REVIEW

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Cancer, Chemistry, and the Cell: Molecules that Interact with the Neurotensin Receptors

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ABSTRACT The literature covering neurotensin (NT) and its signalling pathways, receptors, and biological profile is complicated by the fact that the discovery of three NT receptor subtypes has come to light only in recent years. Moreover, a lot of this literature explores NT in the context of the central nervous system and behavioral studies. However, there is now good evidence that the up-regulation of NT is intimately involved in cancer development and progression. This Review aims to summarize the isolation, cloning, localization, and binding properties of the accepted receptor subtypes (NTR1, NTR2, and NTR3) and the molecules known to bind at these receptors. The growing role these targets are playing in cancer research is also discussed. We hope this Review will provide a useful overview and a one-stop resource for new researchers engaged in this field at the chemistry–biology interface.

Neurotensin (NT) is a short, endogenous peptide that was first isolated from the extracts of bovine hypothalami in 1973 (*1*) and was later prepared synthetically using Merrifield's solid-phase synthesis procedures (*2*). At that time, the physiological role of NT was not understood very well, but it was believed to have a neuronal function as a modulator in the brain and some involvement in regulating hormonal activity (*3*). Furthermore, the observation that NT could induce vasodilation was an early indicator that it may not be restricted to the central nervous system (CNS). In fact, we have since come to learn that NT is intimately involved in a number of important biological processes, including dopamine transmission (*4*), analgesia (*5*), and hypothermia (*6*). Clinical induction of hypothermia may sound an odd strategy, but it can reduce the risk of brain damage in a patient after a cardiac arrest, and a role for NT in this setting is presently being explored (*7*). In the periphery, NT is a peptide of the digestive and cardiovascular systems (*8, 9*). Now, we are beginning to understand its impact in the proliferation of normal and neoplastic cells (*10, 11*). By way of an introduction, we can consider its role by looking at prostate cancer.

Neuroendocrine cells are found in the basal layer of the human prostate. It is not clear at present if they represent cells from the neural crest that have migrated into the embryonic prostate or if they are stem-like cells. Recent data show that in prostate cancer the presence of cells with a neuroendocrine phenotype within a typical prostate adeno-carcinoma can predict the future development of progressive disease, metastasis, and hormone resistance (*12*). Whilst pure neuroendocrine tumors are not common in the prostate, cells with some features of neuroendocrine differentiation, such as the presence of synaptopysin B or chromogranin A, are

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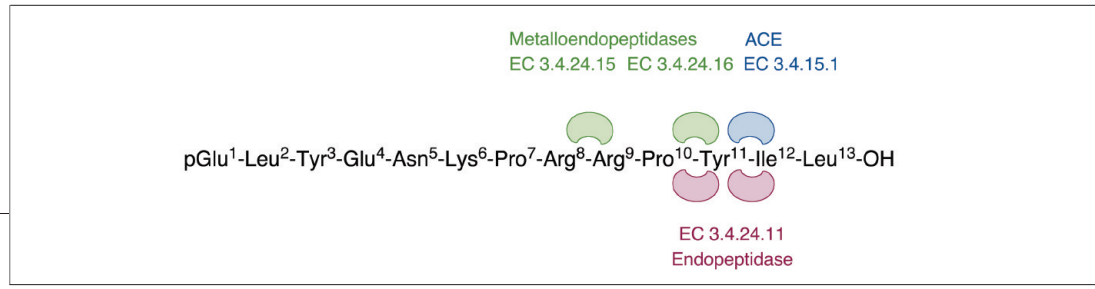


Figure 1. Peptide sequence of neurotensin and the enzymes responsible for its degradation.

found in about a third of cancers. Neuroendocrine tumors are also found in the lung and thyroid. Recent data show that a neuroendocrine signature can correctly subclassify a range of cancers and identify patients at high risk (*13*). NT secretion has been demonstrated in prostate cancer cells and down-regulation of the NT pathway decreases invasiveness making the NT receptor a potentially useful target.

However, before discussing molecular targets, we first need to consider how the active fragment of NT was identified, which leads to an introduction of the NT receptors and how they come to be expressed in cancer. What follows is a discussion about the molecular tools that have been used to distinguish between the NT receptors. To conclude, NT-mediated neoplastic cell proliferation is discussed along with the therapeutic strategies that capitalize from this biological phenomenon.

Identifying the Active Fragment of NT. The *in vivo* use of linear peptides as experimental tools is often limited by their poor metabolic stability. The likely sites for enzymatic cleavage in NT have been taken into consideration and appropriate structural modifications were made to the peptide sequence, which led to analogues that were more resistant to degradation (*14*). It was found that NT is degraded by metalloendopeptidases 24.16 (EC 3.4.24.16) and 24.15 (EC 3.4.24.15) that cleave the Pro10−Tyr11 and the Arg8−Arg9 bond, respectively; endopeptidase 24.11 (EC 3.4.24.11) that cleaves the NT Pro10−Tyr11 and Try11−Ile12 bonds; and angiotensin-converting enzyme (ACE) (EC 3.4.15.1) that cleaves the Try11-Ile12 bond (Figure 1).

The active sequence of NT was finally determined using the usual process of synthesizing and biologically evaluating NT analogues. This rudimentary research that was conducted in the late 1970s and 1980s led to major advances in our understanding of NT agonism. There were several key contributions that underpinned this success and here a few of these are highlighted.

Molecules based on *N*- and *C*-terminal fragments, along with cyclic congeners of NT, were among some of the first NT analogues to be synthesized (*15, 16*). Key to success were the systematic D-isomer substitutions that were made to the NT sequence. This process resulted in unnatural peptides that were tested for their ability to induce hypothermia (*17*). *In vivo*, the relative potency of each analogue was assessed by its ability to lower the body temperature of cold-exposed rats. Similar potencies between analogues with amino acid substitutions occurring in the NT(1−9) sequence were observed (Table 1).

This tolerance suggested that the *N*-terminal sequence was less of a contributor to the biological activity of NT than the *C*-terminal. In contrast, significant reductions in activity were observed for analogues that incorporated single D-isomer substitutions in the NT(10−13) region (Table 1). The D-Tyr11-NT and the D-Phe11-NT analogues had far higher potencies than predicted, which were attributed to stabilization of their tertiary structure and improved resistance to degradation. In a separate study, the Pro10 and Tyr11 residues were found to be essential for the function of NT in stimulating intracellular cyclic GMP formation in murine neuroblastoma cells (N1E-115) (*18*). D-Phe substitution of Tyr11 and (separately) D-Pro in place of the natural isomer led to the complete abolition of NT activity, warranting Pro10 special status as a key spacer unit. Muta-

TABLE 1. Relative potencies of NT D-isomer analogues

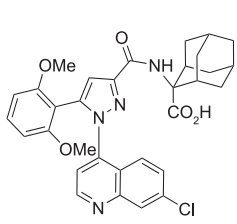
Analogue	Relative potency[a]
[D-Tyr[11]]-NT, [D-Phe[11]]-NT	Significantly more potent (1000)
[D-Lys[6]]-NT, [D-Leu[2]]-NT, [D-Tyr[3]]-NT, [D-Glu[4]]-NT	More potent (110−300)
NT (control), [D-pGlu[1]]-NT, [D-Asn[5]]-NT, [D-Pro[7]]-NT, [D-Arg[8]]-NT, [Phe[3]]-NT, [Phe[11]]-NT, [Cys[2,13]]-NT	As potent (100)
[D-Arg[9]]-NT, [D-Pro[10]]-NT, [D-Ileu[12]]-NT, [D-Leu[13]]-NT, [D-Lys[11]]-NT, [D-Leu[11]]-NT, NT(8−13), NT(9−13), NT-NHMe	Less potent (50−1)

[a]Determined by their ability to lower the body temperature of cold exposed rats (4 °C) after 60 min following intracisternal administration of the peptide.

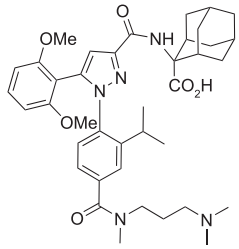
tions of Arg8 and Arg9 to D-Arg or D-Lys (respectively) led to a reduction in agonism, suggesting these residues played an important role in receptor recognition (*19*).

Thanks to this early research and supporting evidence reported elsewhere it is now widely accepted that the active fragment of NT lies in the NT sequence Arg8-Leu13 which is more commonly referred to as NT(8−13) (*20*).

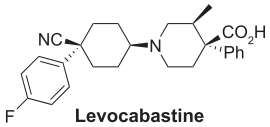
The Molecular Toolbox. Three molecules in particular stand out in the NT literature: SR 48692 (Meclinertant), SR 142948A, and levocabastine as they have been valuable molecular tools and have helped to illuminate the binding properties of the NT receptors.



SR 48692 (Meclinertant)



SR 142948A



Levocabastine

The creative use of this limited yet versatile molecular toolbox has helped pin down much of what is understood about the different receptor subtypes, especially NTR1 and NTR2. This being said, it would be wise to accept that this picture is far from complete, as the intricacies of the human NT signalling network remain to be elucidated in their entirety (*21*). Although these three molecules might perhaps be better placed in the context of strategies to block NT binding, which is considered later, their use is so central to our core insight into NT binding that they warrant their own moniker: the molecular toolbox. The ubiquitous prevalence of these molecules in the NT literature seems to have arisen partly through good fortune in the case of levocabastine. More significant has been the availability of the industry-synthesized agents SR 48692 and SR 142948A. This is an important demonstration of the value and productivity of collaborative efforts between industry and academia. SR 48692 and SR 142948A are products of industry-led lead discovery and drug development, with SR 48692 having recently completed phase II−III clinical trials for small-cell lung cancer (NCT00290953).

