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Pharmacological Studies on Novel Neurotensin Mimetics: Discovery of a Pharmacologically Unique Agent Exhibiting Concentration-Dependent Dual Effects as Antagonist and Agonist

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SUMMARY

We report the development of two novel neurotensin mimetics (mimics 1 and 2). These compounds were rationally designed and synthesized according to the multiple template approach. We present results of experiments designed to define their pharmacological profiles. In radioligand binding assays with murine neuroblastoma clone N1E-115, we determined the equilibrium dissociation constants for these compounds at the neurotensin receptor. The K_d values for mimic 1 and mimic 2 were 3.3 μM and 1.9 μM, respectively. Functionally, both mimetics antagonized the neurotensin-stimulated production of cGMP, with K_d values in the low micromolar range. Interestingly, mimic 2 displayed a dualistic pharmacological profile, which was concentration dependent. At doses in the 10–100 μM range, mimic 2 became a full agonist, stimulating cGMP production in N1E-115 cells with an EC_{50} value of 19 μM. Furthermore, mimic 1 did not antagonize the cGMP response elicited by mimic 2. When the neurotensin receptor was desensitized with a neurotensin receptor agonist, mimic 2 failed to stimulate significant cGMP production. We propose that mimic 2 binds to a higher affinity site when acting as an antagonist and binds to a lower affinity and different site when acting as an agonist. Thus, mimic 2 would appear to represent a unique pharmacological tool to characterize the neurotensin receptor and its diverse binding sites in N1E-115 cells.

NT is a tridecapeptide (*p*-Glu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu) that is widely distributed in the central nervous system and in peripheral tissues of various mammalian species, including humans (1–4). It stimulates the formation of intracellular cGMP (5) and the turnover of PI (6) and increases the intracellular concentration of calcium, leading to increased phosphorylation, in the rat caudate nucleus (7). NT interacts with dopaminergic neurons with binding sites for this peptide. In addition, it has many of the properties found for neuroleptic agents (8). NT also modulates the release of acetylcholine from terminals of cholinergic projection neurons ascending from the basal forebrain to the cerebral cortex (9).

Recent studies suggest that the interaction of NT with its receptor results in internalization of the peptide-receptor complex, which might in turn trigger intracellular events such as the regulation of gene expression (10). This peptide is associated with a variety of other actions, including the production of hypotension (11), the reduction of pain sensation (12), effects on the contractility of various nonvascular smooth muscles (13), and growth stimulation of human colon cancers and human pancreatic cancers (14, 15). Furthermore, the concentration of NT in the cerebrospinal fluid of schizophrenic patients is decreased, compared with controls (16–18). In two populations of schizophrenic patients with subnormal cerebrospinal fluid NT concentrations, treatment with antipsychotic drugs returned NT concentrations to normal levels (19). These findings implicate the involvement of NT in the pathophysiology of schizophrenia and suggest a possible role for NT agonists as therapeutic agents in the treatment of this disease.

Because of these important biological roles for NT and the fact that no nonpeptidic NT agonist has been discovered, we engaged in an effort to develop full or partial nonpeptidic NT mimetics. These would be organic molecules with improved stability and hydrophobicity that could mimic the action of a native peptide and might possess improved selectivity, affinity, and degrees of agonism or antagonism. To efficiently generate such mimetics a strategy was developed, the multiple template approach,[1] for the design of nonpeptidic peptide mimetics at a

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[1] Pang, Y.-P., J. Zaidi, A. P. Kozikowski, B. Cusack, and E. Richelson. Multiple template approach for the design of peptide mimetics: discovery of a neurotensin antagonist and agonist. Submitted for publication.

ABBREVIATIONS: NT, neurotensin; PI, phosphatidylinositol; NT2, [D-Lys^8]neurotensin(8–13); DMSO, dimethylsulfoxide.

stage when information on the topography of the receptor is not available. Two compounds were synthesized (Fig. 1) that were designed, according to this approach, as either a putative NT agonist or antagonist.[2]

In this article, we report the details of the pharmacological studies on these two compounds. One of these compounds, mimic 2, exhibited a unique dualistic behavior that was concentration dependent. At low concentrations it behaved as an antagonist, whereas at higher concentrations it elicited the profile of a full agonist. This novel character for mimic 2 provides a valuable tool for studying the diverse binding sites at the NT receptor in N1E-115 cells.

