In Vitro Analysis of Stable, Receptor-Selective Neurotensin[8−13] Analogues

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A set of neurotensin[8−13] (NT[8−13]) analogues featuring substitution of non-natural cationic amino acids in the Arg(8) position have been synthesized and tested for binding potencies against the three cloned human NT receptors (hNTR-1, hNTR-2, hNTR-3), functional agonism of the hNTR1 and for rat serum stability. Three distinct classes of peptides have been identified: Class 1 features alkyl-Arg analogues at Arg(8), Class 2 features α-azido-cationic amino acids at Arg(8), and Class 3 feature modified Arg(8) and Tyr(11) residues. Most of the peptides maintain or exceed the binding potency of NT[8−13] to hNTR-1. Class 2 analogues exceed the binding potency of NT[8−13] to hNTR-2 with KK19 binding with higher affinity to hNTR-2 than hNTR-1. Peptides with enhanced binding potencies for hNTR-3 were not found. All analogues are functional agonists of the hNTR1 receptor as indicated by phosphoinositide (PI) determination. Serum stability increased with peptide classification where the half-life of Class 1 < Class 2 < Class 3 which are stable to rat serum for > 24 h.

Introduction

Neurotensin (NT), a tridecapeptide first isolated from bovine hypothalamus nearly 30 years ago,[1] has a myriad of biological activities both peripherally and in the central nervous system (CNS). Activities include induction of cyclic nucleotide production,[2,3] phosphatidylinositol turnover,[4] intracellular calcium influx,[5] phospholipase C,[6] and Na^+,K^+-ATPase activation.[7] When administered directly into the CNS, biological effects induced include muscle relaxation,[8] decreased food consumption,[9] catalepsy,[10] decreased locomotion,[11] hypothermia,[12] antinociception,[13] antipsychosis,[14] blockage of disruptions in prepulse inhibition-induced by DA agonists,[15] blockage of amphetamine-induced locomotor hyperactivity,[16] and potentiation of barbiturate-induced sedation.[17]

The biological effects of NT are realized upon binding to one of three cloned NT receptors (NTR-1, NTR-2, or NTR-3) or possibly yet uncharacterized NTRs.[18] NTR-1 and NTR-2 are G protein-coupled receptors maintaining 43% absolute amino acid homology.[18] In contrast, NTR-3, a non-G-protein-coupled receptor, is structurally identical to gp95sortilin.[19] The role of each receptor has yet to be explicitly defined. Recently, two NT antagonists, SR48692 and SR142948A, have been described and utilized to help define the functions of the NTR subtypes.[20,21] SR48692 has shown some selectively for NTR-1 since this compound does not inhibit NT-induced analgesia.[20] More recently, NTR-1 knockout mice have been generated to specifically address the roles of NTR-1.[22] It was reported that NTR-1 mediates the hypothermic, antinociceptic, and motor performance effects of centrally administered NT. However, other groups have demonstrated that the hypothermic and antinociceptive effects of NT are dissociable.[23,24] Hence there is much to learn about the independent and synergistic roles of the NTRs.

An alternative method to decipher the roles of the NTRs is to design and synthesize selective receptor agonists. The C-terminal hexapeptide fragment, NT[8−13] (H−Arg-Arg-Pro-Tyr-Ile-Leu-OH), contains the essential elements necessary for hNTR-1 binding.[25] Therefore, NT[8−13] is the obvious lead compound for NTR agonist design and development. Amino acid substitutions in the Arg(8), Arg(9), Tyr(11), and Ile(12) positions have produced peptides reported to cross the BBB and induce significant CNS effects such as hypothermia, antinociception, attenuation of haloperidol-induced catalepsy, and inhibition of *d*-amphetamine-induced hyperlocomotion.[26−29] Although active transport cannot be dismissed, increased lipophilicity and resistance against proteases appear to be responsible for BBB partitioning and the CNS effects.

Technology developed in our laboratory focuses on the design and synthesis of non-natural, cationic amino acids analogues of Arg and Lys.[30−35] These analogues (Figure 1) feature alkyl groups on, or adjacent to, the cationic portions of the side-chains. It was originally hypothesized that, when incorporated into biologically relevant peptides, these residues would favor the ion pairing versus solvated state thus resulting in peptides with increased binding affinities.[36] We have since demonstrated that these cationic homologues confer increased resistance to protease degradation, as Arg and Lys are often found in recognition motifs for these enzymes.[37] In addition, since hydrophobic compounds typically partition through membrane barriers to a greater extent than hydrophilic compounds,[38] these less polar residues may produce peptides with greater abilities to cross membrane barriers. Finally, altering the