At this point, we would like to draw the reader's attention to a few of the conventions that are used in the NT literature. We also would like to warn of the unavoidable mention of the three receptor subtypes (NTR1, NTR2, and NTR3) in the following section prior to the main discussion on them later. In referring to the species origin of a particular NT receptor, in the case of NTR1, for example, isolated from rat, a small-case letter "r" precedes the receptor abbreviation: rNTR1. Similarly, hNTR1 indicates the receptors have been isolated from human tissues. The biological data accompanying the discussion of the molecules in the toolbox typify the studies that populate much of the NT literature but in fact represent only a small selection.

SR 48692. The interactions between brain NT and dopamine was a focus for the pharmaceutical company Sanofi-Recherche in the early 1990s. As part of their research and development program a high-throughput screening campaign was conducted that was aimed at the discovery of new molecular probes for investigating the role of NT in a neurological setting.

Their efforts culminated in the identification of SR 45398 (*22*), which had micromolar activity (IC_{50} = 40 μM) for the NT receptors expressed in guinea pig brain tissue (*23, 25, 26*). Through a series of structural modifications to SR 45398, the combined effects of a 2,6-dimethoxyaryl substitution pattern, an *N*-linked aromatic moiety, and the adamantyl amide at the 3-position of the pyrazole (and thus the relative orientation of the carboxylic acid) were key to the successful optimization of SR 45398 to give the first non-peptide NT receptor antagonist SR 48692 (*24–28*). The Sanofi synthesis of SR 48692 began with a base-mediated Claisen condensation of a methyl ketone with di-

KEYWORDS

Agonist: A molecule that binds to a specific receptor and triggers a response in the cell mimicking the action of an endogenous ligand.

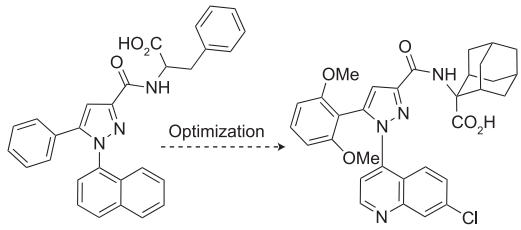
Antagonist: A molecule that binds to a specific receptor yet does not provoke a biological response itself upon binding but blocks or dampens the agonist-mediated response.

Homology and identity: Measures of similarity between protein sequences. Homology implies an evolutionary link and is qualitative, whereas identity is quantifiable.

Site-directed mutagenesis: A process where specific single nucleotide changes within a gene can be made resulting in the change of a specific amino acid within a protein sequence.

Prognostic factor: A biomarker that can be used to estimate the chance of recovery from a disease or the chance of the disease recurring.

Predictive factor: A biomarker used to predict the response of a cancer to a given therapy.



SR 45398 — 40 μM (guinea pig brain)

SR 48692 — 1 nM (guinea pig brain)

methyl oxalate to afford a tricarbonyl (Scheme 1). Subsequent Paal-Knorr pyrazole formation using hydrazine **1** and hydrolysis of the resulting ester produces a carboxylic acid that was converted to the acid chloride. This was then coupled with the adamantyl amino acid **2** to produce SR 48692. Preparation of the unnatural amino acid **2** was not described, but methods for its preparation have been reported elsewhere (*29–31*).

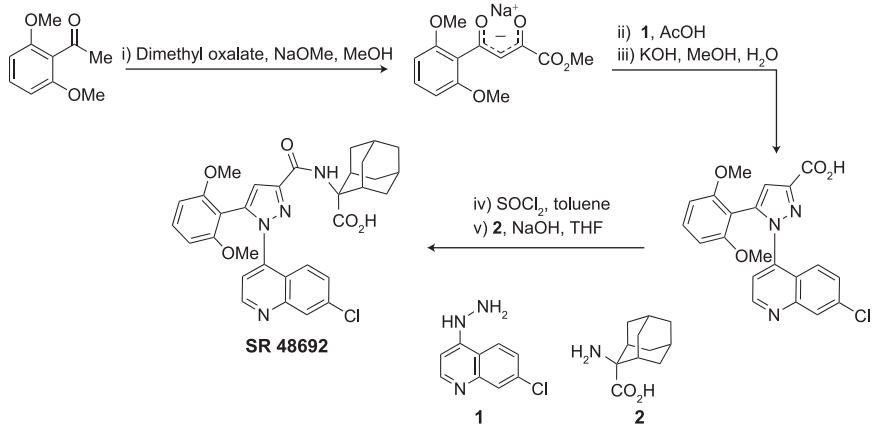
In terms of pharmacological activity, SR 48692 is a selective antagonist for NTR1 with IC_{50} values in the low-nanomolar range. At much higher concentrations, it binds NTR3 in addition to NTR1 (*32, 33*). SR 48692 can also discriminate between the NTR1 and NTR2 receptors, recognizing NTR1 preferentially (*34*). *In vitro*, SR 48692 inhibited the binding of labeled ^{125}I-NT to NTR1 isolated from the brain tissues of several species (guinea pig, rat, mouse, new born and adult human) and in human colon carcinoma (HT-29) cells, all in the low-nanomolar range (4.0−17.8 nM). SR 48692 was also shown to displace labeled NT from NTR2 isolated from mouse and rat brain tissues at higher concentrations (34.8−82.0 nM). Furthermore, the turning behavior of mice induced by intrastriatal administration of NT could be reversed using low doses of SR 48692.

To date, SR 48692 is the only small molecule antagonist of NTR1 with a well-documented pharmacological profile, and news of its progress beyond the clinical trials is awaited.

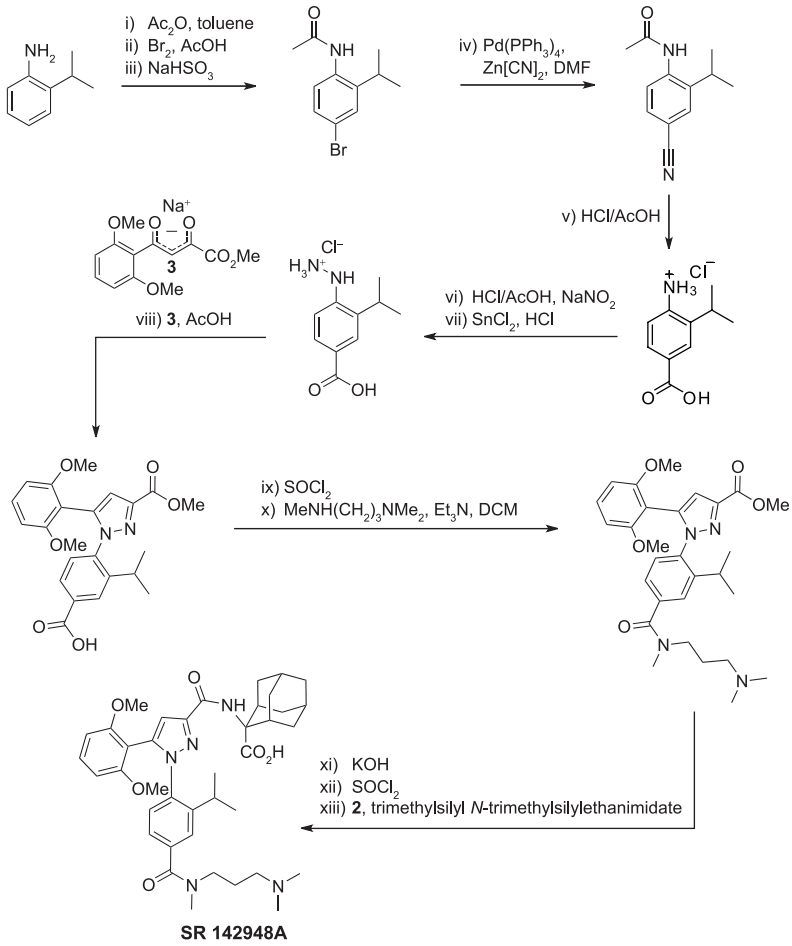
SR 142948A. The search for alternative NT probes based on the core framework of SR 48692 led to the second-generation analogue SR 142948A, which possesses rather different pharmacological properties. It is a selective antagonist of NTR1 (*35, 36*) and a putative agonist for NTR2 (*37*). Its synthesis as given by Sanofi is illustrated in Scheme 2 (*36*).

Unlike SR 48692, SR 142948A exhibits high-nanomolar affinities for NTR1 and NTR2 and binds NTR2 preferentially (*35, 38*). Superimposition studies with levocabastine have revealed common structural elements that could account for this activity (*39*). SR 142948A has also been shown to block the NTR1- and NTR3-mediated growth response to NT (*40*). The most widely reported use of SR 142948A as a NT antagonist has been in the study of rat brains (*41–45*). The revelation that SR 142948A and SR 48692 were agonists of hNTR2 expressed in Chinese hamster ovary (CHO) cells was therefore unexpected; inositol 1,4,5-triphosphate (IP_3)

Scheme 1.