Materials and Methods

Cell culture. Murine neuroblastoma clone N1E-115 cells were cultured in 35 ml of Dulbecco modified Eagle's medium supplemented with 10% (v/v) fetal calf serum (Hyclone Labs, Logan, UT) (medium I). Cells (passage number, <23) were grown in 175-cm^2 flasks (Corning Glass Works, Corning, NY) in a humidified atmosphere of 10% CO_2/90% air at 37°. Subculture was achieved by incubation of cells in a modified Puck's D_1 solution, without antibiotics and phenol red (solution I) (20), and resuspension in medium I. On day 5 after subculture, the cells were fed daily by removal of 20 ml of growth medium and replacement with 20 ml of fresh medium I. Cells were harvested during the stationary phase of growth 14–22 days after subculture.

For use in binding assays and functional assays, cells were harvested by aspiration of culture medium, incubation of the cellular monolayer for 10 min at 37° in solution I, disruption of the layer by agitation of the flask, and collection of cells by centrifugation at 300 × *g* for 1 min at 4°, in a GPR centrifuge (Beckman Instruments, Fullerton, CA). The cellular pellet was resuspended in 10 ml of solution II, consisting of 110 mM NaCl, 5.3 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, 2.0 mM Na_2HPO_4, 25 mM glucose, and 70 mM sucrose, pH 7.35 (340 mOsm), made 0.1% (w/v) in bovine serum albumin. The washed cells were collected by centrifugation, and the cellular pellet was resuspended in at least 2 ml of solution II. Twenty microliters of cell suspension were removed for enumeration of cells in a Hylite counting chamber (Fisher Scientific, Pittsburgh, PA).

NT receptor binding assay. Before distribution to assay tubes, the cellular suspension was diluted in solution II to provide 300,000 cells/assay tube and was equilibrated at 4°. Competition binding assays with [^{3}H]NT (2 nM) and varying concentrations of unlabeled NT, mimic 1, or mimic 2 were carried out with intact N1E-115 cells. Nonspecific binding was determined with 1 μM unlabeled NT in assay tubes with a total volume of 1 ml. Incubation was at 4° for 30 min. The assay was routinely terminated by addition of cold 0.9% NaCl (4 ml), followed by rapid filtration through a GF/B filter strip that had been pretreated with 0.2% polyethylenimine. Methods for cell culture and details of binding assays have been described before (21). The data were analyzed using the LIGAND program (22), as modified by us to calculate the Hill coefficient.

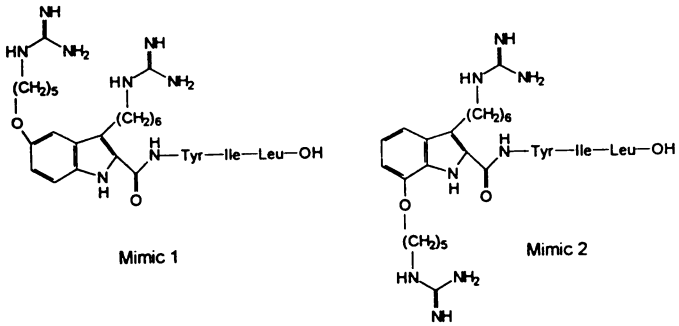

Fig. 1. Chemical structures of mimics 1 and 2.

Assays of cGMP content and PI turnover. N1E-115 cells were harvested for cGMP determination and assay of PI turnover as described above. We have described elsewhere the details of assaying in intact cells the relative changes in cGMP formation, by using a radioactively labeled precursor (23), and of measuring PI turnover (24). To correct for the presence of DMSO, we tested the effects of varying DMSO concentrations on cGMP levels and PI turnover and subtracted these values from the appropriate mimic results (at DMSO concentrations of 1% or less there was no significant effect).

Desensitization experiments. N1E-115 cells were incubated in the absence (control) or presence of NT2 (1 μM) for 15 min at 37° (25). At the end of the incubation, the cells were washed two times with 10 ml of cold solution II and were centrifuged at 300 × *g* for 1 min at 4°. The cellular pellet was resuspended in an appropriate volume of cold solution II to provide 100,000 cells/sample for cGMP assay. After being distributed to assay wells, the cells were placed in a 37° bath for exactly 1 min before stimulation of cGMP production.

