposition of hNTS1 and hNTS2 bound with NT8–13. Discrepancies between the two receptor subtypes can be observed (NTS1 in green, NTS2 in dark pink).

3.6 | Combination of silylated amino acids and reduced pseudopeptide bond

As mentioned above, the introduction of unnatural amino acids into the pharmacophore of NT8–13 is essential both to selectively address binding affinity and to enhance plasma half-life. In this context, the incorporation of silicon-containing amino acids could reinforce the affinity towards the target, as well as the action of proteolytic enzymes could be prevented, resulting in improved bioavailability.[75]

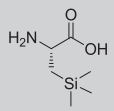
In 2015, Fanelli and co-workers developed a small panel of NT analogues in which two silylated amino acids, trimethylsilylalanine (TMSAla)[76] and silaproline (Sip),[77,78] were introduced (Table 10).[79] The design rationale emerged from the well-known hydrophobic character of the NT binding site, especially the region interacting with residues Ile^{12} and Leu^{13}.[80] Consequently, the presence of hydrophobic residues at positions 12 and 13 of the NT analogues was considered essential, and the introduction of silicon-containing amino acids would lead to greater hydrophobicity than the native carba-analogues.[75,81] With the exception of **63**, Arg^8-Arg^9 to Lys^8-Lys^9 substitution was also performed for all analogues. Introduction of TMSAla at position 13 (**59**) led to improved binding for both receptors compared with NT8–13, with IC_{50} values in the sub-nanomolar range, whereas the preference towards both receptor subtypes was almost unchanged (Table 8). On the other hand, the presence of TMSAla at position 12 (**60**), as well as the double substitution at positions 12 and 13 (**61**), gave significantly higher IC_{50} values, than for NT8–13, reflecting the detrimental effect of these replacements. This discrepancy shows that TMSAla fits well in the deepest region of the binding pocket, whereas its bulky side chain is poorly tolerated at position 12. Incorporation of Sip at position

TABLE 9 Analogues **54**–**58** and relative binding affinities

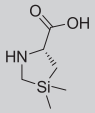
		K_i (nM)			
Cmp	Sequence	hNTS1	hNTS2	SI NTS1/NTS2	hNTS1-R212E K_i (nM)
54	H-Lys-Lys-Pro-**Lys**-Ile-Leu-OH	5700	261.8	21.8	199.2
55	H-Lys-Lys-Pro-**Orn**-Ile-Leu-OH	>10,000	619.5	nd	na
56	H-Lys-Lys-Pro-**His**-Ile-Leu-OH	455.6	474.1	0.96	na
57	H-Lys-Lys-Pro-**Asp**-Ile-Leu-OH	>10,000	4200	nd	>10,000
58	H-Lys-Lys-Pro-**Glu**-Ile-Leu-OH	>10,000	1600	nd	>10,000

Note: Adapted from research articles published by Fanelli and co-workers.[74]
Abbreviations: nd, not determinable; na, not available.

TABLE 10 Sequences, binding affinity and selectivity index of silylated analogues



TMSAla



Sip

Cmp	Sequences	IC_{50}, nM hNTS1	hNTS2	SI NTS1/NTS2
NT8–13	H-Arg-Arg-Pro-Tyr-Ile-Leu-OH	0.82	7.52	0.1
59	H-Lys-Lys-Pro-Tyr-Ile-**TMSAla**-OH	0.02	0.26	0.07
60	H-Lys-Lys-Pro-Tyr-**TMSAla**-Leu-OH	93.8	405	0.23
61	H-Lys-Lys-Pro-Tyr-**TMSAla**-**TMSAla**-OH	15.4	28.9	0.53
62	H-Lys-Lys-**Sip**-Tyr-Ile-Leu-OH	15.2	21.2	0.71
63	H-*N*MeArg-Lys-Pro-Tyr-**TMSAla**-Leu-OH	246	29.7	8.3

Note: Adapted from research articles published by Fanelli and co-workers.[79]

10 (**62**) led to acceptable IC_{50} values in the low nanomolar range towards both NT receptors, whereas (**63**), which carries *N*MeArg[8] and TMSAla[12], exhibited an IC_{50} value of 29.7 nM at NTS2, coupled with a SI of 8.3, resulting in the most selective NTS2 ligand of the series.

The Sip derivative **62** was evaluated in vivo in different pain models. Intrathecal administration of **62** significantly attenuated both acute and tonic pain.[82,83] In particular, a dose-dependent analgesic effect was observed in the tail-flick test, with an increase in tail-flick latency compared to saline-treated animals. At the same time, formalin pain-related behaviours were strongly reduced when rats were pre-treated with **62**. Additionally, analogue **62** showed an interesting ED_{50} value of 2.33 μg/kg in the acetic acid-induced visceral pain model, and a significant anti-allodynic effect in the peripheral neuropathic pain model was observed. Finally, spinal administration of **62** was found to significantly improve the rehabilitation outcomes, such as weight bearing on the injured limb and limb use time. Interestingly, despite the potent binding affinity towards NTS1, analogue **62** did not induce hypothermia, probably because of biased signalling after NTS1 activation, which could recruit different signalling pathways leading to analgesic and/or hypothermic effects.[84]