SCHEME 2.



formation, Ca^{2+} mobilization, arachadonic acid release, and mitogen-activated protein (MAP) kinase activation were all observed (*37*). Whilst the effects of SR 142948A on human tumor cell proliferation may have been studied systematically, this is not evident in the literature. Of related interest, this molecule appears to be a potent antagonist of the cardiovascular effects of NT *in vitro* and *in vivo* (*46*).

Even from this limited selection of experimental results, it should be evident that the pharmacological behavior of this second-generation analogue appears to be less predictable in the study of NTR2 than the analogous SR 48692—NTR1 relationship. When taken together as molecular tools, SR 48692 and SR 142948A constitute a pair of handy non-peptide NT antagonists of different potency with complementary binding preferences.

Levocabastine. The selective histamine H1 receptor antagonist levocabastine is usually used to alleviate symptoms associated with rhinoconjunctivitis (*47*) and is currently marketed as Livostin by Novartis. Despite sharing no structural relationship with NT, many of the properties of NTR2 have been elucidated using levocabastine. It was first shown to selectively inhibit the binding of [^{3}H]-NT to NTR2 in rat and mouse brain (*48*). This ability to discriminate between NTR1 and NTR2, along with its high binding affinity for NTR2 has made levocabastine invaluable in this field. However, such is the

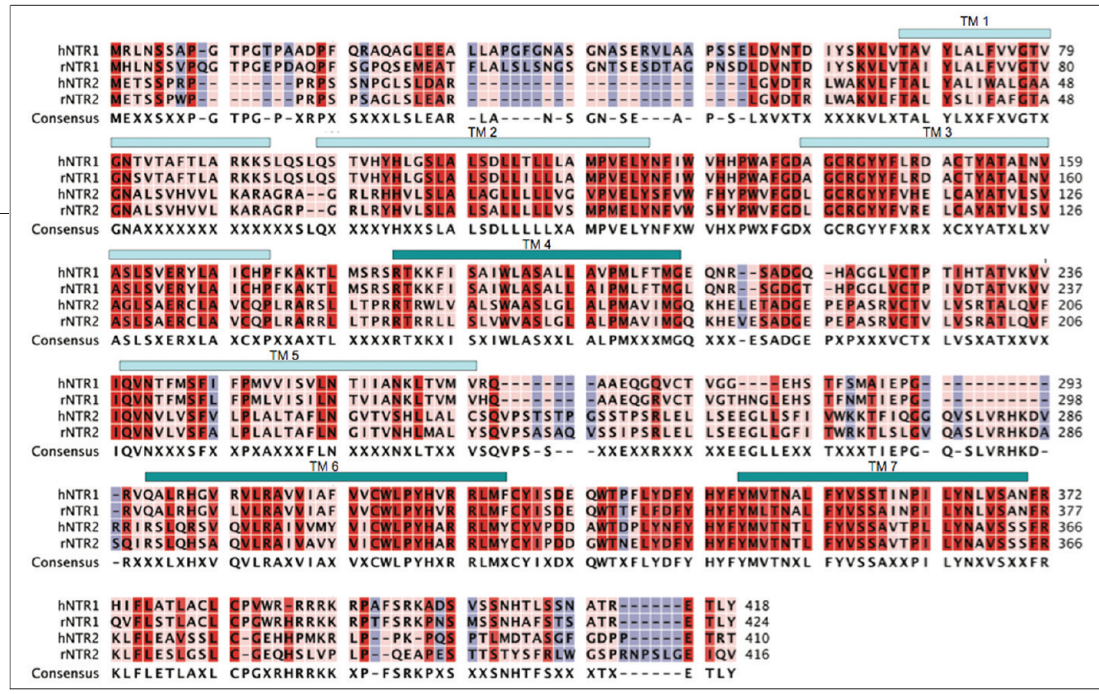


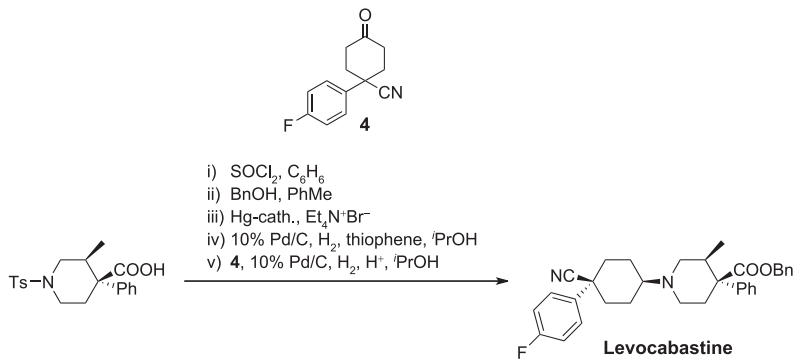
Figure 2. Multiple sequence alignment of rNTR1 (NCBI accession no. NM_001108967.1), hNTR1 (NCBI accession no. NM_002531.2), rNTR2 (NCBI accession no. NM_022695.1), and hNTR2 (NCBI accession no. NM_012344.3). Conservation: dark red (high), pink (medium), and purple (low).

complex behavior of NTR2 when expressed in mammalian cell lines, levocabastine has been shown to act as both an agonist (*49, 50*) and an antagonist (*37, 51*). Downstream effects of levocabastine binding include changes in the concentration of cytosolic calcium ions, activation of MAP kinases, and IP_3 formation (*52*). Inhibition of caspase-3-mediated apoptosis by levocabastine has also been observed in a murine cell line (*53*). Levocabastine does not appear to be capable of modulating the effects of NT in lung (*54, 55*), colon (*46*) and pancreatic cell lines (*56*), which is likely to be linked to the absence of NTR2 receptors in human tumors (*57*). Levocabastine is widely available from commercial sources and its synthesis should not be necessary for those wishing to use it for their research. However, for completeness, a five-step synthesis has been reported by Janssen Pharmaceuticals (*58*) (Scheme 3), and a crystal structure is also available (*59*).

The NT Receptor Family. As previously mentioned, the accepted NT receptor family comprises three subtypes: NTR1, NTR2, and NTR3, these are also widely referred to as NTS1, NTS2, and NTS3 in the literature (*60*). A comparative sequence alignment of NTR1, NTR2, and NTR3 is shown in Figure 2. Very recently, a fourth NT receptor isolated from the brain tissue of bull frogs (bfNTR4) was reported to be successfully antagonized by SR 48692 and SR 142948A (*61*).

The NT receptors bind NT *via* the active fragment; the *C*-terminal hexapeptide sequence NT(8−13). NTR1 and NTR2 are seven-transmembrane (7-TM), G-protein-coupled receptors (GPCRs) that activate multiple signal transduction pathways (*62–64*) (Figure 3). NTR3, which is also called sortilin, is a multiligand type 1 receptor

SCHEME 3.



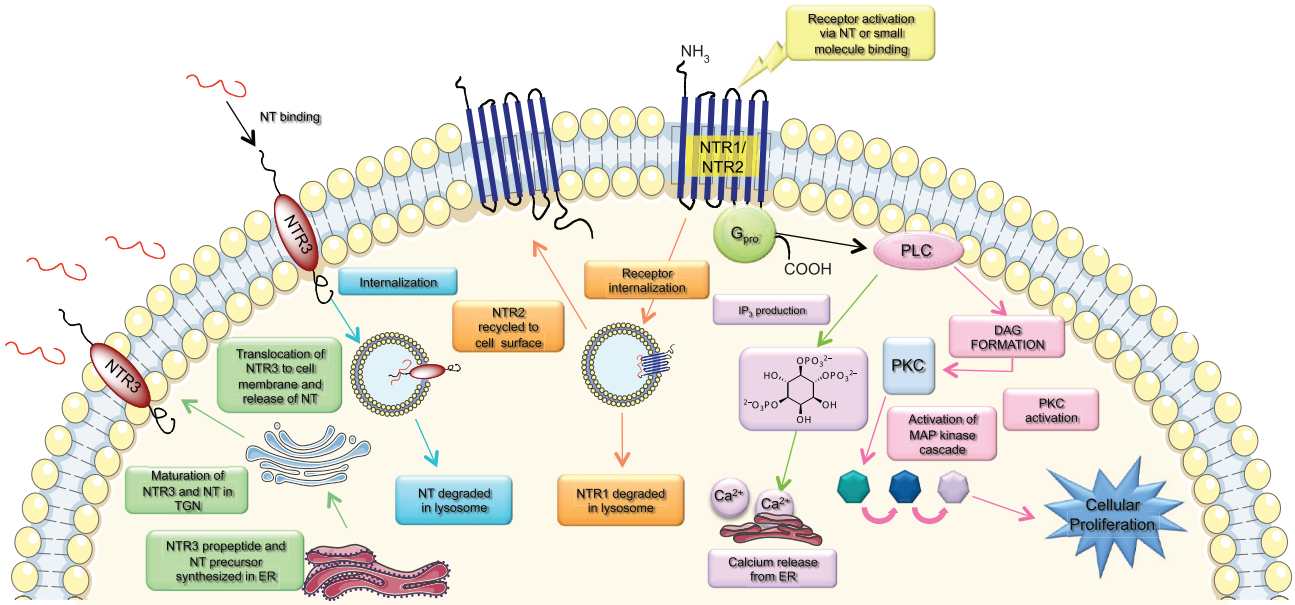


Figure 3. Roles of the NT receptors in the cell. Turquoise, lilac, and pink pathways represent responses to ligand binding. Orange and green pathways represent movement of NT and its receptors within a cell. Only major pathways relevant to this Review are included; others have been omitted for simplicity. (G_{pro}, G protein; PLC, phospholipase C; DAG, diacylglycerol; PKC, protein kinase C; ER, endoplasmic reticulum; TGN, *trans*-Golgi network; IP_3, inositol, 1,4,5-trisphosphate; MAP kinase, mitogen activated protein kinase.)

with a single transmembrane domain. It belongs to a family of receptors with an extracellular/luminal region rich in cysteine residues that share homology with the yeast protein Yps10p (*9, 65*). NTR3 has multiple roles within the cell: scavenging NT from the extracellular fluid, sorting neuropeptides for their release from the cell, and as a membrane receptor to trigger cellular effects (*65*) (Figure 3).