Sources of materials. Mimic 1 and mimic 2 were synthesized according to the procedure of Kozikowski *et al.*[2] NT2 was synthesized by solid-phase methods as described previously (26). [^{3}H]NT (107 Ci/mmol, in ethanol) was obtained from New England Nuclear (Boston, MA). [^{3}H]Guanosine (6.2 Ci/mmol) was from ICN (Irvine, CA), whereas [^{14}C]cGMP (52 mCi/mmol), *myo*-[^{3}H]inositol (18.3 Ci/mmol), and *l-myo*-[^{14}C]inositol-1-phosphate (55 mCi/mmol) were from Amersham (Arlington Heights, IL). Polyethylenimine and bovine serum albumin (A-7906) were supplied by Sigma Chemical Co. (St. Louis, MO), whereas NT was from Boehringer-Mannheim (Indianapolis, IN). All other reagents were analytical grade.

Results

Determination of equilibrium dissociation constants. Both of the mimics were initially solubilized in DMSO, and additional dilutions were made in assay buffer (final DMSO concentration in assays was 10% or less). Under these conditions mimic 1 and mimic 2 exhibited low micromolar affinities (Table 1) for the NT receptor, with mimic 2 being almost twice as potent (Fig. 2). The Hill coefficients for both mimics were close to unity, indicating binding to a single noncooperative receptor site (Table 1).

Effects on intracellular [^{3}H]cGMP production. Mimic 1 at concentrations up to 0.1 mM had no effect as an agonist on the production of intracellular cGMP (data not shown). However, when cells were incubated with 10 μM mimic 1 for 15 min at 37° before stimulation with NT, the dose-response curve for NT-stimulated cGMP production was shifted to the right in a parallel surmountable fashion (Fig. 3A). These results indicated that mimic 1 was a competitive antagonist of NT. We used the dose ratio method (27) for determining the K_d of mimic 1 from the EC_{50} values for NT with and without this antagonist. Using this approach, we derived a K_d of 4200 ± 50 nM (geometric mean ± standard error, five experiments), a value that agreed favorably with the K_d derived from competition binding studies (Table 1).

Mimic 2 exhibited dual effects, which were concentration dependent. When mimic 2 was tested as an antagonist, using the same concentration and conditions as used for mimic 1 (i.e., 10 μM for 15 min at 37°), it exhibited the effects of a competitive antagonist of NT (Fig. 3B). Applying the dose ratio method as described above, we calculated its K_d as 2400 ± 110 nM (geometric mean ± standard error, three experiments). This com-

[2] Kozikowski, A. P., J. Zaidi, D. S. Dodd, Y.-P. Pang, B. Cusack, and E. Richelson. Synthesis of partial nonpeptidic peptide mimetics as neurotensin agonist and antagonist. Manuscript in preparation.

TABLE 1

Binding and biological functions of NT and its partial mimetics in N1E-115 cells

Values are geometric mean ± standard error; number of experiments given in parentheses.

Agent	Stimulation of cGMP, EC_{50}	Competition with [^{3}H]NT binding, K_d	Dose ratio analysis, K_d	Hill coefficient for binding, n_H
	nM	*nM*	*nM*	
NT	0.93 ± 0.08 (13)	8.9 ± 0.6 (15)		0.96 ± 0.01 (15)
Mimic 1	No effect	3,300 ± 100 (5)	4,200 ± 500 (5)	0.96 ± 0.01 (5)
Mimic 2	19,000 ± 1,000 (7)[a]	1,900 ± 200 (4)	2,400 ± 100 (3)	0.98 ± 0.02 (4)

[a] Corrected for DMSO effect.

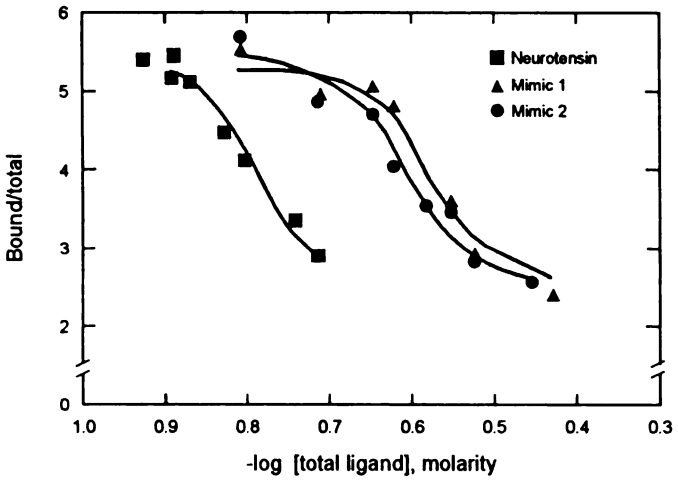

Fig. 2. Competition binding between [^{3}H]NT and the indicated compounds in intact N1E-115 cells. Cells were assayed using 2 nM [^{3}H]NT and varying concentrations of drugs, as described in the text. *Curves* were generated using the LIGAND program. Data points are the means of duplicate determinations and are representative results from one of 15 (NT), one of five (mimic 1), or one of four (mimic 2) independent experiments.