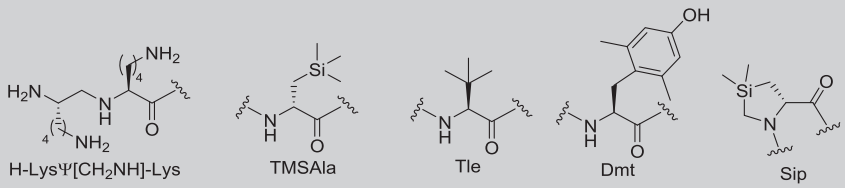
Alongside the introduction of unnatural amino acids, backbone modifications also represent a prized strategy to optimize peptide ligands.[85–87] Of all the properties to be considered in the drug discovery process, plasma stability (to achieve higher exposure) represents a key parameter.[88] Given the limited NT half-life (i.e., <2 min), ideal NT receptor ligands for, *in casu*, potent pain treatment, should show high protease stability. Taking into account the proteolytic action of thimet oligopeptidase at the Arg^8-Arg^9 peptide bond, a series of reduced peptide bond hexapeptides was introduced.[89] In particular, the Arg^8-Arg^9 dipeptide was replaced by the reduced Lys^8-Lys^9 pseudo-peptide bond (i.e., LysΨ[CH_2NH]Lys), as in the 'milestone' NT ligands JMV449 and PD149163 mentioned above (Figure 2), which had a remarkable positive influence on the half-life of the compounds by preventing the first cleavage between the two basic residues.

To explore the role of the pseudo-peptide bond in terms of affinity and selectivity, eight pairs of couples were synthesized, each differing by the presence of the reduced bond, in combination with the insertion of several unnatural amino acids (Table 11). With few exceptions, these NT analogues showed a higher binding affinity for NTS2 than for NTS1. The introduction of a reduced pseudo-peptide bond (**5** vs. JMV449) led to a minimal improvement both in terms of NTS2 affinity and selectivity. With the exception of **70**, the presence of the reduced Lys^8 Ψ[CH_2NH]Lys^9 bond and silylated amino acids TMSAla and Sip at positions 13 and 10, respectively, mainly decreased the affinity for NTS1 (cfr. **59** vs. **65**, **62** vs. **66** and **67** vs. **68**). Incorporation of the pseudo-peptide bond in the moderately selective analogue **54**, afforded a 10-fold improvement in the NTS2 affinity, resulting in ligand **64** with an SI of 254. The presence of the LysΨ[CH_2NH]Lys motif and the incorporation of Lys and TMSAla at positions 11 and 13, respectively, significantly improved binding to NTS2 (i.e., **70**). Finally, introduction of Dmt and Tle at positions 11 and 12, respectively, led to analogues **71** and **72**, which exhibited single-digit K_i values at NTS2, together with limited selectivity. The introduction of D-Trp^{11} likely leads to a conformational change resulting in a moderately strong affinity for NTS1 in the case of the reduced analogue **74**, whereas the unreduced analogue **73** showed a K_i value in the low nanomolar range towards NTS2 along with the highest SI, equal to 423.

With respect to plasma stability, the incorporation of a single pseudo-peptide bond exhibited a half-life value more than fivefold higher than that of native NT8–13 (1.6 min vs. 8.4 min). However, the simultaneous presence of unnatural amino acids led to ligands with very high plasma stability. Compounds **66**, **68**, **70**, **72**, and **74** showed impressive half-life values ranging from 10 to 24 h.

In a subsequent study, the analgesic effect of **65**, **66**, and **68** was evaluated.[46] In the tail-flick acute pain paradigm, compounds **66** and **68** showed maximal antinociceptive responses after 20 min of intrathecal injection in rats. It is important to note that the analgesic effect in this type of pain paradigm was comparable with that observed for an equimolar dose of morphine. In the tonic pain paradigm, **68** showed an analgesic effect similar to that of morphine in reducing pain-related behaviours during the inflammatory phase. In contrast, no effect was observed during the first acute phase. In the peripheral inflammatory pain model induced by complete Freund's adjuvant (CFA) injection, **68** significantly reduced the development of mechanical allodynia at days 3 and 14 (52% and 59% reversal of allodynia, respectively).

