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Identification and Functional Characterization of a Stable, Centrally Active Derivative of the Neurotensin (8−13) Fragment as a Potential First-in-Class Analgesic

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The neurotensin hexapapetide fragment NT(8−13) is a potent analgesic when administered directly to the central nervous system but does not cross the blood−brain barrier. A total of 43 novel derivatives of NT(8−13) were evaluated, with one, ABS212 (**1**), being most active in four rat models of pain when administered peripherally. Compound **1** binds to human neurotensin receptors 1 and 2 with IC_{50} of 10.6 and 54.2 nM, respectively, and tolerance to the compound in a rat pain model did not develop after 12 days of daily administration. When it was administered peripherally, serum levels and neurotensin receptor binding potency of **1** peaked within 5 min and returned to baseline within 90−120 min; however, analgesic activity remained near maximum for > 240 min. This could be due to its metabolism into an active fragment; however, all 4- and 5-mer hydrolysis products were inactive. This pharmacokinetic/pharmacodynamic dichotomy is discussed. Compound **1** is a candidate for development as a first-in-class analgesic.

Introduction

Neurotensin (NT[a]) is a tridecapeptide first isolated from bovine hypothalamus over 30 years ago.[1] The hexapeptide C-terminal fragment NT(8−13) [Arg[8]-Arg[9]-Pro[10]-Tyr[11]-Ile[12]-Leu[13]] was shown to retain the full activity of the parent compound,[2] defining it as the active component of the molecule and the logical lead for development of NT-based therapeutics. Although its effects are not limited to the nervous system,[3,4] NT is known to act as a neurotransmitter/neuromodulator where it induces several important physiological effects in mammals including analgesia[5] and hypothermia.[6] It also has been shown to have potential as an antipsychotic compound based on its blockade of amphetamine-induced locomotor hyperactivity.[7,8] NT exerts its effects through binding of two brain receptors, NTR-1 (also referred to as NTS-1) and NTR-2 (NTS-2), although the relative roles of each receptor in the physiological responses are not well-understood.[9−11] Numerous experiments with both NT and NT(8−13) suggest that all central activities attributable to NT and derivatives only manifest following direct application into the CNS[12] while peripheral administration is ineffectual. This probably is a result of the compounds' polarity and short half-lives in plasma.[13] Hence, NT derivatives that could be delivered peripherally and cross the BBB would have great potential for development as pharmaceutical agents.

Two previously described NT(8−13) analogues (NT1 and NT69L) displayed significant analgesic and antipsychotic effects when injected ip.[14,15] However, various aspects limited their usefulness as a drug including the induction of significant tolerance after a single injection.[16] Research in this laboratory and others has shown that minor changes to the structure of NT(8−13) can effect extreme changes in hypothermic, antipsychotic, or analgesic responses.[12,13,15,17−19] Specifically, modification of the N-terminus of NT(8−13) and substitution of *t*Leu at position 12 produce compounds exhibiting increased stability in blood and antipsychotic activity in rats[19,20] (also unpublished data); the most active of these compounds is ABS201 (**2**)[21] (compound **11** in earlier manuscript).

Compound **2** also exhibits minor analgesic activity. In this paper, structural modification of **2** and subsequent evaluation in various rat models have been used to identify new NT(8−13) derivatives possessing dramatically improved analgesic properties. A library of 44 proprietary modifications to the NT(8−13) parent compound have been screened, with ABS212 (**1**) chosen as the lead compound based on its superior activity in the hot plate model. Compound **1** was further screened in a battery of analgesic models and its pharmaceutical properties were explored in order to evaluate its potential for further development as a first-in-class analgesic.

Results

Synthesis of Analogues. NT and NT(8−13) are pluripotent peptides that have activity as analgesics and antipsychotic

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[a] Abbreviations: NT, neurotensin; BBB, blood−brain barrier; NTS, neurotensin receptor; CNS, central nervous system; MPE, maximum possible effect; PK, pharmacokinetic; PD, pharmacodynamic.