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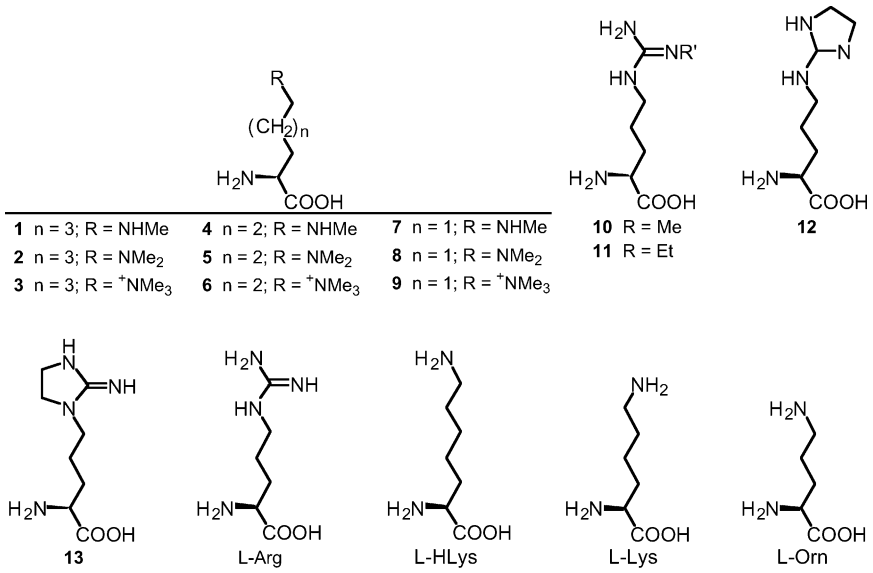

Figure 1. Structures of natural and non-natural cationic amino acids.

Table 1. Amino Acid Sequence and Analytical Data for NT, NT[8−13], and KK1−19

peptide	amino acid sequence													MW (g/mol) obsd[a] (calcd)[b]
	1	2	3	4	5	6	7	8	9	10	11	12	13	
NT	Glu	L-Leu	L-Tyr	L-Glu	L-Asn	L-Lys	L-Pro	L-Arg	L-Arg	L-Pro	L-Tyr	L-Ile	L-Leu	NA[c]
NT[8−13]	-	-	-	-	-	-	-	L-Arg	L-Arg	L-Pro	L-Tyr	L-Ile	L-Leu	NA
KK1	-	-	-	-	-	-	-	N_3-L-Hlys	L-Arg	L-Pro	L-Tyr	L-Ile	L-Leu	829.5 (829.0)
KK2	-	-	-	-	-	-	-	N_3-**1**[d]	L-Arg	L-Pro	L-Tyr	L-Ile	L-Leu	843.5 (843.0)
KK3	-	-	-	-	-	-	-	N_3-**2**	L-Arg	L-Pro	L-Tyr	L-Ile	L-Leu	857.5 (857.1)
KK4	-	-	-	-	-	-	-	N_3-**3**	L-Arg	L-Pro	L-Tyr	L-Ile	L-Leu	871.5 (872.1)
KK5	-	-	-	-	-	-	-	N_3-L-Lys	L-Arg	L-Pro	L-Tyr	L-Ile	L-Leu	815.5 (815.0)
KK6	-	-	-	-	-	-	-	N_3-**4**	L-Arg	L-Pro	L-Tyr	L-Ile	L-Leu	829.6 (829.0)
KK7	-	-	-	-	-	-	-	N_3-**5**	L-Arg	L-Pro	L-Tyr	L-Ile	L-Leu	843.5 (843.0)
KK8	-	-	-	-	-	-	-	N_3-**6**	L-Arg	L-Pro	L-Tyr	L-Ile	L-Leu	857.6 (858.1)
KK9	-	-	-	-	-	-	-	N_3-L-Orn	L-Arg	L-Pro	L-Tyr	L-Ile	L-Leu	801.4 (801.0)
KK10	-	-	-	-	-	-	-	N_3-**7**	L-Arg	L-Pro	L-Tyr	L-Ile	L-Leu	815.5 (815.0)
KK11	-	-	-	-	-	-	-	N_3-**8**	L-Arg	L-Pro	L-Tyr	L-Ile	L-Leu	829.5 (829.0)
KK12	-	-	-	-	-	-	-	N_3-**9**	L-Arg	L-Pro	L-Tyr	L-Ile	L-Leu	843.5 (844.0)
KK13	-	-	-	-	-	-	-	N_3-L-Hlys	L-Arg	L-Pro	L-Tyr	L-*tert*-Leu	L-Leu	829.5 (829.0)
KK14	-	-	-	-	-	-	-	N_3-L-Hlys	L-Arg	L-Pro	L-Trp	L-*tert*-Leu	L-Leu	852.5 (852.0)
KK15	-	-	-	-	-	-	-	N_3-L-Arg	L-Arg	L-Pro	L-Tyr	L-Ile	L-Leu	843.5 (843.0)
KK16	-	-	-	-	-	-	-	**10**	L-Arg	L-Pro	L-Tyr	L-Ile	L-Leu	831.6 (831.0)
KK17	-	-	-	-	-	-	-	**11**	L-Arg	L-Pro	L-Tyr	L-Ile	L-Leu	845.6 (845.0)
KK18	-	-	-	-	-	-	-	**12**	L-Arg	L-Pro	L-Tyr	L-Ile	L-Leu	843.6 (843.0)
KK19	-	-	-	-	-	-	-	**13**	L-Arg	L-Pro	L-Tyr	L-Ile	L-Leu	843.6 (843.0)

[a] An electrospray mass spectrum for each analogue was obtained using a Finnigan LCQ instrument. [b] Molecular weight was calculated using ChemDraw Pro 4.0.1. [c] NA = Peptides were purchased from Peninsula Laboratories and Sigma (St. Louis, MO) [d] Bolded numbers refer to non-natural amino acids as indicated in Figure. 1.

structure groups crucial to the binding of a peptide to its receptor should result in peptides that exhibit enhanced receptor subclass selectivities, as the peptides are likely to differentially interact with the receptor active site topologies.[39]

In the present study, the in vitro evaluation of a series of NT[8−13] analogues incorporating non-natural homologues of Arg and Lys designed and synthesized in this laboratory are reported. Receptor binding affinities, functional agonism, and plasma stability are determined for these analogues. Three classes of peptide analogues are identified with Class 3 analogues emerging as ideal candidates for further evaluation as potential antipsychotics due to their stability in rat serum.