TABLE 11 Pairs of NT8–13 analogues and their binding affinity, selectivity towards NTS2 and plasma stability

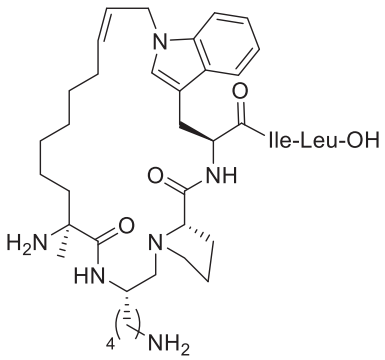


Cmp	Sequence	K_i (nM) hNTS1	hNTS2	SI NTS1/NTS2	Plasma stability $t_{1/2}$
5	H-Lys-Lys-Pro-Tyr-Ile-Leu-OH	4.0	1.1	3.6	1.6 min
JMV449	H-**LysΨ[CH$_2$NH]Lys**-Pro-Tyr-Ile-Leu-OH	2.0	0.31	6	8.4 min
54	H-Lys-Lys-Pro-**Lys**-Ile-Leu-OH	7600	310	25	2.9 min
64	H-**LysΨ[CH$_2$NH]Lys**-Pro-**Lys**-Ile-Leu-OH	6600	26	254	5 h
59	H-Lys-Lys-Pro-Tyr-Ile-**TMSAla**-OH	0.018	0.25	0.1	1.6 min
65	H-**LysΨ[CH$_2$NH]Lys**-Pro-Tyr-Ile-**TMSAla**-OH	2.5	0.55	4.5	2.0 h
62	H-Lys-Lys-**Sip**-Tyr-Ile-Leu-OH	14	21	0.7	4.5 min
66	H-**LysΨ[CH$_2$NH]Lys**-**Sip**-Tyr-Ile-Leu-OH	300	130	2.3	22 h
67	H-Lys-Lys-**Sip**-Tyr-Ile-**TMSAla**-OH	55	16	3.4	3.5 min
68	H-**LysΨ[CH$_2$NH]Lys**-**Sip**-Tyr-Ile-**TMSAla**-OH	610	24	25	20 h
69	H-Lys-Lys-Pro-**Lys**-Ile-**TMSAla**-OH	710	76	9.3	2.8 min
70	H-**LysΨ[CH$_2$NH]Lys**-Pro-**Lys**-Ile-**TMSAla**-OH	150	1.5	100	10 h
71	H-Lys-Lys-Pro-**Dmt**-**Tle**-Leu-OH	55	2.4	24	4.6 min
72	H-**LysΨ[CH$_2$NH]Lys**-Pro-**Dmt**-**Tle**-Leu-OH	57	1.4	79	>24 h
73	H-Lys-Lys-Pro-**D-Trp**-Ile-**TMSAla**-OH	3600	8.5	423	10 min
74	H-**LysΨ[CH$_2$NH]Lys**-Pro-D-**Trp**-Ile-**TMSAla**-OH	55	3.5	16	19 h

Note: The unnatural amino acids and pseudo-peptide were shown. Adapted from research articles published by Previti and co-workers.[89]

3.7 | Macrocyclic analogues

Unlike linear peptides, macrocycles usually feature enhanced conformational homogeneity, which may improve both the pharmacodynamic and pharmacokinetic properties of the peptide.[90,91] Early SAR studies, aimed at developing cyclic NT analogues, showed promising results in terms of binding affinity towards both NT receptors, as well as improved plasma stability and significant analgesic effects.[92–95] Among these, promising results were obtained with the nonselective cyclic NT8–13 analogue JMV2012 (c[Lys-Lys-Pro-Tyr-Ile-Leu-Lys-Lys-Pro-Tyr-Ile-Leu]): Indeed, this cyclic analogue showed potent antinociceptive and hypothermic effects after peripheral administration, suggesting an appreciable crossing of the BBB.[93] More recently, the development of the first NTS2-selective macrocyclic ligand has been reported.[96] Compound CR-01-64 (**75**) (Figure 4) bears an *N*-allylated Trp^{11} residue, which is essential both for selectivity towards NTS2 and for the cyclization. The macrocyclization itself was performed using a ring-closing metathesis (RCM) reaction between a side chain-anchored allyl group of Trp and the olefin in the unnatural amino acid replacing Lys^8, namely Fmoc-*(S)*-2-amino-2-methyldec-9-enoic acid. The resulting 23-membered macrocycle **75** showed a very favourable K_i value at NTS2 (K_i = 7.0 nM), whereas the affinity towards NTS1 was found to be considerably lower (K_i = 871 nM). The macrocyclization limits the action of peptidases, compared with linear NT ligands, both in rat plasma and cerebrospinal fluid, **75** exhibiting half-life values greater than 24 h in both biological fluids.



75
NTS1, K_i = 871nM
NTS2, K_i = 7.0 nM
SI NTS1/NTS2 = 124

FIGURE 4 Chemical structure, binding affinities, and SI of macrocycle **75**. Adapted from research articles published by Chartier and co-workers[96]