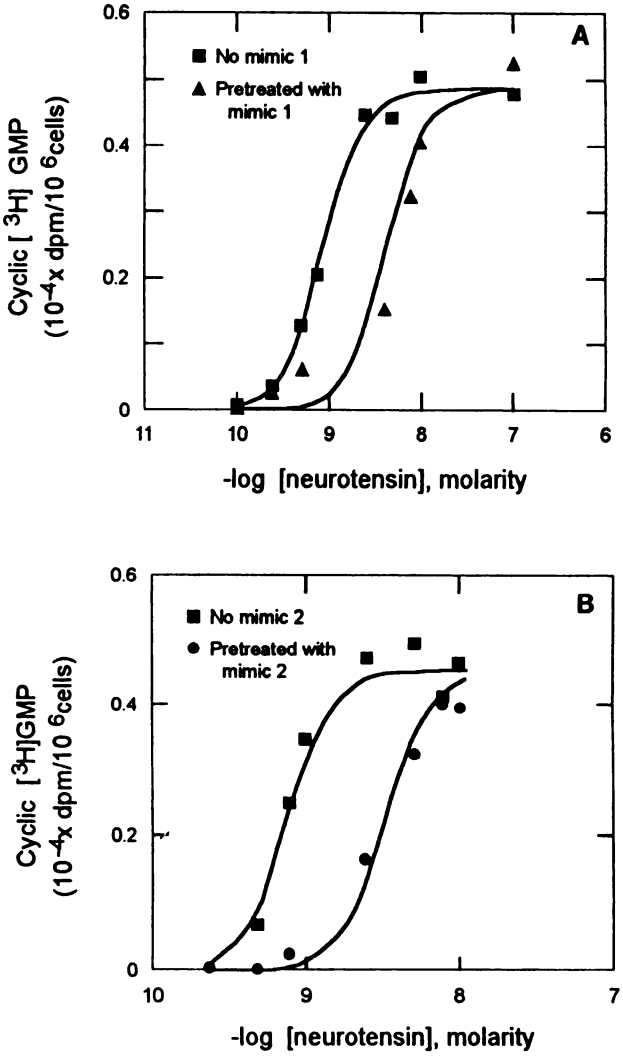

Fig. 3. Dose-response curves for NT-stimulated [^{3}H]cGMP formation in intact N1E-115 cells that were either untreated or pretreated with mimic 1 (10 μM) (A) or mimic 2 (10 μM) (B) for 15 min at 37°. Data are the means of duplicate determinations from which the average of duplicate basal values has been subtracted (basal levels of [^{3}H]cGMP, in dpm/10^6 cells: A, 670 for control and 540 for mimic 1-treated cells; B, 800 for control and 530 for mimic 2-treated cells). The data presented are representative results from one of five (mimic 1) or one of three (mimic 2) independent experiments.

pared favorably with its K_d calculated from binding studies (Table 1).

Additionally, at concentrations of 10–100 μM mimic 2 caused a cGMP response that was dose dependent (Fig. 4), suggesting that it was an agonist at the NT receptor. We could not be certain of its maximal response, because at concentrations greater than 50 μM the effects of the solvent, DMSO, interfered with the accurate determination of maximal stimulation. However, additional experiments using ethanol (0.25% final concentration) as a solvent revealed that mimic 2 was a full agonist. Its EC_{50} was 19 μM (Table 1). Interestingly, mimic 1 did not antagonize the effects of mimic 2. With mimic 1 as an antagonist under the same conditions as before (10 μM for 15 min at 37°), the EC_{50} values for mimic 2 without and with mimic 1 were 17.7 ± 0.8 μM and 21.4 ± 0.8 μM, respectively (geometric mean ± standard error, three experiments). There was no significant difference between these two values ($p > 0.05$ by Student's t test).