Results

A set of 19 peptides containing non-natural amino acid residues substituted for Arg(8) (KK1−19) were synthesized and purified. Two peptides, KK13−14 feature additional non-natural amino acid substitutions at Tyr(11) and Ile(12).[29] The choice of residues substituted was based on providing a range of position (8) side-chain structures with and without N-terminal azido substitution (to remove the positive charge from the α-amino group) and to provide steric hindrance at the two sites of cleavage of the parent peptide, Arg(8)-Arg(9) and Trp-(11)-Ile(12).[37] Structures and analytical data for these peptides are summarized in Table 1.

Competitive Binding Assays. Each peptide was evaluated in competitive binding assays against the three cloned hNTRs, with binding affinities defined as the concentration of peptide required to inhibit 50% of the binding of ^{125}I-Tyr$^{(3)}$-NT to each hNTR (IC_{50}, Table 2).

hNTR-1. Substitution of the Nα amine of Arg(8) in NT[8−13] with an azide (KK15) did not adversely affect binding to the hNTR-1 binding when compared to NT-[8−13]. Substitution of the Arg(8) in NT[8−13] with

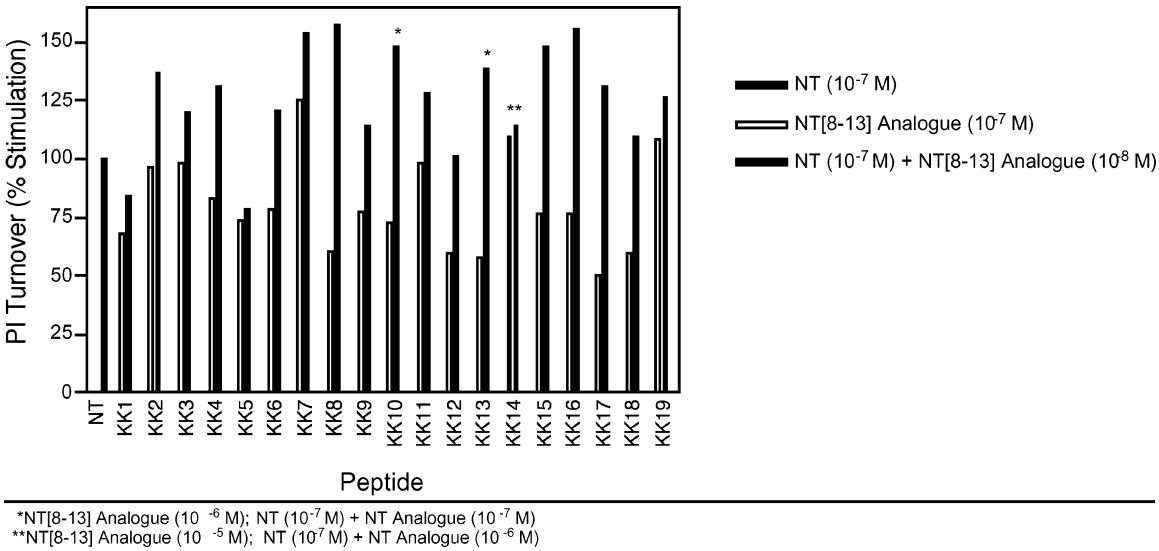

Figure 2. Effect of NT[8−13] analogues on PI production in cells transfected with hNTR1. COS-7 cells were transfected with hNTR1 and incubated for 15 min at 37 °C with the determined amount of NT[8−13] analogue alone or in combination with NT as described in the Experimental Section. Results are expressed as percent PI turnover with NT defined as 100% stimulation.

Table 2. Binding Affinities of NT and KK1−19 to HNTRs

	binding affinity (nM)		
peptide	hNTR1[a]	hNTR2[a]	hNTR3[b]
NT	0.4 ± 0.1	4.0 ± 1.1	440 ± 14
NT[8−13]	0.3 ± 0.1	2.7 ± 0.2	640 ± 40
KK1	0.38 ± 0.07	0.7 ± 0.2	1200 ± 55
KK2	0.52 ± 0.15	6.9 ± 1.3	400 ± 23
KK3	0.28 ± 0.10	3.5 ± 0.7	700 ± 80
KK4	0.51 ± 0.15	3.1 ± 0.9	550 ± 20
KK5	0.30 ± 0.14	2.1 ± 0.4	512 ± 8
KK6	0.34 ± 0.11	1.8 ± 0.2	1,150 ± 125
KK7	0.4 ± 0.2	2.8 ± 0.1	1,620 ± 50
KK8	1.6 ± 0.2	3.2 ± 0.1	3500 ± 200
KK9	0.59 ± 0.18	3.0 ± 0.3	1400 ±80
KK10	0.24 ± 0.08	3.5 ± 0.3	640 ± 35
KK11	0.36 ± 0.15	5.1 ± 0.2	480 ± 20
KK12	0.34 ± 0.1	5.3 ± 1.0	2000 ± 100
KK13	5.71 ± 0.37	11.1 ± 0.1	4000 ± 350
KK14	133 ± 4.5	500 ± 38	53 700 ± 2700
KK15	0.4 ± 0.1	0.9 ± 0.2	1740 ± 40
KK16	0.30 ± 0.05	0.67 ± 0.07	720 ± 25
KK17	0.25 ± 0.07	0.3 ± 0.1	480 ± 20
KK18	0.3 ± 0.1	0.78 ± 0.15	510 ± 35
KK19	1.2 ± 0.7	0.23 ± 0.05	590 ± 50