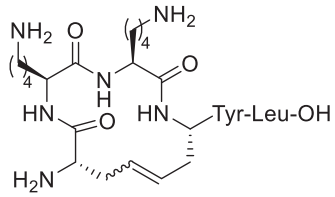
In acute pain models, intrathecal administration of **75** resulted in a significant increase in tail-flick latency compared to saline-treated rats, and an established ED_{50} of 11.1 μg/kg. The intrathecal injection of an

equimolar dose of morphine showed a weaker analgesic effect than **75**. Notably, co-administration of morphine and **75** (1 mg/kg and 30 μg/kg, respectively) led to a marked increase in tail-flick latency compared with a single injection of individual compound, suggesting a synergistic/additive analgesic effect. In the formalin-induced inflammatory pain model, **75** reduced the nociceptive behaviours (i.e., paw licking, biting and lifting) at a dose of 30 μg/kg and showed an ED_{50} value equal to 7.1 μg/kg. Finally, the macrocycle exhibited a potent analgesic effect in the CFA-induced chronic inflammatory pain model, inducing more than 80% of pain attenuation compared with saline-treated rats. No hypothermia was observed when **75** was administrated at the maximum analgesic dose tested (60 μg/kg), nor were any other effects mediated by NT receptor activation. In contrast, intravenous injection (0.01 mg/kg) of the macrocycle resulted in a mild transient hypotensive response, which lasted an average of 2 min. The hypotensive effect observed was dose-dependent: Injection of 0.1 mg/kg of **75** induced a moderate but less pronounced triphasic hypotension than that of native NT and did not extend beyond 15 min.

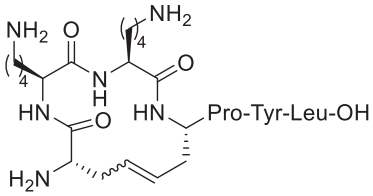
In parallel to the development of the first NTS2-selective macrocycle, a meticulous and rational SAR study led to the identification of structurally novel NTS2-selective macrocyclic NT analogues.[97] Initially, an alanine scan and various substitutions and deletions were carried out, from which the truncated linear NT analogue NT8–12 showed a K_i value of 620 nM towards NTS2, and no binding to NTS1 up to 100 μM was observed (SI > 160). On top of this, the truncated analogue [Ile^{12}]NT8–12 showed a further improvement in binding affinity towards NTS2 (K_i = 391 nM), with an SI greater than 255.

Based on these findings, 14 macrocycles were rationally designed, varying the C-terminal moiety, and the macrocyclization was performed in the *N*-terminal fragment. In particular, macrocyclization was achieved through RCM between two allylGly residues, appropriately inserted along the peptide backbone. Among the macrocycles, four showed promising results both in terms of affinity and selectivity (Figure 5). Compound **76**, which was macrocyclized between positions 7 and 10, exhibited K_i value in the lower nanomolar range for NTS2 (K_i = 50 nM), whereas no binding to NTS1 up to 100 μM was observed. Besides the cyclization, ligand **76** is a truncated analogue in which the residue at position 13 was removed, and Ile^{12} was replaced by Leu. Analogues of **76** bearing Ile^{12}-Leu^{13}, Leu^{12}-Ile^{13} and Ile^{12} without the 13th amino acid were found to be less active with no detectable binding at NTS2 up to 10 μM. The promising properties of ligand **76** could be due to the conformationally constrained Lys^8-Lys^9 fragment and the steric hindrance imposed by amino acid side chains at position 12. Hypothetically, the two basic amino acids Lys^8-Lys^9 and Glu179 in ECL2 of NTS2 could interact. In contrast, the latter residue is present as Arg212 in NTS1, resulting in a dramatic loss of affinity. It also appeared that the γ-branching pattern of Leu is essential for NTS2 binding, compared with the β-branching in Ile at position 12. Incorporation of Pro (compound **77**) between the macrocycle and Tyr^{11} did not improve the affinity towards NTS2 but resulted in a fourfold increase in K_i value. The stereochemistry of allylGly was also evaluated and only the analogue **78**, which features the D-allylGly residue at position 7, showed comparable results with macrocycle **76**. Finally, catalytic hydrogenation of the linker led to analogue **79**, which displayed a moderate affinity towards NTS2 (K_i = 90 nM), indicating that a more rigid linker plays a marginal role in the binding affinity of both NT receptors.

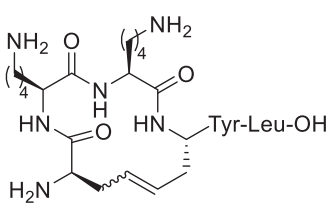
With respect to plasma stability, compound **76** exhibited a half-life value of 15 min, approximately 10-fold higher than that of native



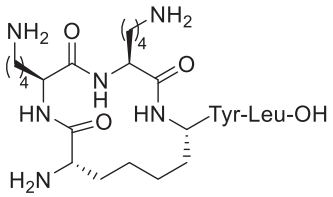
76
NTS1, K_i = >100 μM
NTS2, K_i = 0.05 μM
SI NTS1/NTS2 = >1600



77
NTS1, K_i = >100 μM
NTS2, K_i = 0.2 μM
SI NTS1/NTS2 = 600



78
NTS1, K_i = >100 μM
NTS2, K_i = 0.1 μM
SI NTS1/NTS2 = 900



79
NTS1, K_i = >100 μM
NTS2, K_i = 0.09 μM
SI NTS1/NTS2 = >1100

FIGURE 5 Macrocyclic NT analogues and binding data (with SI). Adapted from research articles published by Chartier and co-workers[97]

NT, probably because of the different secondary structure imposed by the macrocycle. Similar results were obtained when stability in cerebrospinal fluid was evaluated. Indeed, a half-life value higher than 24 h was observed, which is somewhat better than that of native NT, which settles around 15 h.