In brief, NTR1 has a subnanomolar (high) affinity for NT, and NTR2 has a nanomolar (lower) affinity for NT. In addition, NT acts as an agonist toward NTR1-mediated pathways, yet it can act as either an antagonist or an agonist at NTR2, depending on levels of expression and the NTR2-mediated pathway in operation (*64*). The ability of NTR3 to bind multiple ligands has made it much more complex to study (*66*).

NTR1. The high-affinity, levocabastine-insensitive receptor. NTR1 is the most extensively characterized subtype of the NT receptors. Purification from rat (*52*), bovine (*67*), and mouse (*68, 69*) brain tissues and subsequent cloning and expression of rat (*70*) and human (*71*) NTR1 receptors have allowed its physical, biological, and pharmacological properties to be investigated. A large-scale (3−10 mg) purification process of NTR1 from whole cells has been described that provided suitable quantities of the receptor, in this case, for antibody generation (*72*).

Human NTR1 (hNTR1) is a 418-amino-acid 7-TM protein with 84% identity to rNTR1. It has been isolated from human colon carcinoma (HT-29) cells that bound radiolabeled NT strongly (*71*), and this binding could be inhibited by the neuropeptide neuromedin N and SR 48692. In the same study, Northern blot analysis confirmed hNTR1 to be localized in both human brain cells and colon cells.

In the absence of a crystal structure of the NT−receptor complex, NMR experiments have been able to determine the backbone conformation of NT bound within the receptor (*73–75*), and by combining data assimilated through mutagenesis, inhibition studies, and molecular modeling, a mode of binding of NT to NTR1 has been proposed (*76*). Site-directed mutagenesis of wild-type rNTR1 receptor expressed in COS cells identified key residues involved in NT binding (*77*). Mutations within transmembrane domain 6 (TM6) (R327 M and R327E) and TM7 (Y347A) led to complete loss of af-

TABLE 2. Effects of site-directed mutagenesis on the binding of NT to NTR1

Location	NT binding reduced	NT binding abolished	Refs
TM4	M208A	–	*77, 80*
TM6	F331A	R327M, R327E	*77, 78, 80*
TM7	Y349A, Y351A	Y347A	*77, 80*
E1	–	D139G, R143G	*77, 80*
E3	E337G, W339A, F344A	–	*80*

finity for radiolabeled NT binding. Mutations within E1 (D139G and R143G) also led to loss of NT binding (*78*). Further residues have been implicated in reduced affinity of NT to mutant rNTR1. These findings are summarized in Table 2 and illustrated in Figure 4.

A binding site for NT located in the E3 loop of NTR1 has been proposed following computer modeling studies based on the affinities of truncated NT(8–13) analogues for rNTR1 (*79*). These predictions were refined to include mutagenesis data putting TM6 and TM7 residues within the binding site (*80*). The model emphasizes several characteristics required for NT binding: R327 makes strong ionic interactions with the *C*-terminal carboxylic acid of NT; Y347 makes strong π–π stacking interactions with Tyr11 of NT; F344 and W339 make weak π–π stacking interactions with Tyr11; F331 and M208 make hydrophobic interactions with Ile12 and Leu13, respectively; and finally, F331 makes cation–π interactions with Arg9 of NT.

Binding of SR 48692 at NTR1. Through the use of [^{3}H]-labeled SR 48692 and mutants generated by site-directed mutagenesis, several residues essential for antagonist binding were revealed (*77*). No binding to the mutant receptor was detected when residues within

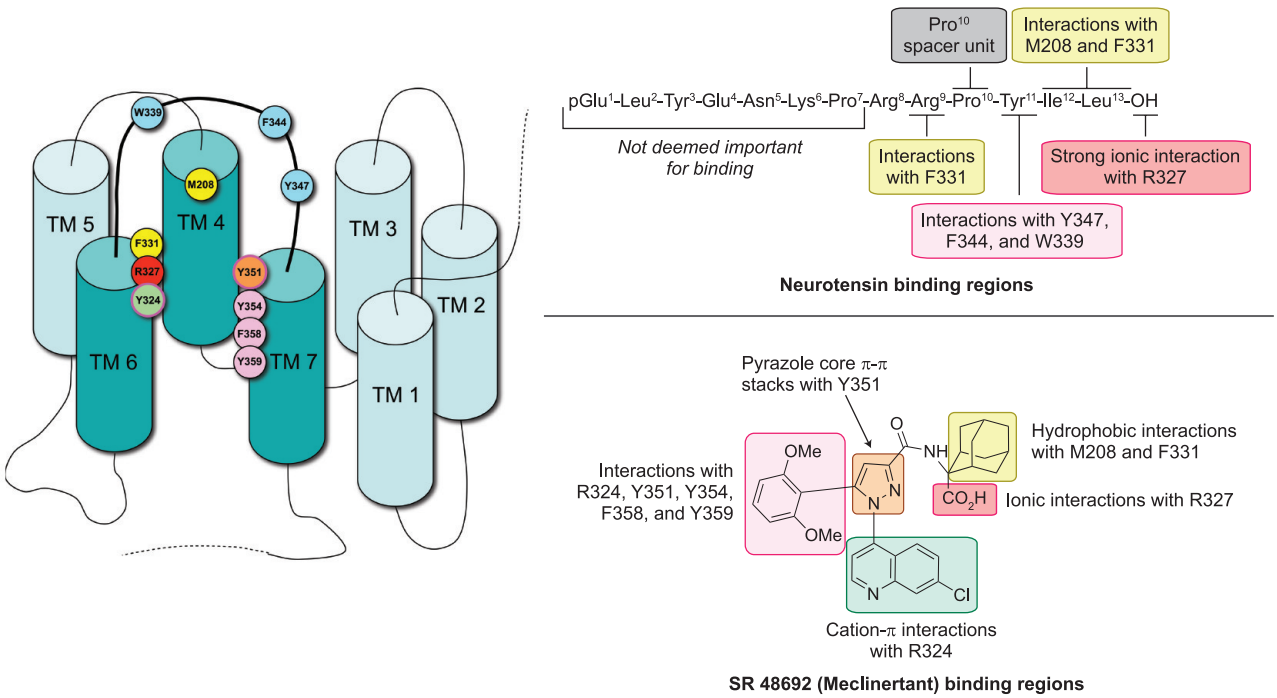


Figure 4. NT and SR 48692 binding sites in rNTR1.

TABLE 3. Effects of site-directed mutagenesis on the binding of [^{3}H]-SR 48692 to NTR1

Location	[^{3}H]-SR 48692 binding reduced	[^{3}H]-SR 48692 binding abolished	Refs
TM4	—	M208A	*77, 80*
TM6	—	Y324A, F331A, R327M, R327E	*77, 80*
TM7	T354A, F358A	Y351A, Y359A	*80*

TM4 (M208A), TM6 (Y324A, R327M, R327E, F331A), or TM7 (Y351A, Y359A) were mutated. In addition, two mutations of TM7 (T354A, F358A) led to significant reductions in affinity (Table 3).

The active conformation of the adamantyl carboxylic acid in SR 48692 was deduced in separate synthesis studies (outside the scope of this Review) that involved introduction of an asymmetric center at the amino acid to give two enantiomers (*24*). Taken together with molecular modeling experiments (*23*) and site-directed mutagenesis data (*77*), a model for SR 48692 binding to NTR1 has also been proposed (Figure 4). SR 48692 binds close to the membrane interacting strongly with TM4, TM6, and TM7. The key interactions of NTR1 with SR 48692 fall into the following groups: M208 and F331 interact hydrophobically with the adamantyl moiety of SR 48692; R327 forms a strong ionic interaction with the carboxylic acid group; Y351 makes $\pi-\pi$ stacking interactions with the pyrazole core; Y324 makes $\pi-\pi$ stacking interactions with the chloroquinolinyl group; Y351 makes $\pi-\pi$ stacking interactions with the dimethoxyphenyl moiety; and hydrogen bonding is present between the dimethoxyphenyl ring and residues Y351, Y324, Y354, and F358. Comparing these interactions with the NT binding site, it becomes possible to see how binding of SR 48692 to the receptor can occlude NT from its binding pocket within NTR1 (*76*).