[a] Cell membranes transfected with either hNTR-1 or hNTR-2 were incubated with 0.4 nM ^{125}I-Tyr$^{(3)}$-NT (2000 Ci/mmol) and various concentrations of unlabeled NT, or the indicated NT[8−13] analogue. For ^{125}I-Tyr$^{(3)}$-NT binding to hNTR-1 (0.2 nM = 200 000 cpm/assay) total binding = 20 000, nonspecific binding = 650 for 10 μg of protein. For ^{125}I-Tyr$^{(3)}$-NT binding to hNTR-2 (0.3 nM = 300 000 cpm/assay) total binding = 3500 and non specific binding = 650 for 30 μg of protein. Nonspecific binding was determined in the presence of 1 μM unlabeled NT and represented less than 5% of the total binding. [b] Whole cells transfected with hNTR-3 were incubated with 0.4 nM ^{125}I-Tyr$^{(3)}$-NT in the presence of 0.8 mM 1,10-phenanthroline and increasing concentrations of NT, or the indicated NT[8−13] analogue. For ^{125}I-Tyr$^{(3)}$-NT binding to hNTR-3 (0.4 nM = 400 000 cpm/assay) total binding = 8500 and nonspecific binding = 2500 for 70 μg of protein performed on whole cells. Nonspecific binding was determined with 1 μM unlabeled NT.

α-azido analogues of ω-alkylated or nonalkylated Hlys, Lys, Orn, and Arg (KK1−12, KK15) and ω-alkylated Arg analogues (KK16−19) resulted in binding affinities for hNTR-1 (IC_{50} = 0.24−1.6 nM) that maintain or exceed the binding affinity of NT[8−13] (0.3 nM). Disubstituted peptides, KK13 and KK14, exhibited a significant decrease in hNTR-1 binding (5.71 nM and 133 nM respectively) compared to NT[8−13]-hNTR-1 binding.

hNTR-2. Monosubstituted, α-azido NT[8−13] analogues KK1−12 bind to hNTR-2 with varied affinities ranging from 0.71 to 5.3 nM. Peptides containing ω-alkylated Arg residues in the Arg(8) position (KK16−19) bind hNTR-2 with higher affinities (IC_{50} = 0.23−0.67 nM) than that of NT[8−13], with KK19 exhibiting 10-fold better binding. In addition, two α-azido NT[8−13] analogues (KK1 and KK15) exhibited binding affinities 3-fold better than NT[8−13]. Like hNTR-1, hNTR-2 binds disubstituted NT[8−13] analogues, KK13 and KK14, with lower affinities when compared to NT-[8−13].

hNTR-3. All peptides exhibited binding affinities greater than 2 orders of magnitude lower than hNTR-1. hNTR-3 did not accept the α-azido substitution as readily as hNTR-1 or hNTR-2. Many of our peptides exhibited binding affinities greater than NT[8−13]. However, only KK2 (IC_{50} = 400 nm) exhibited a binding affinity exceeding that of NT (IC_{50} = 440 nM).

Phosphoinositide Determination. PI turnover was measured in COS-7 cells expressing hNTR1. Results are summarized in Figure 2. All analogues demonstrated significant agonism of the receptor with KK19 producing the largest amount of PI turnover (126%) compared to NT. Three other analogues KK2, KK3, and KK7 also demonstrated PI production that meets or exceeds PI turnover produced by NT. Analogues KK10, KK13, and KK14 required increased incubation concentrations in order to stimulate PI turnover in the range of the other NT[8−13] analogues.

Degradation Analysis in Rat Blood Serum. Each of the peptides were dissolved in rat serum, and degradation rates were determined using a quantitative MALDI assay developed in this laboratory.[37] Representative plots of signal intensity ratios versus time are illustrated in Figure 3. A linear regression analysis of each peptide degradation profile was performed (GraphPad Prism) to determine the in vitro $t_{1/2}$ that are summarized in Table 3. Peptides featuring substitution of ω-alkylated Arg derivatives (**10**−**13**) in the Arg(8) position (KK16−19) inhibited, but did not prevent, aminopeptidase cleavage of the Arg(8)-Arg(9) bond. In

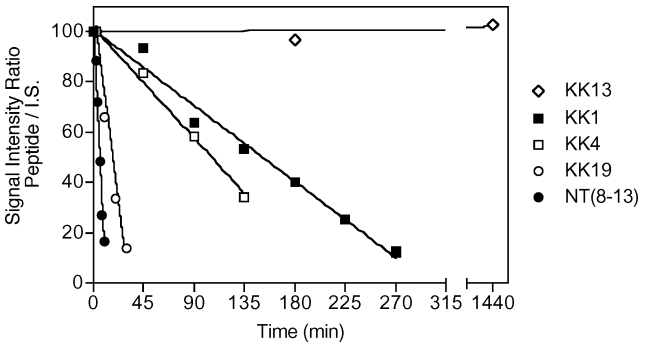

Figure 3. In vitro degradation of NT[8−13] and representative NT[8−13] analogues. MALDI-TOFMS was utilized to quantify relative rates of in vitro degradation according to a modified method of Kokko and Dix (2002). Data points represent group means for triplicate measurements. Lines represent a linear regression analysis over time.