The antinociceptive properties of compound **76** were subsequently evaluated in three different pain models. In an acute thermal pain test, macrocycle **76** exhibited potent antinociceptive effects after intrathecal injection, with an ED_{50} value of 43.8 μg/kg, reaching 98.5% of the maximal possible effect at a dose of 150 μg/kg. The observed ED_{50} value of ligand **76** is consistent with that reported for the NTS2-selective linear NT79. Despite a higher ED_{50} value than a previously reported NTS1-targeted macrocycle,[95] the analgesic effect of compound **76** in acute and chronic inflammatory pain models resulted in a significant decrease in nociceptive behavioural episodes in both phases. Finally, compound **76** exhibited antiallodynic properties in a chronic inflammation pain model, covering not only its analgesic properties but also its hypotensive and hypothermic effects. Intravenous administration of ligand **76** (0.1 mg/kg) did not result in a reduction in blood pressure, and no reduction in body temperature was observed after intrathecal injection at 150 μg/kg.

Consequently, interesting SAR studies have led to NTS2-selective macrocycles. Macrocycle **75** could represent a promising lead compound for the development of antinociceptive agents. This compound exhibited a single-digit nanomolar K_i value towards NTS2 with appreciable inter-NTS selectivity (SI = 124), and further SAR studies could lead to an improved binding affinity for NTS2, relative to NTS1. Additionally, this macrocycle showed impressive stability in rat plasma and cerebrospinal fluid (half-life values > 24 h). On the other hand, the macrocycles reported in Figure 5 showed a sub-micromolar binding affinity for NTS2, with SI values up to 1600, although significantly lower plasma stability was observed compared with **75**.

4 | SAR OVERVIEW AND IN VIVO EFFICACY

In this review, we summarized the SAR studies in which NTS2 ligands have been developed. As a summary, we report in Figure 6 the most promising structural features that lead to NTS2-selective ligands. The well-known Arg^8-Arg^9 replacement by the Lys^8-Lys^9 dipeptide does not significantly influence the selectivity towards NTS2. Both basic

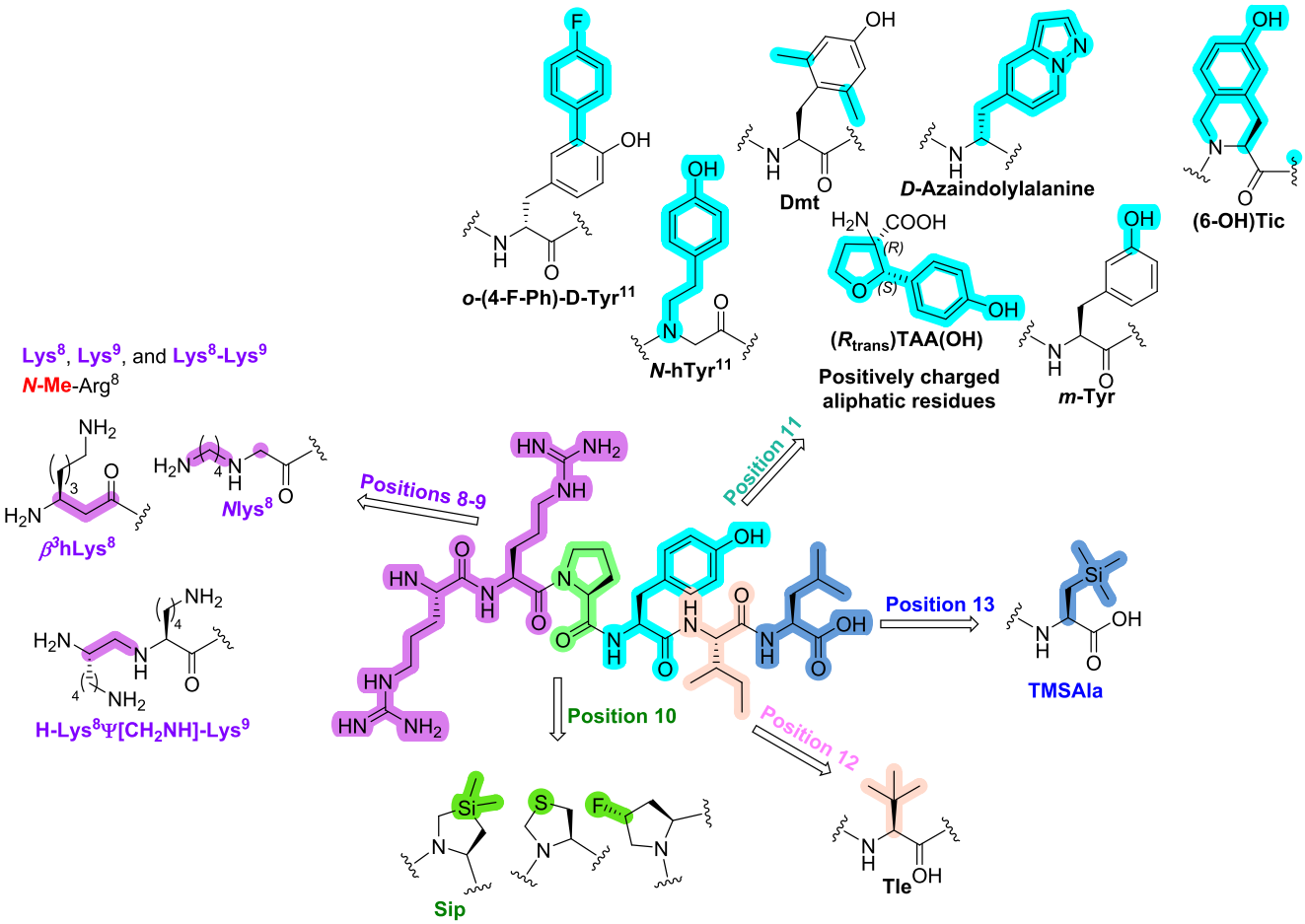
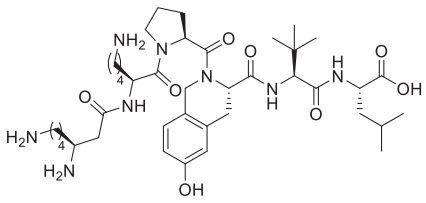