NTR2. The low affinity, levocabastine-sensitive receptor NTR2 is also a 7-TM GPCR; it is 410 amino acids in length and shares 38% identity with hNTR1 and 79% identity with rNTR2 (*37*). NTR2 was first detected (*81*) and its existence later confirmed by inhibition studies in rat and human brain cells (*48*). This later study found that 60% of [^{3}H]-NT binding in human brain cells could be inhibited by levocabastine, implying a high level of expression. Since its detection, NTR2 has been cloned and expressed from rat (*82*), mouse (*83*), and human (*84*) brain tissues. Furthermore, binding studies have shown NTR2 has a lower affinity for NT compared with that of NTR1 (*37*). SR 48692 and SR 142948A effectively inhibit binding of NT to hNTR2; in the same study, levocabastine (which has no effect on the interaction of NT with hNTR1) also inhibited NT binding to NTR2 (*37*). Unlike NTR1, several studies have shown NTR2 to be constitutively active (*51, 83*). However, this activity was not reported in CHO cells (*37*). These results reflect the complex pharmacological properties of NTR2 that appear to be species-dependent.

NTR3. The sortilin transmembrane protein is the third NT receptor and is a 100-kDa protein that has been isolated and characterized from mouse (*68*) and human (*85*) brain tissues. It is 100% identical to the human gp95/sortilin protein (*86, 87*) and has numerous endogenous ligands. NTR3/sortilin is synthesized as a propeptide (110 kDa) which is cleaved to the mature receptor (100 kDa) in the *trans*-Golgi network by the propeptide convertase, furin (*88*). The propeptide has a relatively low affinity for NT (K_d 10−15 nM), whereas NT has high-nanomolar affinity toward NTR3 (K_d = 0.3 nM) (*68, 86*). A recent 2-Å resolution crystal structure of the sortilin Vps10 domain in complex with NT revealed the *N*-terminal domain as the first example of a 10-bladed β-propeller structure (*89*). This propeller domain forms a tunnel in which NT binds to blade 6 through its *C*-terminal residues. Unlike NTR1 and NTR2, evidence suggests that the majority of NTR3/sortillin (90−95%) is located within intracellular compartments (*90*). The significance of this distribution is not well understood, although a review by Mazella summarizes some of the putative roles of NTR3 (*65*).

Interactions of NTR1, NTR2, and NTR3. Many GPCRs are known to form homo- and/or heterodimers. Although the NT signalling pathways are not fully understood (*91*), there is evidence that interactions between the NT receptors may be required for their normal functioning. For example, in one study NTR1 and NTR2 were shown to form heterodimeric structures when co-expressed in COS cells, and although dimerization did

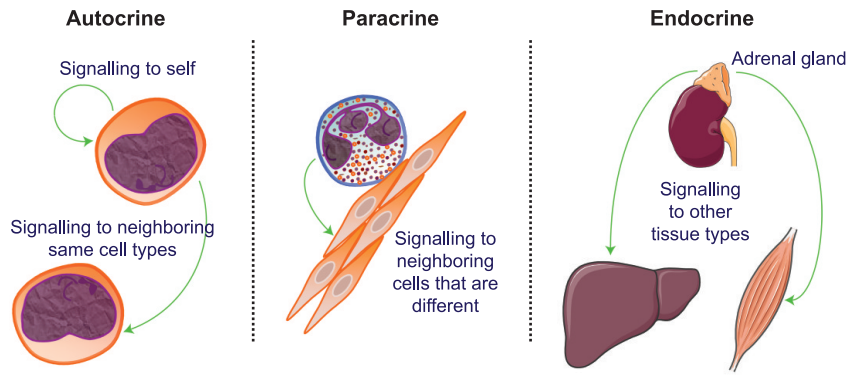


Figure 5. Autocrine, paracrine, and endocrine signalling. For autocrine signalling, a cell secretes a hormone (or chemical messenger) that binds to receptors on the same cell (or similar ones), leading to changes in the cell. For paracrine signalling, target cells are near the signal-releasing cell, but they belong to different cell types. In endocrine signalling, the signal-releasing cell, usually located in a specialized organ, is situated far apart from the target cells and usually uses the circulatory system to deliver the signal molecule or hormone.

not seem to affect affinity for NT, down-regulation of NTR1 was significantly reduced (*92*). Similarly, hNTR1 and hNTR3 appear to form heterodimers; for example, experiments that have analyzed NT-induced phosphorylation of MAP kinases Erk1/2 suggest this receptor complex is involved in the NT signalling pathway (*93*).

The Role of Neurotensin in Cancer. The role of NT in cancer has piqued a great deal of interest in the past few years, as there is now compelling evidence that NTR1 is overexpressed in a number of human cancers (*10, 11*). There is also mounting evidence that NTR3 may also be involved in cancer progression (*46*).

Outside of the brain and the CNS, NT promotes the growth of gastroenteropancreatic tissues (colon, pancreas, and stomach) and the adrenal cortex *in vivo*, and the proliferation of dispersed cells (fibroblasts and hepatocytes) in culture (*94, 95*). NT is secreted by cells in the ileum in response to fat in the small intestine and modulates gut motility. While these functions are vital for the maintenance of a healthy bowel, it can become a powerful weapon for cells that step outside the normal cell cycle and become tumorigenic. In fact, the release of NT has been implicated as a possible cause of cancer with high dietary fat intakes. NT release is promoted by unsaturated acids (linoleic, oleic) not by saturated (stearic) fatty acids (*96*). There are several tumor that cells can both secrete NT and express NT receptors (*97–99*) and this adaptation allows tumor cells to stimulate their own growth in an autocrine manner, as well as the growth of other neoplastic cells using paracrine signalling (Figure 5).

Understanding the mechanisms underlying these processes in the case of NT would open up considerable opportunities for designing drugs to control aberrant NT receptor expression or processes for keeping the system in check. There is a model for NT-induced carcinoma growth (*10*), and although it is not yet complete, it suggests that the basic pathways for different cancers are similar.

Cancer and NT-Mediated Growth. Cancer is characterized by the uncoordinated proliferation of a group of cells (neoplasm). With the majority of neoplasms, their growth is often promoted by mitogens in an autocrine, paracrine, or endocrine fashion, as illustrated in Figure 5.

Overexpression of NT and its receptors is widely implicated in progression of the disease to a more aggressive phenotype. Expression levels of NTR1 in different cancer subtypes vary not only between cancers but also between the methods used to detect expression levels, for example, RT-PCR (*100, 101*), autoradiography on tissue sections (*102*), and mRNA expression (*46*). Due regard for these variations is encouraged (*10*).

We now look in more detail at NT and its receptor expression in the development and progression of cancer and point out where opportunities may lie for intervention or exploitation.

Breast Cancer. This is the most common cancer in women, with ~1.3 million cases diagnosed annually.

Despite all the effort that goes into early detection and treatment, it still takes the life of ~500,000 women per year. The risk of developing breast cancer increases substantially with age, with 80% of cases occurring in women over the age of 50. In the U.K., incidence has risen by 57% in the past 25 years. Thankfully, the 5-year survival rate has much improved.

Breast cancer is a heterogeneous disease and includes tumors with diverse morphological and biological profiles. The two most prevalent pathological forms of breast cancer (invasive ductal and lobular) originate from premalignant hyperplastic lesions that carry considerable risk of developing into fully invasive carcinomas. More recently, the use of gene expression arrays has enabled the identification of distinct molecular subgroups, and currently there are six subtypes of breast cancer that differ in their prognosis and response to treatment (*103*). The use of prognostic and predictive markers on a case-by-case basis in its treatment is unique in the oncology setting. Along with improved breast screening programs, these strategies account for the increase in survival rates. However, despite advances in genomics, there are still only three biological molecular markers currently used in the management of breast cancer: the estrogen receptor (ER), the progesterone receptor, and human epidermal growth factor receptor 2 (HER-2). The status of these biomarkers is routinely monitored in patients. A fourth predictive biomarker, BRCA1, may be used in the clinic in the near future (*104*).

Biomarker screening is primarily done by immunohistochemistry, which allows a tailored approach to treatment regimens. Antiestrogen therapy in the form of the aromatase inhibitor tamoxifen (an ER antagonist) can be given to patients with ER-positive tumors to inhibit the mitogenic effects of estrogen (*105*). Introduced recently, Herceptin (a monoclonal antibody that acts on the HER-2 receptor) is used in combination with other chemotherapeutic agents to inhibit tumor proliferation in HER-2 receptor-positive tumors (*106*). The clinical value of these biomarkers highlights the importance of identifying new prognostic and predictive factors, not only in breast cancer but for all cancers where it is appropriate.

NTR1 is not expressed in normal human epithelial cells or mammary tissue epithelial cells. Production of NT by a non-invasive *in situ* ductal carcinoma of the breast was first reported over 2 decades ago (*107*). More recently, treatment of mammary adenocarcinoma (MCF-7) cells (which have been shown to overexpress NTR1) with the pseudopeptide NT agonist JMV449 (H-Lysψ(CH_2NH)Lys-Pro-Tyr-Ile-Leu-OH) inhibited cellular apoptosis by up-regulating the expression of the anti-apoptotic Bcl-2 gene (*108*).

Another study has confirmed that NT promotes breast cancer progression and is involved in cellular migration, invasion, and tumor growth (*109*). Among the breast carcinomas studied, 91% were NTR1 positive, and in 30% of the invasive ductal carcinomas, NT was expressed along with NTR1. Furthermore, the use of RNAi (to deplete NTR1 expression) or administration of SR 48692 resulted in a significant decrease in the volume, weight, and growth rate of breast cancer xenografts in nude mice.