Table 3. Degradation Half-Lives of KK1−19[a]

peptide	plasma $t_{1/2}$[a] (min)
NT[8−13]	5.9 ± 0.2
KK1	153 ± 4
KK2	211 ± 6
KK3	126 ± 18
KK4	103 ± 10
KK5	136 ± 6
KK6	128 ± 6
KK7	127 ± 2
KK8	128 ± 30
KK9	134 ± 27
KK10	131 ± 8
KK11	126 ± 9
KK12	216 ± 33
KK13	stable > 24 h
KK14	stable > 24 h
KK15	174 ± 7
KK16	14.2 ± 0.3
KK17	31.5 ± 0.6
KK18	15.9 ± 1.6
KK19	16.5 ± 0.6

[a] Rat serum containing 1.07 mM of peptide was incubated at 37 °C in a water bath. Aliquots were removed at predetermined times and precipitated in alcohol (3:1 MeOH:EtOH). The alcoholic solution was combined with the internal standard and added to the matrix (α-CHCA) and spotted, on the target plate preheated to 31 °C on a hot plate, with 0.5 μL of the mixture. The samples were analyzed by MALDI-TOFMS. Half-lives were determined from triplicate measurements subjected to linear regression over the time course.

contrast, peptides which incorporated an azide in the Arg(8) α-position (KK1−15) completely resist aminopeptidase cleavage between the Arg(8)-Arg(9) bond. These peptides are degraded via an alternate route through cleavage of the Tyr(11)-Ile(12) bond resulting in a di- and tetrapeptide, the latter of which is not degraded further. Concomitant substitution of an azide in the Arg-(8) α-position and *tert*-Leu in the Ile(11) position (KK13−14) confers full resistance to peptidase activity in rat serum.

Discussion

A set of NT[8−13] analogues which feature non-natural, cationic amino acids in the Arg(8) position have been characterized. Many of these analogues maintain or exceed hNTR binding affinities compared to NT[8−13] while exhibiting drastically increased plasma stabilities.

Regarding hNTR binding, several key observations can be made. First, analogue KK15 differs only from NT[8−13] in that an azide is substituted in place of the α-amine in the Arg(8) position. Incorporation of the azide did not adversely affect binding affinity to the hNTR-1, which demonstrates that substitution of this lipophilic group for the hydrophilic amine does not adversely affect overall potency at the hNTR-1. Moreover, the azido substitution enhances binding to hNTR-2 3-fold. A variety of other α-azido peptides (KK1−12) that feature variations in the Arg(8) side chain length and structure also maintain high affinities for the hNTR-1. Furthermore, NT[8−13] analogues that feature ω-alkylated Arg analogues (KK16−18) meet or exceed NT[8−13] in hNTR-1 binding. This finding further demonstrates the elasticity of hNTR-1 to accommodate an array of substitutions in the Arg(8) position. While other groups have demonstrated the ability of NTR-1 to bind peptides featuring non-natural and D-amino acids in the Arg(8) position,[40] until these peptides were introduced,[39,41] no group has exploited the elasticity of the hNTR-1, while maintaining potency and increasing the lipophilicity of the peptide. In contrast, modification of residues 11 and/or 12 of NT[8−13] were not accepted as readily as the modifications to the N-terminus, as indicated by the significant drop in binding affinities of KK13 and KK14. This finding is in agreement with results reported by other groups that have incorporated l-Trp and l-*tert*-Leu in the Tyr(11) and Ile(12) positions, respectively,[40,42,43] and likely reflects either a negative influence of the groups on the binding conformer population or the introduction of adverse steric effects. Moreover, all analogues tested were confirmed to act as functional agonist at the hNTR1 as demonstrated by PI turnover. This is significant because hNTR1 is hypothesized to mediate the effects of NT as it corresponds to the potential for treating schizophrenia.

The best hNTR-2 binding analogues (KK15−19) were 3- to 12-fold more potent than NT[8−13]. KK19 in particular binds better to hNTR-2 than hNTR-1 despite the significant inherent differences in NT binding of these receptors (IC_{50} of 0.4 and 4.0, respectively). Therefore, KK19 represents a first-generation precursor which, with further study, may develop into a unique biochemical tool used to define the role of hNTR-2 in physiology and the development of selective hNTR-2 agonists having the potential for use in pain management.[23] Although hNTR-3 has been implicated in the growth response of human cancer cells to neurotensin,[44] the physiological roles this receptor are significantly less characterized than hNTR-1 and -2. Identifying selective NTR-3 agonists and antagonists would thus represent a major achievement leading to the elucidation of its biological functions. Unfortunately, no peptides in the series studied herein exhibited significantly enhanced hNTR-3 binding or enhanced selectivity compared to hNTR-1 or hNTR-2, although, as with the other two receptors, a fairly wide range of affinities were observed.