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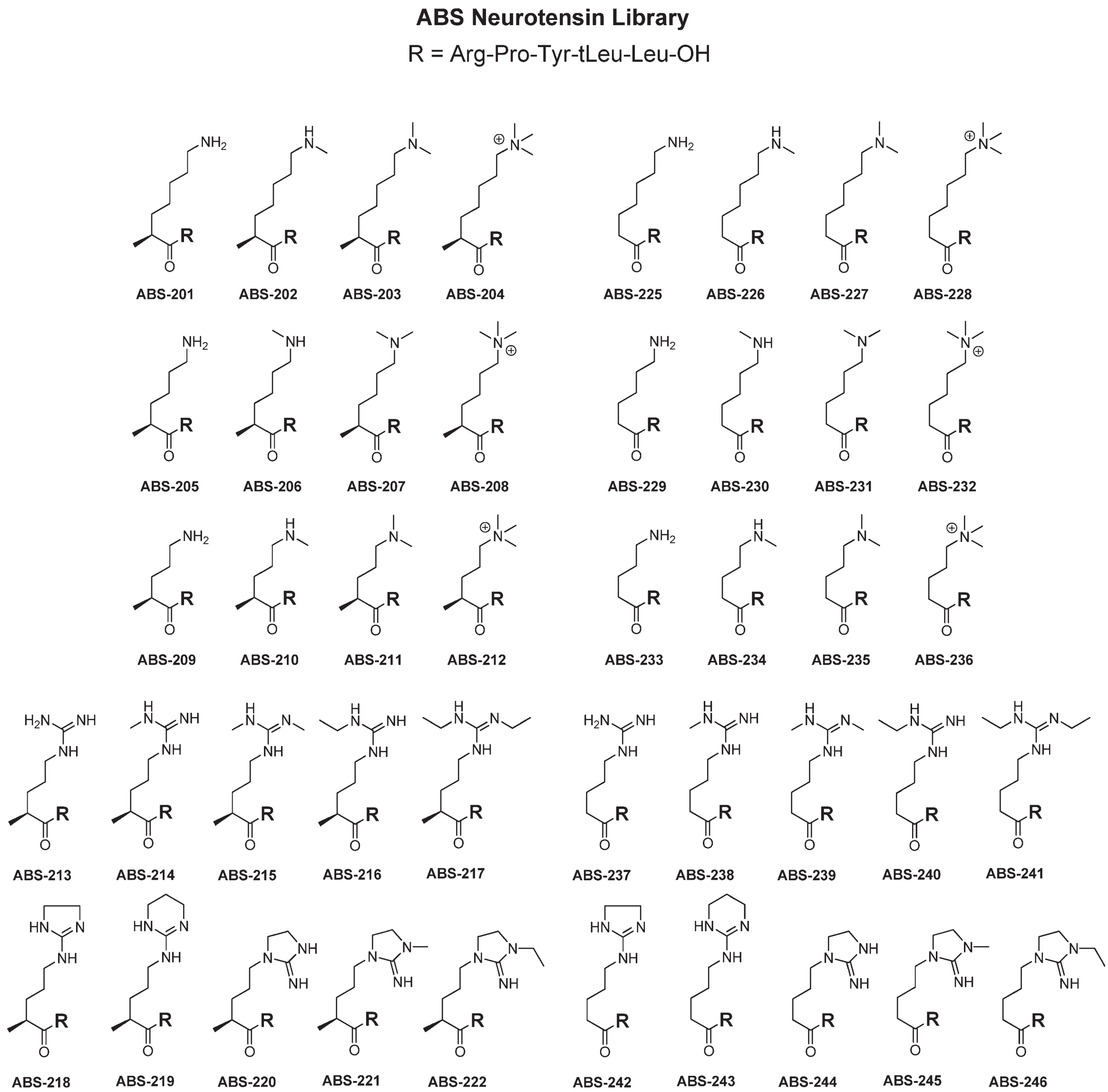

Figure 1. Structures of cationic natural and non-natural amino acids substituted for Arg[8] in NT(8−13).

and hypothermic agents only when administered directly into the CNS. Previously, we developed a derivative of NT(8−13), **2**, that has potent antipsychotic but weak analgesic properties when dosed ip or iv.[13] This analogue possesses several significant alterations from the parent compound including a modified N-terminus and Arg[8] residue, and substitution of *t*Leu for Ile.[12] The primary result of these alterations was to block peptidase-catalyzed hydrolysis at Arg[8]-Arg[9] and Tyr[11]-Ile[12], respectively.[22] To expand upon previous findings, a series of 43 additional analogues were synthesized that feature further modification of the N-terminus and Arg residue with the anticipation that we would uncover compounds featuring changes/improvements in their relative antipsychotic/analgesic profiles. The structures of the new peptides are given in Figure 1.

