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A STRUCTURE-AFFINITY STUDY OF THE AMINO ACID SIDE-CHAINS IN NEUROTENSIN : N and C TERMINAL DELETIONS AND ALA-SCAN

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Abstract : We report a systematic N- and C-terminal deletion and Ala-scan study which quantifies the involvement of each neurotensin amino acid side-chain for the in vitro binding interaction with the neurotensin receptor. The conclusion is that Tyr^{11} and Leu^{13} are the most important and there appears to be some synergy between the binding interactions of these two side-chains.

Neurotensin (NT) is a tridecapeptide which was first isolated from bovine hypothalamic extracts [1] and has been identified as one of the brain gut peptides. It is widely distributed in the CNS where it appears to act as a neurotransmitter/neuromodulator and in peripheral tissues where it has many of the properties of a hormone/cellular mediator [2-4].

Neurotensin (NT) = $pGlu^{1}$-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu^{13}-OH.

The objective of the present study was to investigate the role that the amino acid side-chains of neurotensin play in the in vitro receptor binding interaction between this peptide and its receptor. Our strategy was to systematically prepare N- and C-terminal deletion peptides to identify the shortest continuous peptide fragment that retains full peptide receptor binding affinity and then to sequentially replace each amino acid within this minimum fragment with a single Ala residue. L-Ala was selected for this "scan study" because its amino acid side-chain is a single methyl group and it is less likely to severely alter the main chain conformation than a Gly-scan. Similar Ala-scans have been performed on other peptides to probe the importance of the amino acid side-chains in the receptor binding interactions of the N-terminus of galanin [5], the neuropeptide-Y derivative NPY (1-4)aca(25-36) [6], human growth hormone [7], interleukin-8 [8], and also to probe the molecular recognition of inhibitory peptides of cAMP-dependent protein kinase [9].

In order to preserve the neutral amide environment found in the backbone of neurotensin we decided that the deletion peptides should be acetylated (for N-terminus deletion) or amidated (C-terminus deletion).

Table 1. Neurotensin receptor binding affinities for N- and C-terminal deletion peptides.

Peptide[a]	NT receptor binding affinity (K_i, M)[b]	
Neurotensin (1-13)	1.7×10^{-10}	(0.9 - 2.0)
Ac-Neurotensin (2-13)	1.1×10^{-10}	(0.7 - 1.6)
Ac-Neurotensin (3-13)	2.1×10^{-10}	(1.8 - 2.3)
Ac-Neurotensin (4-13)	1.2×10^{-10}	(0.8 - 1.8)
Ac-Neurotensin (5-13)	1.3×10^{-11}	(1.0 - 2.1)
Ac-Neurotensin (6-13)	2.4×10^{-11}	(2.3 - 2.5)
Ac-Neurotensin (7-13)	1.0×10^{-10}	(0.7 - 1.4)
Ac-Neurotensin (8-13)	1.3×10^{-10}	(1.1 - 1.6)
Neurotensin (8-13)	1.8×10^{-11}	(1.4 - 3.3)
Neurotensin (8-12)NH_2	1.2×10^{-5}	(0.8 - 1.7)
Neurotensin (8-13)NH_2	1.0×10^{-8}	(0.8 - 1.1)

[a]The peptides were prepared by solid phase peptide synthesis using Fmoc methodology, and purified to >95% by reverse phase HPLC. Identity was confirmed by amino acid analysis and FAB mass spectrometry.

[b]K_i represents the concentration producing half-maximal inhibition of specific binding of [I^{125}]-Tyr^3-NT(1-13) to neurotensin receptors in neonatal mouse whole brain (minus cerebellum). The values given are the geometric mean and the range from at least 3 separate experiments. Non-specific binding was defined with 1×10^{-6}M NT (1-13).

Table 2. Neurotensin receptor binding affinity for the Ala-scan peptides together with the change in binding affinity (K_i/1.8×10^{-11}M) and the change in free energy of binding ($\Delta\Delta G$) for each Ala-scan peptide compared to NT(8-13).