One of the many obstacles that peptide-based drug candidates must circumvent is degradation by peptidases of the vasculature. Therefore, determining the relative stabilities of NT[8−13] and analogues in blood serum is a key prerequisite for the design and synthesis of NT[8−13] analogues with pharmacological or clinical

potential. Inspection of the degradation $t_{1/2}$ data for KK1−19 in Table 3 enables the identification of three distinct classes of peptides:

Class 1: Peptides maintaining $t_{1/2}$s between 10 and 35 min (KK16−19).

Class 2: Peptides maintaining $t_{1/2}$s between 100 and 220 min (KK1−12 and KK15).

Class 3: Peptides stable to serum peptidases for greater than 24 h (KK13−14).

These classes reflect a distinct pattern. Class 1 consists of all of the NT[8−13] analogues that contain *N*-alkyl Arg residues (KK16−19) substituted in the Arg-(8) position. Like NT[8−13],[38] degradation of these peptides proceeds primarily through the cleavage of the Arg(8)-Arg(9) bond, albeit less quickly than NT[8−13]. Substitution of non-natural, alkyl Arg residues **10**−**13** (Figure 1) into the Arg(8) position increases the $t_{1/2}$ of NT[8−13] 2.5- to 5.5-fold. Class 2 contains all of the NT-[8−13] analogues that contain a single α-azido acid substitution (KK1−12 and KK15) in the Arg(8) position. Substitution of α-azido acid analogues in the Arg(8) position completely blocks peptidase cleavage of the Arg-(8)-Arg(9) bond, which demonstrates that the α-amine is a key recognition element for serum peptidases. These substitutions increase the $t_{1/2}$ of NT[8−13] 18- to 38-fold by shifting the degradation mechanism to the slower cleavage of the Tyr(11)-Ile(12) bond.[38] Class 3 contains all of the NT[8−13] analogues that contain multiple substitutions at the Arg(8) and Ile(12) position (KK13−14). These peptides are stable to peptidase degradation over 24 h in rat serum. As a result, it can be concluded that concomitant substitution at the Arg-(8) and Ile(12) positions with non-natural amino acids completely prevents peptidase degradation in rat serum. Prior studies have demonstrated the Arg(8)-Arg(9) and the Tyr(11)-Ile(12) bond to be major sites of peptide cleavage in the brain;[45−50] hence, it is hypothesized that these peptides will be resistant to peptidases in the brain. Modifications of this method in order to quantify NT[8−13] and KK1−19 in brain homogenate are currently under investigation.

This in vitro stability and hNTR binding data provoke further investigation into the design and synthesis of novel NT[8−13] analogues that are both resistant to peptidase degradation yet more selective for a specific NTR. The three peptides of greatest interest for exploring the effects of hNTR-1 versus -2 are KK12, which has a 16-fold greater affinity for hNTR-1 over -2, KK19, which has a 5-fold greater affinity for NTR-2 over -1, and KK17, which binds equally (within standard error) to both receptors. This provides a matrix by which we can begin to address the potential roles of the two receptors and different biological effects if other mitigating factors (relative access to receptors, for example), can be ruled out. KK12, KK17, and KK19 also can be used as templates on which to design second-generation analogues anticipated to feature higher degrees of selectivity for each receptor subtype. The strategy is simply to substitute the non-natural residue at position 8, which is different for the two analogues, with the identical structure in which the steric bulk of each R-group is increased through the standard series. KK12 features a trimethylammonium ion at position 8 side-chain; thus, the sequence monoethyldimethyl-, diethyl-monomethyl-, and triethylammonium ions should lead to greater hNTR-1 selectivity with, hopefully, not a serious loss of overall receptor binding. Larger alkyl groups should also be explored if binding is maintained in the latter series. Conversely, KK17 manipulation through increasing the size of the ethyl group on position 8 ethyl-Arg should provide more potent hNTR-1 and hNTR-2 agonists. The logic of this strategy is supported by data in Table 2 − going from control → KK16 → KK17 (where Arg(8) → Me-Arg(8) → Et-Arg-(8)) results in a small improvement of NTR-1 binding and a large improvement in NTR-2 binding. Conversely, modifications of KK19 via the addition of alkyl groups (methyl, ethyl, isopropyl) on the side may result in peptides that further exploit hNTR-2 preference for alkylated side chains resulting in more selective hNTR-2 agonist. Other structural changes can be made to increase the selectivity for hNTR-2. Placing an α-azido group in the Arg(8) position of KK19 may also lead to a more hNTR-2 selective peptide, based on the observation that, with KK15, an increase in hNTR-1 binding was not observed versus parent, in contrast to the 4-fold binding increase seen with hNTR-2. This will also have the advantage of significantly stabilizing the peptide from degradation by converting it from a class 1 to a class 2 peptide (see above). Since substitution of Trp-(11) and *tert*-Leu(12) for Tyr(11) and Ile(12) did not lower the binding affinity to as great of an extent when binding to hNTR-2 versus hNTR-1, incorporating one or both of these substitutions in combination with the above substitution in the Arg(8) position is expected to provide peptides with even higher selectivity for hNTR-2. Ultimately, completion of these studies should result in second-generation compounds that will allow further characterization of the active-site topography of the hNTRs while producing stable compounds with greater selectivities for individual receptors which can be used to further define the relative contribution of each hNTR to the observed pharmacological effects.