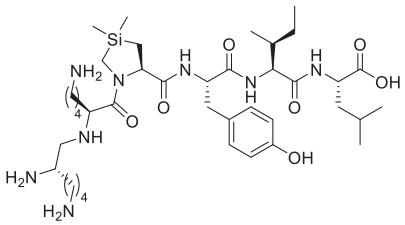
FIGURE 6 Representation of the most promising substitutions and/or modifications on the NT8–13 segment to reach high affinity towards NTS2

amino acids can be introduced at these positions, and mono-substitution at both positions is well tolerated. The introduction of β^3-hLys- at position 8 improved both binding towards NT receptors and plasma stability. The presence of the reduced amide bond LysΨ[CH_2NH]Lys leads to analogues with impressive plasma stability (>24 h). In general, ligands carrying this backbone modification show a higher SI (NTS1/NTS2) when compared with the corresponding backbone-unmodified peptides. The development of *N*-hTyr[11] peptoid-peptide hybrids resulted in NTS2-selective ligands with high binding affinity and high SI values. Introduction of a 4-F-phenyl ring at the *ortho* position of D-Tyr orients the binding affinity towards NTS2. At position 8, the introduction of *N*MeArg and *N*lys is well-tolerated: Incorporation of these residues and *N*-hTyr[11] leads to potent and selective NTS2 derivatives. At position 10, the introduction of various heteroatoms on the proline ring leads to promising results only in the presence of multiple NT8–13 modifications. With a few exceptions, substitution of Pro is poorly tolerated in terms of affinity towards NTS2. Introduction of thio-Pro and (*R*)-4-F-Pro, along with *N*-hTyr and *N*MeArg at positions 11 and 8, respectively, yields the most interesting NTS2-selective ligands. The introduction of silylated amino acids at positions 10 and 13 enhances the binding affinity towards both receptors, as well as plasma stability. Incorporation of conformationally constrained residues at position 11 is productive both in terms of affinity towards NTS2 and selectivity. The presence of various decorated tetrahydrofuran (TAA) analogues leads to potent and selective NTS2 ligands. Similarly, the presence of (6-OH)Tic at position 11 affords potent and selective analogues towards NTS2. Incorporation of Tyr derivatives, such as *meta*-Tyr and Dmt, is well-tolerated. The presence of Tle at position 12 results in ligands with improved selectivity towards NTS2. Generally, a single modification slightly improves half-life values, whereas two or more modifications along NT8–13 resulted in very stable ligands, with half-life values >24 h.

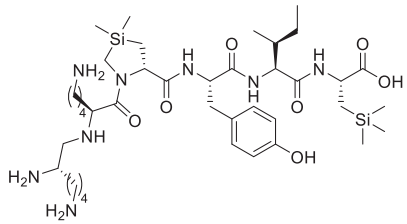
The in vivo evaluation of promising NT ligands in different pain models is summarized in Figure 7. In formalin murine pain models, intrathecal administration of the NTS2-selective ligand **53** led to a significant analgesic effect over a 60-min period, with an ED_{50} value of 1.4 nmol, which is slightly better than morphine, and without impact on blood pressure and body temperature. In the same pain model, the NTS2-selective analogue **52**, which differs from **53** only by the presence of Lys instead of β^3-hLys at position 8, showed a shorter antinociception effect. This discrepancy was hypothesized to be due to the significant difference in terms of plasma stability (**53**: >24 h vs. **52**: 4.4 min). In the tail-flick (acute pain) model, **66** and **67** exhibited a similar analgesic effect as an equimolar dose of morphine. Additionally, the silylated analogue **68** showed an analgesic effect comparable to that of morphine in the tonic pain model, coupled with a significant