Compound	Structure	Neurotensin receptor binding affinity (K_i, M)[b]	K_i, M / 1.8×10^{-11}M	$\Delta\Delta G$[a]
NT(8-13)	Arg^8-Arg^9-Pro^{10}-Tyr^{11}-Ile^{12}-Leu^{13}	1.8×10^{-11} (1.4 - 3.3)	1	0
[Ala^8]-NT(8-13)	Ala-Arg-Pro-Tyr-Ile-Leu	2.5×10^{-10} (1.5 - 5.0)	14	1.5
[Ala^9]-NT(8-13)	Arg-Ala-Pro-Tyr-Ile-Leu	2.1×10^{-9} (0.9 - 6.8)	120	2.8
[Ala^{10}]-NT(8-13)	Arg-Arg-Ala-Tyr-Ile-Leu	9.2×10^{-10} (0.6 - 1.5)	50	2.3
[Ala^{11}]-NT(8-13)	Arg-Arg-Pro-Ala-Ile-Leu	1.1×10^{-7} (0.8 - 1.5)	6100	5.1
[Ala^{12}]-NT(8-13)	Arg-Arg-Pro-Tyr-Ala-Leu	4.5×10^{-9} (1.0 - 11)	250	3.2
[Ala^{13}]-NT(8-13)	Arg-Arg-Pro-Tyr-Ile-Ala	2.0×10^{-7} (1.5 - 2.3)	11000	5.4
[$Ala^{11,13}$]-NT(8-13)	Arg-Arg-Pro-Ala-Ile-Ala	3.9×10^{-6} (2.5 - 5.8)	220000	7.2

Footnotes : [a]$\Delta\Delta G$ = {ΔG (binding)/kCal.mol^{-1} for NT (8-13)} - {ΔG (binding)/kCal.mol^{-1} for Ala derivative} ($\Delta G = -RT \ln K_i$). [b]see footnote[b] table 1

In this respect the present study differs from those previously reported for neurotensin [10-13] which have unprotected amino or carboxy groups in the deletion peptides. The results (table 1) show that the hexapeptide C-terminal fragment Ac-NT(8-13) ($K_i = 1.3 \times 10^{-10}$M) retains similar binding affinity to NT (1-13) ($K_i = 1.7 \times 10^{-10}$M) indicating that neither the amino acid side-chains on residues (1-7) nor their amide backbone contributes to receptor binding. If the C-terminal residue of the hexapeptide is amidated or deleted the binding affinity is reduced considerably (77,92000-fold reduction in binding affinity respectively). This is in agreement with other reports that the full in vitro smooth muscle activity of NT resides in the 8-13 fragment [10-13]. Removal of the N-acetyl protecting group to give NT(8-13) improves the binding affinity 7-fold ($K_i = 1.8 \times 10^{-11}$) and this high affinity hexapeptide was used as the starting point for the Ala-scan.

The results of the Ala-scan (table 2) indicate that the i-Pr group of Leu^{13} and the phenolic ring in Tyr^{11} are particularly important for binding. Deletion of these side-chains decreases the binding affinity by 11000- and 6100-fold respectively. Interestingly the three carbon atoms of the Leu side-chain appear to contribute 5.4 kCal/mol to the free energy of this binding interaction. This is considerably more than the average of approximately 1 kCal/mol/methylene group that has been observed in other molecular recognition phenomena [14,15] and more than is observed when the three carbon atoms are removed from the Ile^{12} side-chain (250-fold decrease in binding affinity, 3.2 kCal/mol). An explanation of this discrepancy may be that the $Leu^{13} \rightarrow$ Ala substitution decreases binding interactions of other groups in the molecule (see below).

The basic guanidine side-chains at Arg^8 and Arg^9 contribute 14- and 240-fold respectively to the binding. This indicates that they are less important than Tyr^{11} and Leu^{13}.

The binding changes observed for these Ala-scan derivatives may be due to factors other than deletion of a group which is itself directly involved in binding. For example, the increased conformational flexibility of the amide backbone in the $Pro^7 \rightarrow$ Ala peptide may contribute to its decreased binding energy (50-fold, 2.3 kCal/mol).

In the right hand column of table 2 we report the difference between the free energy of the binding interaction for the Ala-scan derivatives versus the free energy of the binding interaction of NT(8-13)($\Delta\Delta G$ values, kCal/mol). The sum of the $\Delta\Delta G$ values for the six Ala-scan peptides in table 2 is 20.3 but the ΔG value for the binding of NT (8-13) itself is only 14.4 kCal/mol. This led to the speculation that the Ala replacements may decrease the binding interactions of more than just the amino acid side-chain which is replaced. This speculation, together with our observation of the unusually high $\Delta\Delta G$ for $Leu^{13} \rightarrow$ Ala led us to replace both the Tyr^{11} and Leu^{13} in one peptide, [$Ala^{11,13}$]NT(8-13). The change in the free energy of

the binding interaction of this compound ($\Delta\Delta G$ = 7.2 kCal/mol) is less than the sum of the $\Delta\Delta G$ values for [Ala^{11}]NT(8-13) and [Ala^{13}]NT(8-13)(10.5 kCal/mol). This indicates that there is some synergy between the binding interactions of these two side-chains.

This is the first systematic study which quantifies the importance of each amino acid side-chain in the peptide-neurotensin receptor binding interaction. Our results are in agreement with previous reports which have demonstrated the importance of Tyr^{11} [13], and Leu^{13} [11,12].

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