Conclusion

In summary, a series of NT[8−13] analogues which contain non-natural cationic amino acid homologues of Arg and Lys were synthesized and binding affinities determined for the known hNTRs, for functional agonism of hNTR1 and for peptide stability in serum. Identified in this report are three classes of peptides based on serum stability and hNTR binding. Class 1 peptides (KK16−19) feature ω−alkylated Arg residues in the Arg(8) position, confer limited serum stability, and bind to hNTR-1 and hNTR-2 with high affinity. KK17 and KK19 have been chosen as leads to further exploit selectivity for hNTR-2 over hNTR-1. Class 2 peptides (KK1−12, KK15) feature an azido group substituted for the α−amine in the Arg(8) position, confer a significant degree of serum stability and bind to hNTR-1 with high affinity. Second generation peptides are currently being designed to increase the selectively for hNTR-1 over hNTR-2. Class 3 peptides (KK13 and KK14), which feature an azido group substituted for the α−amine in the Arg(8) position as well as *tert*-Leu inthe Tyr(11) position, confer complete resistance to serum peptidases. Because these peptides are stable and are functional agonist, they are currently being investigated

in our laboratory for in vivo activity as potential antipsychotics.

Experimental Section

Starting Materials. NT was from Peninsula Laboratories and NT(2−13) was synthesized by Neosystem. ^{125}I-Tyr$^{(3)}$-NT was prepared and purified as described previously.[51] The expression plasmid containing the cDNA from the human NTR-3 was prepared as described previously.[19] The pcDNA3-human hNTR-1 and hNTR-2 plasmids were gifts from Dr. Patrick Kitabgi (Valbonne, France). Dulbecco's modified Eagle's medium was from Life Technologies Inc, 1,10-phenanthroline from Sigma France, and fetal calf serum from Roche Diagnostics. NT[8−13] and Kemptide (H-Leu-Arg-Arg-Ala-Ser-Leu-Gly-OH) were purchased from Sigma (St. Louis, MO). NT-[9−13] was purchased from Bachem (Torrence, CA). α-Cyano-4-hydroxycinnamic acid (α-CHCA) was purchased from Aldrich (Milwaukee, WI). Rat serum was purchased from Bioreclamation Inc. (Hicksville, NY). Amino acids for peptide synthesis were purchased from Advanced Chem Tech (Louisville, KY).

Synthesis of Non-Natural Amino Acids. Non-natural amino acids analogues of Arg and Lys (Figure 1) protected for incorporation into Merrifield-based peptide synthesis schemes were synthesized as described previously by our laboratory[30,31,33−35] and others.[52]

Peptide Synthesis and Purification. Peptides were synthesized and purified as previously described.[39,41] Purity was assessed utilizing three separate analytical HPLC systems.

System 1. RP-HPLC analysis was performed on a Waters dual pump HPLC system equipped with a Vydac (C18, 10 mm × 250 mm) column. The solvent system consisted of 0.1% TFA in water (solvent A) and 0.1% TFA in acetonitrile (solvent B). Samples (approximately 1−2 mg) were eluted with a linear gradient from 0% to 50% B over 30 min at a flow rate of 3.0 mL/min and detected by UV absorbance at 280 nm.

System 2. RP-HPLC analysis was performed using a Waters dual pump HPLC system equipped with a Bakerbond (C18, 4.6 mm × 250 mm) column. The solvent system consisted of 0.1% TFA in water (solvent A) and 0.1% TFA in 83% acetonitrile (solvent B). Samples (approximately 10−50 μg) were eluted with a linear gradient from 5% to 50% B over 30 min at a flow rate of 1.0 mL/min and UV detection at 220 nm.

System 3. RP-HPLC analysis was performed on an ABI chromatograph (model 130A, Applied Biosystems) with an Aquapore 300 column (C8, 2.1 mm × 30 mm). The solvent system consisted of 0.1% TFA in water (solvent A) and 0.1% TFA in 80% acetonitrile (solvent B). Approximately 1−2 μg of peptide was injected and separated at 100 μL/min with a gradient of 5% to 50% B. UV detection was at 230 nm. All peptides analyzed were greater than 95% pure in all three systems.

Mass Spectroscopy. An electrospray mass spectrum for each peptide (M^+ and $[M + 2H]^{2+}$ ions) was obtained using a Finnigan LCQ instrument and indicated the correct molecular weight for each peptide.

hNTR Binding Assays. Transient transfections were performed with 1−5 μg of recombinant vector by the DEAE-dextran precipitation method.[53] Binding assays were performed either on cells plated in 12-mm cell culture dishes or on cell homogenates approximately 60 h after transfection.