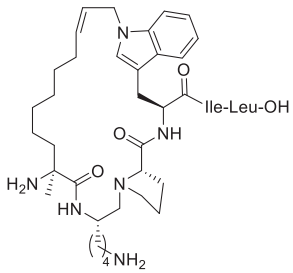
53
NTS1, K_i = 3786 nM
NTS2, K_i = 2.9 nM
SI NTS1/NTS2 = 1324
Relevant analgesic effect in formalin model of pain
ED_{50} = 1.4 nmol (3.5 μg/kg)
$t_{1/2}$ = > 24 h



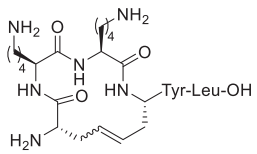
66
NTS1, K_i = 300 nM
NTS2, K_i = 130 nM
SI NTS1/NTS2 = 2.3
Comparable analgesic effect of an equimolar dose of morphine in tail-flick acute pain paradigm
$t_{1/2}$ = 22 h



68
NTS1, K_i = 610 nM
NTS2, K_i = 24 nM
SI NTS1/NTS2 = 25
Comparable analgesic effect of an equimolar dose of morphine in tail-flick acute pain paradigm and in tonic pain paradigm
$t_{1/2}$ = 20 h



75
NTS1, K_i = 871 nM
NTS2, K_i = 7.0 nM
SI NTS1/NTS2 = 124
Acute pain model, ED_{50} = 11.1 μg/kg
Formalin-induced inflammatory pain model, ED_{50} = 7.1 μg/kg
Potent analgesic effects in chronic inflammatory pain model
$t_{1/2}$ = > 24 h



76
NTS1, K_i = >100 μM
NTS2, K_i = 0.05 μM
SI NTS1/NTS2 = > 1600
Acute thermal pain, ED_{50} = 43.8 μg/kg
Significant antinociception effects in acute and chronic inflammatory pain models
$t_{1/2}$ = 15 min
$t_{1/2}$ in cerebrospinal fluid = 15 h

FIGURE 7 Chemical structures and biological data of the most promising NTS2-selective ligands giving way to potent analgesic effects. Half-life refers to plasma unless specified otherwise.

reduction in the development of mechanical allodynia in the chronic inflammatory pain paradigm. The in vivo biological evaluation of NTS2-selective macrocycles showed promising findings in terms of analgesic effects. Macrocycle **75** exhibited (i) antinociception effects comparable to morphine in tail-flick acute pain paradigm and synergistic/additive analgesic effects when co-administrated with morphine and (ii) a significant reduction of nociception behaviours in the formalin-induced inflammatory pain model. Similarly, derivative **76** exhibited potent antinociception in the acute thermal pain model, quite similar to that reported for **NT79**, and a significant decrease in nociceptive behaviours was observed in both acute and chronic inflammatory pain paradigms, without concomitant drop in blood pressure and body temperature.

5 | CONCLUSIONS

Overall, the collected data described here represent a solid starting point for the development of new series of NT analogues selectively targeting NTS2. Of note, previous studies have also reported the development of opioid/non-opioid hybrids able to target two different systems involved in pain regulation, as has been done for combined NT and opioid pharmacophores.[6,98,99] Consistent with the advantages presented by receptor subtype-selective ligands, new opioid-neurotensin hybrids (OPNT) should selectively target NTS2, in order to avoid the undesirable NTS1-mediated side effects.

Finally, given the hydrophilicity properties of all NT ligands, BBB permeability and accessibility of therapeutic concentrations in the CNS still pose great challenges to the development of NT analogues as antinociceptive agents. Indeed, the presence of a non-fenestrated capillary endothelium, as well as a number of different intracellular efflux pumps and tight intercellular junctions, prevents easy diffusion into the BBB.[100–102] Among the possible approaches for improving brain permeability, conjugation of brain-penetrant peptides with NT provides BBB penetration resulting in analgesic effects.[103] In particular, the peptide Angiopep-2 (also known as An2), is one of the ligands for the multiligand LDL receptor-related protein-1 (LRPL1), which is expressed at the luminal membrane of brain capillary endothelial cells.[104] Considering that An2 can cross the BBB through LRP1 receptor-mediated transcytosis, the An2 penetrating peptide sequence was conjugated with NT sequences (cfr. An2-NT conjugate: ANG2002).[103] After systemic administration in mice, the conjugate An2-NT achieved therapeutic concentrations resulting in antinociceptive effects. This kind of approach, commonly known as the Trojan horse approach, could be useful to improve the BBB permeability of promising NTS2-selective ligands with proven analgesic properties. In conclusion, effective and safe pharmacological alternatives to opioids are currently in high demand for pain management and are expected to have a significant impact on the opioid crisis.

DEDICATION

This work is dedicated to the career and lifetime achievements of Prof. Eric Marsault.