Prostate Cancer. A similar picture has emerged in prostate cancer research. The rates of incidence of this male cancer vary widely between countries and ethnicities, with it being far more prevalent in Europe and the U.S. than in Asia. The incidence of prostate cancer has tripled in recent years, which is largely due to improved detection through the widespread use of the PSA (prostate-specific androgen) test. As an illustration, taking 2005 data from the U.K., around a quarter of all cases of cancer diagnosed in men were prostate cancers. That is ~34,000 men, and ~40% of those diagnosed were under 70 years of age. However, death rates have not changed greatly in the past decade, and every year ~10,000 men in the U.K. still die as a result of their cancer.

For many, the anatomy of the prostate is illusive. It is a gland that sits in the pelvis surrounded by the rectum and the bladder. Its function is not entirely understood, but it produces some seminal fluid and facilitates sperm motility. It is composed of branching glands with ducts, lined mostly with secretory epithelial and basal cells, plus a few scattered neuroendocrine cells. The epithelial cells in the healthy prostate are androgen-dependent for growth and produce PSA. A stroma surrounds the prostate, consisting of fibroblasts, smooth muscle, nerves, and lymphatics. Stromal-epithelial interactions produce growth factors that are important for growth and development of the normal prostate. However, these growth factors are also implicated in the development of prostate cancer (*110, 111*).

Prostate cancers express the androgen receptor and depend on an appropriate concentration of the ligand testosterone to proliferate—thus, most patients can re-

ceive antiandrogen therapy, for example, the drug bicalutamide (*112*). In some cases, however, the cancer becomes refractory and stops responding to treatment. In doing so, the cancer acquires a markedly more aggressive neuroendocrine phenotype (*13, 113*). Elevated levels of neuroendocrine markers in the serum have been correlated with androgen independence (*114, 115*).

LNCaP and PC3 are two cell lines used widely in prostate cancer research (*116*). It has been known for some time that the androgen-sensitive human prostate cancer cell line, LNCaP, produces and secretes NT after androgen withdrawal (*117*), and resistance to bicalutamide can be induced by long exposure of the cell line to the drug *in vitro* (*12*). In turn, this induces neuroendocrine differentiation and production, and secretion of NT, which further induces cell proliferation and invasion. Inhibiting NT expression using siRNA may prevent this. Interestingly, NTR3 was the only NT receptor expressed in LNCaP cells and its expression increased greatly on cell differentiation (*46*).

PC3 cells, a model of late-stage androgen-independent prostate cancer, express NTR1 and bind ^{125}I-NT with high affinity. Unlike LNCaP cells, PC3 cells do not express NT precursor mRNA and neither contain nor secrete NT. However, *in vitro* NT was able to stimulate DNA synthesis in PC3 cells, thereby increasing the number of cells, further supporting an endocrine/paracrine role for NT (*118*). Monitoring NT levels for signs of a resistance response in this subset of cancer patients could be very useful in the future.

The case for more extensive research into NT and its role in cancer is not limited to breast and prostate cancer. In lung, colorectal and pancreatic cancer studies administering NT and NT antagonists also appears to produce significant effects.

Lung Cancer. NT has an autocrine/paracrine effect in lung cancers, especially in small-cell lung cancer (SCLC). Human SCLC (NCI-H209) cells express NT receptors and secrete NT, inducing autocrine stimulation of their own growth (*54*). For example, xenograft experiments in nude mice found that the orally administered SR 48692 inhibited tumor growth up to 99% (*119*). NT is present in measurable amounts in about half of the SCLC cell lines, and ~75% of human SCLCs and non-SCLC cell lines have been shown to express NTR1 (*120, 121*).

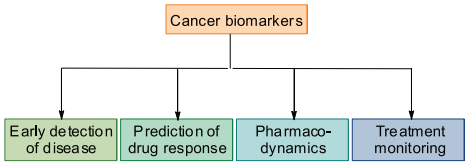
Colon Cancer. Inflammatory bowel diseases such as ulcerative colitis and Crohn's disease carry an increased risk of patients developing colorectal cancer (*122*). The interactions between normal levels of NT and NTR1 are thought to have a dual role in intestinal inflammation, regulating both the progression of the inflammatory process and its recovery (*123*). In common with the cancers discussed above, NTR1 is also overexpressed in colorectal cancer (*124*). This could explain some of the following observations: murine colon cancers (MC26) had a growth response to NT stimulation (*125*); a similar pattern was observed in rats (*126*); mice xenografted with human colon carcinoma (SW480) and treated daily with NT experienced up to a 255% increase in tumor volume; whereas daily dosing with SR 48692 alone reduced both the development and size of tumors (*127*).

Pancreatic Cancer. Ductal adenocarcinoma of the pancreas (PDAC) comprises 90% of all human pancreatic cancers and is associated with a grim 5-year survival rate of 3−5% (*128*). Two independent studies have demonstrated that ~75−90% of resected pancreatic cancers express NT receptors (*100, 129*), whereas in chronic pancreatitis or normal pancreatic tissues these receptors were not found (*129*). Multiple studies have linked the presence of NT receptors to the initiation and development of PDACs. NT stimulated mitogenic signalling pathways and DNA synthesis in PANC-1 cell lines, and the human pancreatic (MIA PaCa-2) cancer cell line were stimulated to grow both in the presence of NT *in vitro* and in nude mouse xenograft models (*130*). Although these growth effects are mediated through NTR1 stimulation, a recent study concluded that NT-induced migration of PDACs *in vitro* occurs *via* NTR3-activated pathways (*131*). SR 48692 blocks the stimulatory effect of NT on the xenografted tumors when it is administered subcutaneously to mice (*56*).

Pituitary Adenoma. These cancers are usually benign, but because they are intracranial, they can often be fatal because of location, expanding size, and (sometimes) inappropriate hormone expression (*132*). The expression of NT in pituitary adenomas is significantly higher in functioning (hormone-producing) adenomas than in non-functioning adenomas or normal pituitary tissues. Elevated levels of NTR3 expression have been observed in both functioning and non-functioning adenomas compared to the normal pituitary. By contrast, expression of NTR1 and NTR2 mRNA was not detected in either of the adenoma types or in normal tissue. The elevated expression of NTR3 and NT by pituitary adenoma

cell types that do not express NT in the normal pituitary suggests that NT autocrine/paracrine stimulation (mediated by NTR3) may be a mechanism associated with the tumorigenesis of functioning pituitary adenomas (*133*).

Biomarkers. A biomarker (biological indicator) is a quantifiable substance present in serum or tissues that can be associated with an increased risk of disease. A major obstacle to progress in cancer research is identifying signature biomarkers that predict who will benefit from a particular targeted therapy (*134*).



Biomarker quantification in a clinical setting enables some degree of prediction of disease progression, treatment programs, and a patient's likely response to treatment. The identification of new cancer biomarkers and their routine monitoring in clinic is somewhat of a holy grail, for these reasons as well as the potential for intervention at a less advanced and more manageable stage of the disease (*135*). As mentioned previously, measurement of the ER and Her-2 tissue biomarkers has become standard in the treatment of breast cancer, and these two markers alone can be credited with increased survival rates. But there does appear to be a disconnection between biomarker identification in the laboratory and practical methods for its measurement arriving in the clinic. In the post-genomics era, new tissue biomarkers in cancers ranging from pancreatic (*136*) to ovarian cancer have in fact been identified in the laboratory (*137*). However, as witnessed in breast cancer, translating these findings from the laboratory to clinic is either very slow or in some cases virtually nonexistent (*138*). The use of NT has been investigated as a serum biomarker in cancer without much success (*139, 140*), but as a tissue biomarker, NTR1 shows greater promise, particularly as a prognostic factor in androgen-refractory prostate cancers.

In addition, a recent study has provided further compelling evidence that NT/NTR1 could be a useful prognostic biomarker for head and neck squamous cell carcinomas (HNSCC) (*141*). Microarray analysis of surgically resected tumors identified 42 genes that were overexpressed in HNSCCs. Of these genes, only high expression of NT was directly correlated with a lower distant metastatis-free survival rate. Further analysis revealed that patients with high NT and NTR1 expression exhibited a higher rate of distant metastasis (worse prognosis) than other groups. The fact that this synergy appears to influence metastatic potential raises the possibility that NT/NTR1 could be used as a molecular target in the antimetastatic therapy of patients, as well as a possible HNSCC predictive marker.

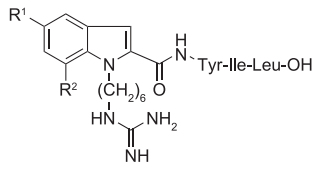
Molecules That Bind the NT Receptors. Molecular targets traditionally require chemical agents or other molecules that bind in specific locations on a receptor that precipitate a desired biological effect. If we focus on NTR1, these molecules fall into one of two categories: peptides and small molecules. In the first of these categories, NT-like peptides are not limited to mirroring the agonist effects of NT. These peptides have provided a foundation on which to build a new array of imaging tools for cancer and novel probes to study the NT network. In contrast, the emergence of improved or alternative small molecules that bind to the NT receptors since the 1990s has not been as quick-paced. At present, nothing yet beats SR 48692 in terms of NTR1 antagonism. This is not due to failed attempts; it appears simply to be an area of inactivity. We shall now review the first of these categories in roughly chronological order.