Analysis of Analogues in the Hot Plate Model. As an initial screen for analgesic activity, all peptides were evaluated in the hot plate analgesic model using a standard dosing of 10 mg/kg ip. This value was an approximate molar equivalent dose to 5 mg/kg morphine to provide a potency benchmark. Figure 2 shows a typical pharmacodynamic (PD) response curve comparing **1** to morphine and saline. For

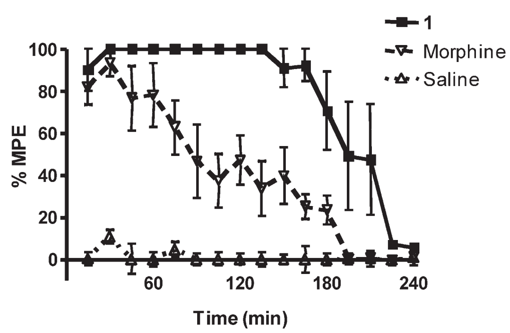

Figure 2. Compound **1** benchmarked with morphine in the hot plate model. Rats were dosed ip with **1** at 10 mg/kg, morphine at 5 mg/kg, or sterile saline and analyzed at the indicated time points in the hot plate model. Individual points indicate the mean ± SEM; $n \geq 6$ for all treatments.

morphine the response reaches 100% of the maximal possible effect (MPE) after 15−30 min and then decreases in a time-dependent manner, returning to baseline by 200 min. When dosed with **1**, the time at 100% MPE was greatly prolonged and did not begin to diminish significantly for 180 min.

Table 1. Comparison of Peptides in the Hot Plate Assay[a]

compd	m/e (obsd/calcd)	MPE	time at MPE (min)	AUC
201 (2)	802.60/802.02	100.00	45	9928
202	816.63/816.04	100.00	75	12182
203	830.65/830.07	60.94	0	4281
204	844.72/845.10	19.90	0	1898
205	788.60/787.99	86.00	0	11015
206	802.64/802.02	55.80	0	6001
207	816.65/816.04	84.90	0	7004
208	830.68/831.08	67.20	15	8788
209	774.60/773.96	89.40	0	9507
210	788.60/787.99	82.40	0	12105
211	nd	nd	nd	nd
212 (1)	816.59/817.05	100.00	135	18084
213	816.56/816.00	48.52	0	3515
214	830.60/830.03	100.00	75	8874
215	844.69/844.06	63.30	0	6106
216	844.63/844.06	74.48	0	12400
217	872.72/872.11	10.00	0	781
218	842.65/842.04	79.31	0	5793
219	nd	nd	nd	nd
220	nd	55.00	0	3569
221	856.71/856.07	76.07	0	7462
222	nd	nd	nd	nd
225	788.67/787.99	100.00	*60	8416
226	802.65/802.02	59.32	0	5345
227	816.75/816.04	29.00	0	3985
228	830.79/831.08	55.70	0	3853
229	774.61/773.96	48.00	0	3520
230	788.64/787.99	100.00	30	5733
231	nd	nd	nd	nd
232	816.69/817.05	84.00	0	11109
233	760.62/759.94	51.69	0	3585
234	774.33/773.96	100.00	120	17320
235	788.40/787.99	100.00	90	11180
236	802.42/803.02	100.00	60	7022
237	802.44/801.98	50.86	0	4039
238	816.48/816.00	100.00	75	8396
239	830.51/830.03	92.28	0	5152
240	830.55/830.03	37.36	0	2148
241	858.68/858.08	95.68	15	7425
242	828.57/828.01	76.40	0	7506
243	842.63/842.04	79.62	0	6637
244	828.69/828.01	82.80	0	6110
245	nd	nd	nd	nd
246	856.69/856.07	80.48	0	7119

[a] Three major end points are compared: (1) the maximal possible effect (MPE) that was obtained with that drug, (2) the time that the maximal effect was maintained, and (3) the area underneath the pharmacodynamic response curve.