When we compared the K_d of mimic 2 with its EC_{50} (Table 1), the observed results did not fit with occupancy theory. An EC_{50} value 10-fold higher than the K_d suggested that mimic 2 was acting 1) not through a receptor-mediated event, 2) at another receptor, or 3) at another lower affinity site on the NT receptor. Three types of experiments were carried out to determine which of the three possibilities was most likely.

cGMP response time after stimulation. We examined the time course for the ability of mimic 2 to stimulate cGMP production (Fig. 5). The response peaked at 30 sec after stimulation and then declined rapidly (data not shown). This type of time course is similar to that of other agonists acting at any of the receptors mediating cGMP synthesis by these cells.

PI turnover. The ability of mimic 2 to stimulate the synthesis of a second messenger different from cGMP was exam-

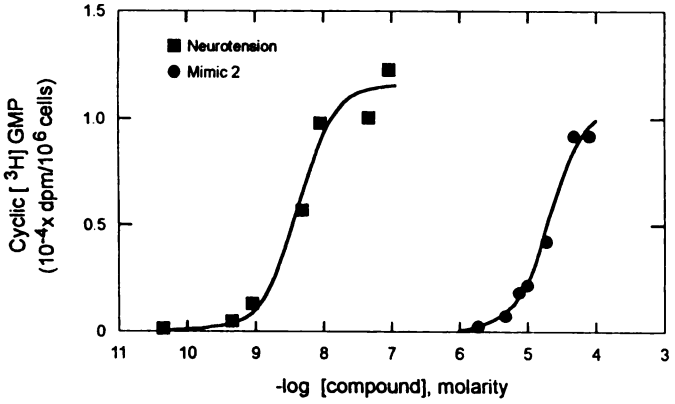

Fig. 4. Effect of agonist concentration on stimulation by NT and mimic 2 of intracellular cGMP synthesis in clone N1E-115 cells. The data presented are from one experiment and are representative of five independent experiments in which the concentration-response curves were obtained for cGMP formation. Each data point is the average of duplicate determinations from which the average of duplicate basal values has been subtracted (basal levels of [^{3}H]cGMP, in dpm/10^6 cells: 800 for control cells and 530 for treated cells).

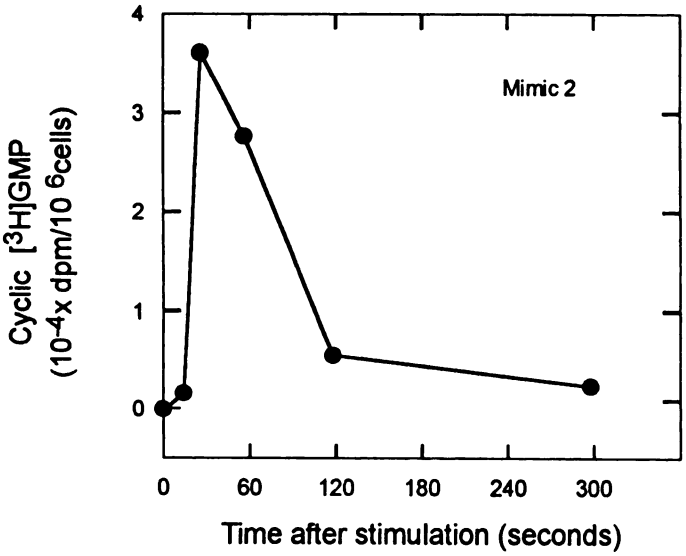

Fig. 5. Relationship between time after stimulation of cGMP production by mimic 2 (50 μM) as an agonist and maximal response in N1E-115 cells. The data presented represent the mean values of two independent experiments, each determined in duplicate. The basal values were subtracted from the data (mean basal levels of [^{3}H]cGMP, in dpm/10^6 cells: 15 sec, 160; 30 sec, 1700; 60 sec, 900; 120 sec, 800; 300 sec, 700).

ined. The NT receptor in N1E-115 cells is known to be linked to PI turnover (6). With mimic 2 as an agonist, we found stimulation of PI turnover. Mimic 2 (50 μM) stimulated PI turnover 1.44 ± 0.02-fold (mean ± standard error) over basal levels, compared with the maximal NT concentration (0.1 μM), which stimulated turnover 1.60 ± 0.03-fold over basal levels (three experiments). There was no significant difference between these two values ($p > 0.05$). Due to the shortage of mimic 2, we were unable to define a complete dose-response curve for PI turnover.