Binding Experiments on Cell Homogenates (hNTR-1 and hNTR-2). Homogenates from cells transfected with either the hNTR-1 or the hNTR-2 plasmids were prepared as previously described.[54] Cell membranes (10−50 μg) were incubated in 250 μl of 50 mM Tris-HCl, pH 7.5, containing 0.1% bovine serum albumin (binding buffer) with 0.4 nM ^{125}I-Tyr$^{(3)}$-NT (2000 Ci/mmol) and various concentrations of unlabeled NT, or the indicated NT analogues. After 20 min at 25 °C, incubation media were filtered through cellulose acetate filters (Sartorius). Filters were rinsed twice with 3 mL of ice-cold binding buffer and counted in a Packard γ-counter (counting efficiency, 80%). For ^{125}I-Tyr$^{(3)}$-NT binding to hNTR-1 (0.2 nM = 200 000 cpm/assay) total binding = 20 000, nonspecific binding = 650 for 10 μg of protein. For ^{125}I-Tyr$^{(3)}$-NT binding to hNTR-2 (0.3 nM = 300 000 cpm/assay) total binding = 3500 and nonspecific binding = 650 for 30 μg of protein. Nonspecific binding was determined in the presence of 1 μM unlabeled NT and represented less than 5% of the total binding.

Binding Experiments to Whole Transfected Cells (hNTR-3). Cells (2×10^5) were equilibrated for 10 min in an Earle's Tris-HEPES buffer, pH 7.5, supplemented with 0.1% glucose and 0.1% bovine serum albumin. The equilibration buffer was then replaced by 250 μL of binding buffer containing 0.4 nM ^{125}I-Tyr$^{(3)}$-NT in the presence of 0.8 mM 1,10-phenanthroline and increasing concentrations of NT, or the indicated NT analogues, for 30 min at 37 °C. At the end of incubation, cells were washed twice with 0.5 mL of equilibration buffer. Cells were harvested with 0.1 N NaOH and counted in a γ-counter. For ^{125}I-Tyr$^{(3)}$-NT binding to hNTR-3 (0.4 nM = 400 000 cpm/assay) total binding = 8500 and nonspecific binding = 2500 for 70 μg of protein performed on whole cells. Nonspecific binding was determined with 1 μM unlabeled NT.

Phosphoinositides Determination. Twenty four hours after transfection with hNTR1, cells were grown in 12-well plates for 15 to 18 h in the presence of 1 μCi of [^{3}H]myoinositol (ICN Biomedicals, Ovsay, France) in a serum-free Ham's F10 medium. Cells were washed with Earles's buffer, pH 7.5 (25 mM HEPES, 25 mM Tris, 140 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$, and 5 mM glucose) containing 0.1% bovine serum albumin and incubated for 15 min at 37 °C in 900 μL of 30 mM LiCl in Earles's Buffer. NT was then added at the indicated concentrations for 15 min. The reaction was stopped by 750 μL of ice-cold 10 mM HCOOH, pH 5.5. After 30 min at 4 °C, the supernatant was collected and neutralized by 2.5 mL of 5 mM NH_4OH. Total [^{3}H]PI were separated from free [^{3}H]inositol on dowex AG-X8 (Bio-rad, Hercules, CA)[55] chromatography by eluting successively with 5 mL of water and 4 mL of 40 mM and 1 M ammonium formate, pH 5.5. The radioactivity contained in the 1 M fraction was counted after addition of 5 mL of Ecolume (ICN Biomedicals).

Degradation Analysis in Rat Blood Serum. Determination of in vitro degradation rates of NT[8−13] and KK1−19 were performed according to a modified method of Kokko and Dix.[37] Briefly, rat serum (290 μL) was placed in a microcentrifuge tube and warmed to 37 °C in a water bath. To this was added 10 μL of peptide in saline (1.07 mM, final concentration), mixed via vortex and incubated at 37 °C. Aliquots (20 μL) were removed at predetermined times and added to 80 μL of alcohol (3:1 MeOH:EtOH), vortexed for 30 s and centrifuged (Sigma C 5605, 2000 g) for 10 min. Next, 1 μL of the alcoholic solution was combined with 1 μL of the internal standard (I.S., Kemptide, 1.30 nmol in saline) and added to 6 μl of matrix (50 mM, α-CHCA in 70% ACN and 0.1% TFA). The target plate, preheated to 31 °C on a hot plate, was spotted with 0.5 μL of the mixture. When dry (20 s/sample), the target plate was removed from the hot plate, allowed to cool to RT, and analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS).

MALDI-TOFMS. MALDI-TOFMS was performed using a Voyager-DE System 1040 mass spectrometer (Applied Biosystems). A nitrogen laser (337 nm) was used for ionization. The accelerating voltage was set to 20 000 V; grid voltage was set to 94% of the accelerating voltage; guide wire voltage was 0.05% of the acceleration voltage; and delay time was 100 ns. The low mass gate was set at 300 Da; laser rep rate was set to 3.0 Hz. Data were accumulated between 300 and 1000 Da. Each data point is the summation of 300 laser shots to the center of the sample.

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Appendix

Abbreviations: All natural amino acids used are of the L-configuration; ACN, acetonitrile; BBB, blood−brain barrier; α-CHCA, α-cyano-4-hydroxycinnamic acid; Da, Daltons; ETOH, ethanol; hNTR, human neurotensin receptor; *tert*-Leu, *tert*-butyl-glycine; MALDI-TOFMS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; MeOH, methanol; NT, neurotensin; NT[8−13], neurotensin[8−13]; RP-HPLC, reverse-phase high-pressure liquid chromatography; TFA, trifluoroacetic acid.

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