Early NT Analogues. The concept of using peptides as therapeutics 30 years ago was peppered with reservations such as the delivery problems associated with poor oral bioavailability and the issues of CNS penetration. Thankfully, many of these hurdles have now been overcome or can be circumvented. The agonist properties of peptidic NT analogues may not be of immediate value in cancer research, but their ability to interact strongly with the NT receptors has clear advantages in the field of tumor imaging.

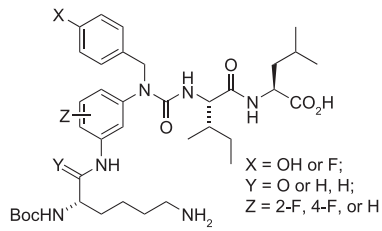
Much of the work on the early NT analogues was done before the identification of the NT receptor subtypes. Therefore, it was not possible for researchers to deduce which subtype compounds bound to; either compounds did or did not have a "good affinity for the NT receptors". The first attempts at making more stable NT analogues resulted in moderately active compounds. Among these were a series of NT(8−10)-substituted compounds containing indole-2-carboxylates with guanidine appendages. These had micromolar binding affinities for the NT receptors (*142–144*). An alkylation at

the 3-position of the indole was initially reported and later reassigned (*145*).

Another series incorporated 3-substituted anilines as replacements for the NT(10−11) fragment resulting in analogues that had low to submicromolar binding affinities for the NT receptors (*146*). The lipophilic *N*-terminal Boc protecting group was not removed before performing the assays because it was reported to increase the molecule's hydrolytic stability and it lent itself to improved penetration of the blood−brain barrier (*147*).



Indole-2-carboxylate analogues

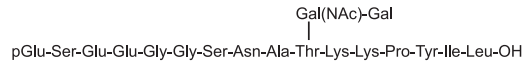


Aniline analogues

Modern Peptidomimetics. In fast-forwarding to the present day, it is clear that there is a renaissance in peptide research. Perhaps the most prominent vanguard is the development of peptide therapeutics. This is not altogether surprising given the advances in peptide manufacturing, for example, directed evolution, metabolic pathway engineering, and bioprocess technologies, plus the improvements that have been made in peptide drug delivery. Together, these impressive developments have managed to re-energize the field considerably. In short, peptides are finding numerous medicinal applications: as carriers or prodrugs for cytotoxic drugs in molecular delivery systems (*148–152*); as radiopharmaceuticals for imaging (*153*); and as drugs or leads (*154–156*). Peptide-based pharmaceuticals now comprise a significant proportion of the current drugs in trials, with a considerable number already on the market, including the natural peptides (insulin, vancomycin, oxytocin, and cyclosporine) and synthetically produced peptides, for example, the mammoth enfuvirtide (MW 4491.90) (*157*) and eptifibatide (MW 831.96). This shatters the widely held notion of "drug-likeness". Furthermore, there are thought to be at least a further 300 peptides in phase II and III clinical trials, with another 400 or more in advanced preclinical studies (*158*). Judging by these forecasts, the peptide drug pipeline is healthy, with new therapies on the horizon for cancer, infection, and pain management.

Many peptides bear similarity to NT in both structure and function, and it would not be possible to cover them all in this Review. Suffice to say that for the most part they are agonists. Agonism of NTR1 is implicated in the antipsychotic-like effects of NT. While inherently of interest, NT agonists are not the tools of choice for cancer research, unless we consider tumor imaging, which we shall come to. Some of these include endopeptidase-resistant analogues (*159*), the widely used NTR1 agonist PD149163 (*160, 161*), and newer nonpeptidic analogues (*162*). The CNS effects of NT analogues were reviewed in 2006 (*163*), and these references may provide useful starting points for those interested in this area.

It is worth highlighting one particular peptide, first as a reminder of the role natural products play in drug discovery (*164–166*) and second for its exoticism.

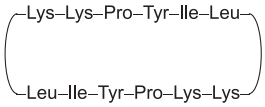


Contulakin G

The venomous cone shell *Conus geographus* is a species of predatory marine cone snail. The conotoxin contulakin G is a 16-amino-acid peptide that bears a species-specific *O*-glycosylated threonine residue. It is unusual in that it is the first invertebrate NT peptide to have been discovered and have its structure elucidated (*167*). It acts as an agonist at mammalian NTR1 receptors (*168*). In terms of development, contulakin G (CGX-1160) has passed phase I clinical trials in spinal cord injury patients, and its wider therapeutic potential is being explored (*169*).

Cyclic Peptides. Cyclic NT analogues pertaining to cancer research have not been reported. However, the medicinal potential represented by these molecules has

not gone unnoticed in the field of non-opioid analgesia (*170*). In 2008, a cyclic NT dimer was reported in which lysine had been exchanged for the arginine residues in NT(8−13) (*171*).



Lys-substituted cyclic dimeric analogue of the NT(8–13) fragment

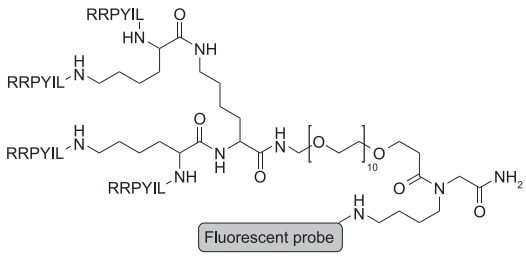
The required linear hexamer (prepared using standard solid-phase synthesis procedures) was dimerized in a head-to-tail fashion using a high monomer concentration (100 mmol L^{-1}). This was effected using the coupling agent HBTU in the presence of triethylamine, affording the protected cyclic peptide in 92% yield. This cyclic analogue may be a future lead given that it was selective for the NTS2 receptor. It also produced an *in vivo* analgesia, suggesting it crossed the blood–brain barrier.

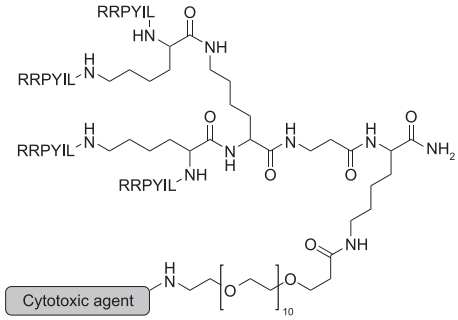
Chemical methods for cyclizing linear peptides into their corresponding macrocycles (which can sometimes prove difficult to accomplish in the laboratory) have been reviewed (*172*). *In vivo*, cyclic peptides are often more stable than their linear counterparts because they have greater resistance toward degradation.

Dendrimeric Peptides. Another approach aimed at improving biostability involves creating branched (dendrimeric) NT analogues. For example, a tetra-branched form of NT(8−13) was shown to be stable for 24 h at 37 °C in human plasma and serum (*cf.* 2 h for linear NT(8−13)) (*173*).

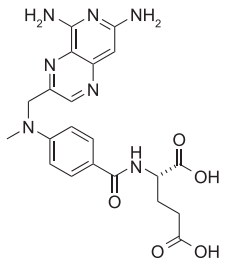
This principle was used to design "peptide bullets" for delivering cytotoxic and imaging agents to tumor cells. In this study, NT4 conjugates were compared to the free drug counterpart in nude mice bearing human colon cancer (HT-29) tumor xenografts. Most notably, conjugation of the chemotherapeutic molecule methotrexate (MTX) with NT4 produced a >60% reduction in tumor weight (*cf.* 10% for free MTX), and conjugation significantly reduced nonspecific toxicity. *In vitro* receptor binding and internalization using the fluorescent probe conjugates were investigated.

Imaging Tools. *Peptide Radiopharmaceuticals.* The oncology-driven development of radiolabeled receptor-binding peptides has seen major developments in recent years (*174–176*) with new radiopharmaceuticals such as the labeled peptide hormone somatostatin in clinical practice (*177*). Radiopharmaceuticals are used in the diagnostic imaging and radionuclide therapy of cancer. Due to the short half-lives of radioisotopes, their manufacture and administration must be completed very rapidly. This requires fast, clean, and efficient processes that minimize reagent quantities and reaction steps. The synthesis of radiolabeled conjugates involves modifying a given bioactive peptide to improve its metabolic stability. Neither the modification of its sequence nor inclusion of a chelator (linker segment) must affect its ability to bind the target receptor. Bifunctional coupling agents used for radiolabeling and target-specific delivery of metallic radionuclides were reviewed in 2008 (*178*).