Drug effects after NT receptor desensitization. Finally, we tested the effects of specific desensitization of the NT receptor on the ability of mimic 2 to stimulate cGMP synthesis. Recently, we reported the ability of an NT analog (NT2) to desensitize and down-regulate NT receptors (25). When NT2 (1 μM) was preincubated with cells for 15 min, NT caused no stimulation of cGMP; that is, the NT receptors were desensitized. The response of desensitized cells to NT (1 μM) stimulation was 0.54% of that of untreated cells (Table 2). Using mimic 2 as an agonist under conditions that caused desensitization, we found that cGMP production was 4.8% of the value derived for untreated cells.

Discussion

The binding data and the antagonism of the cGMP response mediated by NT revealed that mimic 1 was a NT antagonist. Our results showed that mimic 2 had a novel dualistic behavior. At the NT receptor it was an agonist at high concentrations and an antagonist at low concentrations. There are many examples in the literature of compounds that both stimulate and antagonize a receptor. However, these compounds (called partial agonists), unlike mimic 2, are not capable of eliciting the maximal response. Instead, the response to a partial agonist is below that of a full agonist. Furthermore, addition of a partial agonist at a fixed concentration to dose-response assays for a full agonist causes a parallel shift of the dose-response curve to the right, as expected for a competitive antagonist. Importantly, the EC_{50} for the response of the partial agonist and its K_d for blocking a full agonist are the same.

Mimic 2, in two different types of assays (binding and dose response), at low concentrations acted as a competitive antagonist of NT. However, at about a 10-fold higher concentration it was a full agonist in eliciting a cGMP response. It should be noted that maximal cGMP stimulation was essentially the same for cells pretreated with and without mimic 2. Therefore, desensitization did not contribute to the effects we observed. The time course for stimulation of cGMP by mimic 2 was consistent with that found for agonists acting at NT receptors (5) and several other receptors on these cells that mediate the synthesis of this second messenger. Taken together, the observed cGMP response and the ability of mimic 2 to stimulate PI turnover strongly suggest that mimic 2 acts at a receptor site. Finally, we have demonstrated in previous experiments that NT-induced desensitization is homologous and specific (28). Therefore, block of the stimulating effect of mimic 2 on cGMP formation by specific desensitization of NT receptors is strong evidence that mimic 2 works by activating these receptors on clone N1E-115 cells.

To explain the observed results, we propose that mimic 2 acts at two different sites. It acts at a low affinity site on the NT receptor as an agonist and at a higher affinity site as an antagonist. The low affinity nature of this agonist site may

TABLE 2

Effects of mimic 2 and NT on NT2-induced desensitization of cGMP production at the NT receptor

Values are mean ± standard error (three experiments in each case).

Condition	[^{3}H]cGMP production (−basal)	
	Stimulated with NT (1 μM)	Stimulated with mimic 2 (50 μM)
	dpm/10^6 cells	
Untreated cells	2600 ± 600	500 ± 150
Cells treated with NT2 (1 μM) for 15 min at 37°	14 ± 14 (0.54%)[a]	24 ± 18 (4.8%)[a,b]

[a] Percentage of respective control.
[b] Corrected for DMSO effect.

have precluded our detecting it in our binding assays, which used rapid filtration to separate bound from free radioligand. More likely, mimic 2, a smaller molecule than NT, acts at a second site that is not accessible to NT. Competition binding curves that showed complete competition between mimic 2 and [^{3}H]NT, with Hill coefficients of unity, support this point. However, to test this hypothesis adequately, ^{3}H-labeled mimic 2 would be necessary, as well as a fast method for separation of bound from free radioligand.

It is reasonable to speculate that ligands can induce a conformational change in the NT receptor by binding to more than one site on this receptor. Mimic 2 was a full agonist at NT receptors at high concentrations and a competitive antagonist of NT at low concentrations. We think that binding to its antagonist site has no effect on its activation of the NT receptor by binding to its agonist site. This conclusion is strengthened by the fact that mimic 1 did not antagonize the cGMP response elicited by mimic 2.

Although both mimics have low micromolar potencies at the NT receptor, the efficiency of developing active agents at the NT receptor is significant. This is because another recently reported NT antagonist was the result of structural modifications of a lead compound discovered by screening thousands of compounds (29). We designed and tested only two candidates. Both of the designed compounds proved to have NT binding potencies. Most importantly, using mimic 2, we hypothesize the presence of an additional low affinity NT binding site that can stimulate cGMP production. Additional experiments will be needed to substantiate this hypothesis. Additionally, mimic 2 provides us with a unique pharmacological tool that can be used to define further the activation domains of the NT receptor. Finally, the development of mimic 2 as well as mimic 1 can provide insight for additional structural modifications, in search of a full nonpeptidic NT agonist.

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