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CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

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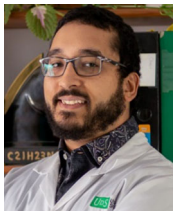
AUTHOR BIOGRAPHIES

Dr. Santo Previti completed his PhD in Chemical Sciences at the University of Messina (Italy) in 2017. Subsequently, Dr. Previti went for a first postdoctoral in Montpellier (France) with Dr. Florine Cavelier at the Institut des Biomolécules Max Mousseron (IBMM). During this period, he was involved in the development of neurotensin derivatives targeting NTS1 (radiopharmaceuticals) and NTS2 (antinociceptives). As a second postdoctoral training, Dr. Previti went to the Research Group of Organic Chemistry with Prof. Steven Ballet at the Vrije Universiteit Brussel (VUB, Brussels, Belgium), where he was involved in the development of hybrid opioid/non-opioid multitarget ligands. He is currently a postdoctoral researcher with Prof. Maria Zappalà at the University of Messina (Italy), where he deals with the development of peptide-based inhibitors of viral and protozoan cysteine proteases.



Michael Desgagné obtained his BSc degree in Biochemistry from the University of Sherbrooke (Québec, Canada) in 2017, where he discovered a great passion for organic chemistry. He then joined Eric Marsault's lab for his graduate studies, working on the development of macrocyclic agonists of the NTS2 receptor in view of improved pain treatments. Since 2020, he is working on translating peptides into small molecules targeting the neurotensin receptors, an endeavor under the supervision of Philippe Sarret and Pierre-Luc Boudreault.

Prof. emer. Dirk Tourwé obtained his PhD degree at the Vrije Universiteit Brussel (VUB, Brussels, Belgium) in 1974. He became director of the Research Group of Organic Chemistry in 1995 and has been professor emeritus since 2012. Starting from a profound interest in synthetic methodology, his current research efforts focus on the design and use of conformationally constrained amino acids as a tool to obtain selective and proteolytically stable peptides.

Dr. Florine Cavelier is CNRS Research Director at the Institute of Biomolecules Max Mousseron (IBMM) in Montpellier (France). She obtained her PhD in Organic Chemistry at the University of Montpellier in 1989 working on lanthionines, thioether-bridged peptides with antibiotic properties. She spent 2 years as a Royal Society Fellow at the Dyson Perrins Laboratory (with Prof. Jack Baldwin, Oxford), where she studied the biosynthesis of iso-penicillin. Next, she obtained an academic position at the National Center for Scientific Research (CNRS) and was promoted to Research Director in 2003. She currently heads the "Stereoselective synthesis and unnatural amino acids" team at IBMM. She was a board member (2007–2022) and president (2011–2013) of the "French Group of Peptides and Proteins." She was elected French representative of the European Peptide Society Council (2012–2020) and was appointed to the EPS Executive Committee as Scientific Affairs Officer (since 2020). Her research interests focus on unnatural amino acids (incl. silicon-containing amino acids) able to modulate the properties of biologically active peptides and increase their proteolytic stability.

Prof. Philippe Sarret received his PhD from the University of Nice Sophia Antipolis (Nice, France) in 2000. After his doctoral training, Prof. Sarret spent 4 years as a postdoctoral fellow at the Montreal Neurological Institute (McGill University, Montreal, Quebec, Canada) with Dr. Alain Beaudet. In 2004, he obtained a tenured faculty position at the University of Sherbrooke (Quebec, Canada), where he holds the Tier 1 Canada Research Chair in Neurophysiopharmacology of Chronic Pain. As director of the Sherbrooke Pharmacology Institute (since 2012) and director of the Neuroscience Research Center (since 2006), he has made several key discoveries in the field of G protein-coupled receptors (GPCRs) in pain modulation, from the optimization of novel pain-relieving compounds, their characterization in functional assays using relevant in vitro cell models, to the validation of their physiological actions in preclinical animal models using behavioral phenotyping tests. His efforts are currently focused on the development of non-opioid analgesics capable of overcoming the current opioid addiction and overdose epidemic, with the goal of providing adequate pain relief and improving the quality of life of patients living with severe pain conditions.

Prof. Steven Ballet completed his PhD at the Vrije Universiteit Brussel (VUB, Brussels, Belgium) in 2007. Directly following his PhD training, Dr. Ballet went for a first postdoctoral stay in Australia at the University of Adelaide with Professor Andrew Abell. During this stay, he applied ring-closing and cross-metathesis reactions on amino acid and peptide substrates. RCM was used to stabilize ("staple") the helical conformation of alpha and beta peptides. As a second postdoctoral training, Dr. Ballet went to the Institut de Recherches Cliniques de Montréal (IRCM, Montreal, Canada) for a specialized training in the opioid peptide field. Together with Prof. Peter W. Schiller, he designed bifunctional opioid ligands with dual MOR/DOR agonist profiles and hybrid opioid/non-opioid multitarget ligands. Since 2010, Dr. Ballet is appointed as a faculty member at his alma mater, where he pursues his efforts in the peptide and peptidomimetic field. Selected research topics involve injectable peptide hydrogels for sustained release of bioactive peptides, the synthesis of turn/helix/loop-based protein mimetics, and transition metal-catalyzed, late-stage derivatization of peptides (e.g., in aqueous media).

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