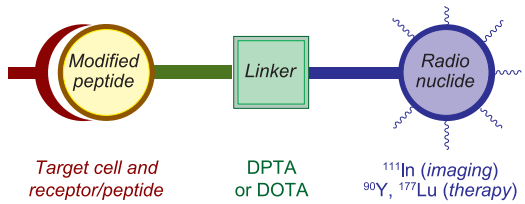


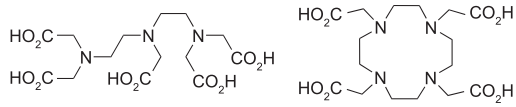


NT4 conjugates



Methotrexate





Diethylenetriamine-pentaacetic acid (DPTA) **Tetraazacyclododecane-tetraacetic acid (DOTA)**

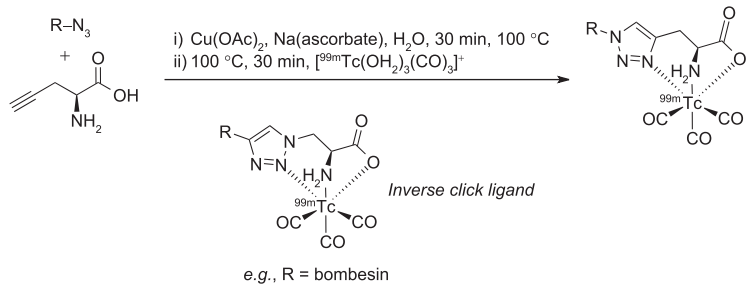
Radiolabeled Neurotensin. A signature of cancer is an overexpression of regulatory receptors, and in a number of human cancers, as discussed, the expression of NTR1 is up-regulated. It is therefore no great leap to envisage how radiolabeled NT analogues could be of great value in the diagnosis and treatment of NTR1-positive cancers. Indeed, stabilized NT analogues coupled to linkers, for example, DTPA for indium-111 labeling for imaging cancers and DOTA for labeling analogues with β-emitting radionuclides, for example, lutetium-177 and yttrium-90, have already been developed for exocrine pancreatic cancer therapy (*179*).

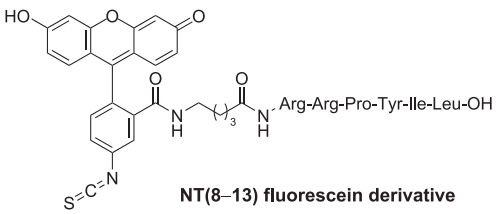
Internalization of receptor-bound technetium-99m radiolabeled analogues of NT and whole-cell binding assays on small-cell lung cancer cells (*180*) and human colon carcinoma cells (HT-29) has also been studied (*181, 182*). The introduction of the bulky [^{99m}Tc(CO)$_3$] chelator complex at the end of the NT(8−13) analogues did not appear to influence receptor recognition. The chemistry required to make these compounds involves Cu(I)-catalyzed [3 + 2] cycloaddition of alkynes and azides to form 1,2,3-triazoles in water. This versatile concept (*183*) has been extended to provide a one-pot strategy for obtaining technetium-99m radiolabeled conjugates and related rhenium complexes (Scheme 4) (*184*). Bombesin, thymidine, carbohydrates, and phospholipids have been derivatized as either azides or alkynes and reacted with either L-propargyl glycine or L-azido alanine to form 1,2,3-triazole-4-yl alanines. Very recently a NT-based ^{99m}Tc/^{188}Re tracer was reported to have such highly specific binding to NTR1 that, on internalization, 50% remained trapped for up to 24 h (*185*).

Much of the above reflects the focus on labeling ligands that bind to NTR1. An exception is a radiofluorinated [^{18}F] analogue of NT69L that binds NTR3 with nanomolar affinity (*186*). This probe was initially developed to study the involvement of these receptors in neurodegenerative diseases such as Alzheimer's or Parkinson's disease. Probes of a similar type could benefit targeted diagnosis/therapy of certain cancers, for example, pituitary and pancreatic ductal adenocarcinomas, where elevated expression of NTR3 is a common feature. However, for receptor-mediated imaging/therapy it is important that the radioprobe is internalized following binding to the receptor on the cell membrane.

Fluorescent NT Probes. Fluorescein derivatives of NT(8−13) containing 5-aminovaleroyl spacer units have demonstrated good receptor binding affinity for NTR1 expressed in human colon cancer (HT-29) cells. Cellular internalization was evident with fluorescence microscopy at 525 nm (*187*).

Scheme 4.



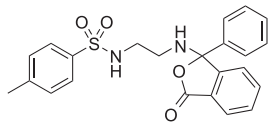


NT(8–13) fluorescein derivative

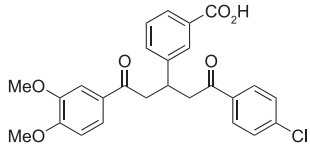
Other receptor internalization studies have used [Nα-fluoresceinyl thiocarbamyl (FTC)-[Glu[1]]NT] (*188*), [Nα-fluoresceinyl-NT(2−13)], and a photoreactive derivative (*189*). A fluorescent-tagged boron-dipyrromethene dye derivative of NT, Nα-BODIPY-NT(2−13), has also been developed (*190*). One study using fluorescently labeled NT concluded that NTR1 is internalized on binding NT and subsequently degraded within the cell (*190*), whereas alternative work has suggested NTR1 (*191*), NTR2 (*192*), and NTR3 (*65*) recycle to the cell surface, thereby maintaining sensitivity to NT agonism.

Small Molecule Antagonists. In addition to SR 48692, levocabastine, and SR 14948A, a handful of other small molecules feature in the public domain that emerged around the same time (*193*). Unfortunately, none have proven as effective as the "molecular toolbox", but these molecules are useful in a sense, as they reflect the pharmaceutical industry's interest in developing selective and orally active NT receptor antagonists, for example, Pfizer's UK-73093, Merck's L-734836, and Parke-Davis's PD-156425.

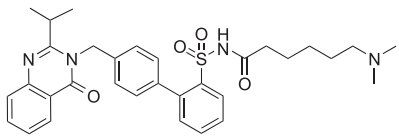
The sulfonamide UK-73093 was one of the first nonpeptide small molecules to emerge. It had low micromolar binding affinities for bovine and mouse NT receptors and behaved as an antagonist without any agonist activity; its binding affinity for rat NT receptors was 10-fold weaker (*194*). In retrospect, we now know that rat and human NTR1 share significant homology. Therefore, it is not surprising that UK-73093 had no effect on human frontal cortex NT receptors: a portentous species-specific activity for UK-73093 was suggested at the time. The simple synthesis of UK-73093 was achieved in one step from *o*-benzoyl benzoic acid and *N*-(2-aminoethyl)-4-methylbenzene sulfonamide in toluene under Dean−Stark conditions (*195*). A second sulfonamide, L-734836, was identified as a selective and competitive antagonist of NT with a binding affinity for NT receptors in rat and HT29 cells in the midnanomolar range (*15*). Lastly, structure–activity relationships of a series of tri-



UK-73093



PD156425

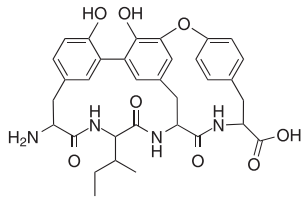


L-734836

arylpentanediones led to the identification of PD156425, which had a nanomolar binding affinity for the mouse NT receptors (*196*). The stereochemistry was not defined in the report. The more potent compounds in the series were shown to be NT antagonists by their ability to inhibit NT-induced Ca^{2+} mobilization in HT-29 cells.

So where do we begin if we want to expand or discover new antagonists of the NT receptors? Do we search for optimum combinations of stabilized cyclic peptides or peptidomimetic structures that could be developed into NTR1 antagonists? Would they have realistic potential in cancer therapeutics? These imagined structures are not dissimilar to certain naturally occurring macrocycles abundant in nature. Could these be a future source of inspiration for chemists? An excellent example is RP-66453. It is a secondary metabolite that was isolated from *Actinomycetes* strain A 9738 in 1998 (*197*). It belongs to a family of macrocycles that include the vancomycin class of antibiotics. Aryl-aryl and biaryl ethers as those contained in RP-66453 are a common structural feature of strained macrocycles.

In binding studies using guinea pig brain tissues that do not express NTR2, RP-66453 bound strongly to NTR1. Structural modifications to RP-66453, it is claimed, has led to NT antagonists of potential use in the treatment of psychosis and Alzheimer's and Parkin-



RP-66453

son's diseases (*198*). It is not apparent from the literature if its effects in cancer cells have been investigated. What is interesting about the structure of RP-66453 is that it is a peptidic secondary metabolite that could be regarded as a constrained *C*-terminal analogue of NT(8−13). The first total synthesis of all *S*-configured diastereoisomer of RP-66453 was reported in 2003 (*199, 200*). The route featured an intramolecular Suzuki−Miyaura coupling forming the biaryl bond, bringing together in a convergent manner two moieties, one derived from commercially available (*S*)-dopa and (*S*,*S*)-isoleucine and the other an (*S*)-iodotyrosine derivative.

The isolation, activities, and synthesis of naturally occurring cyclic peptides containing biaryl and/or biaryl ether linkages have been thoroughly reviewed (*201*). Considering the prevalence of these structures in nature, our skill at being able to synthesize them in the laboratory, and their considerable biological profiles, there is a lot of scope for investigating these fascinating structures further, especially in the context of NT antagonism and cancer research.

Outlook. Despite the considerable progress made in recent years in identifying cancers that overexpress NT receptors, as well having gained a much better understanding of the effects that NT has on cancer cells *in vitro*, there still remains a lot to piece together regarding these processes *in vivo*. Our opinion, garnered from the literature, is that this area is truly a complicated landscape, one that could be brought into higher resolution through the combined efforts of chemists and biologists and a renewed appetite for drug discovery. In the near future, antagonism of NTR1 may become a more widely accepted therapeutic strategy for cancer management. Furthermore, monitoring biomarkers for significant tumoral changes associated with the NT network also has significant potential and would be worthy of more extensive exploration.

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