The remaining compounds in Figure 1 also were tested in this model.

In order to facilitate a meaningful comparison of relative potency of all compounds tested, Table 1 reports three major end points of the model: (1) the maximal effect (as a percentage of the MPE) obtained with the given compound, (2) the time that 100% MPE was maintained, and (3) the area underneath the PD response curve. Analysis of that table indicates that **1** was the most efficacious of the analogues and thus was chosen for further study.

Potency of 1 with Various Routes of Administration. Potency studies were undertaken to determine the EC_{50} of **1** and to evaluate whether it differed as a function of the route of administration. As shown in Figure 3, the EC_{50} of **1** was 2.64 mg/kg when delivered iv. Significant differences were not found when the drug was administered ip, sc, or im.

Effect of 1 in Other Models of Pain. To evaluate the potential usefulness of **1** as a general analgesic, its effectiveness in four additional rat models of pain (acetic acid writhing, tail flick, formalin, and Chung models) was determined. As shown in Figure 4, **1** was quite potent in the acetic acid writhing assay with an EC_{50} of 0.16 mg/kg (approximately 10-fold more potent than the hot plate). This increase in potency relative to the hot plate may be a result of the high sensitivity of this assay.[23,24] In the tail flick assay (Figure 5A), **1** significantly increased the latency of tail withdrawal (indicated at the % MPE) in a manner similar to morphine. A dose response analysis revealed an EC_{50} of 1.84 mg/kg, very similar to what was seen in the hot plate assay (Figure 5B). Figure 6A presents a typical PD response curve in the formalin model. Compound **1** again was significantly more potent than morphine in this model. The dose response curve (Figure 6B) indicated an EC_{50} of 2.05 mg/kg, again similar to the hot plate model. In the Chung model of neuropathic pain (Figure 7, performed at a single dose of 10 mg/kg) **1** elicits a maximal response within 15 min which is maintained for over 1 h before beginning to fade.

Effect of 1 on Hypothermia. A well-known central effect of NT and its active derivatives is the reduction of core body temperature (hypothermia). Figure 8A is a typical PD response curve obtained with **1** that demonstrates a significant and sustained (>4 h) drop in temperature. Interestingly, the dose response curve of the area under the PD curves (Figure 8B) gave an EC_{50} of 0.78 mg/kg, significantly lower than the EC_{50} associated with central pain mediation. This is likely due to the hypothermic and analgesic effects being modified through different NTRs.

Lack of Induction of Tolerance by 1. Previously reported NT analogues have been shown to induce tolerance after as little as a single injection.[16] In order to assess potential development of tolerance to **1**, rats were injected with 10 mg/kg daily for 12 consecutive days. On the indicated days (Figure 9) rats were analyzed in the hot plate analgesia model immediately following injections. As seen in Figure 2, initial injections resulted in a strong and sustained increase in response latency in this assay, again demonstrating the significant analgesic activity of this compound. However, even after 12 daily injections, the animals still displayed a similar PD response, clearly showing that tolerance is not developing to **1** over the time course of the experiments.

Binding to NTRs. To ensure that **1** is binding to appropriate receptors with sufficient affinity to generate the PD responses, binding assays were performed with rat NTR-1. As shown in Figure 10A there was dose-dependent binding of **1** to rat NTR-1 with a K_D of 23.31 nM. Equivalent experiments could not be performed with cloned rat NTR-2, as various investigators have not been able to obtain stable constructs (E. Richelson, personal communication). However, the binding to human NTR-1 and NTR-2 was assessed and demonstrated EC_{50} of 10.6 and 54.2 nM, respectively. For virtually all NT derivatives, their relative affinities for human versus rat NTRs remain consistent.[25,26]

Calcium Mobilization. To ensure that binding to receptor actually results in activation, the flux of calcium (a well established second messenger response) was measured in the rat NTR-1 expressing cells in response to increasing concentrations of **1**. As shown in Figure 10B **1** activated calcium flux with an EC_{50} of 27.9 nM, a concentration very similar to its binding affinity.

PK Analysis. As a further step toward understanding the mechanism of action of **1**, a PK analysis was performed.

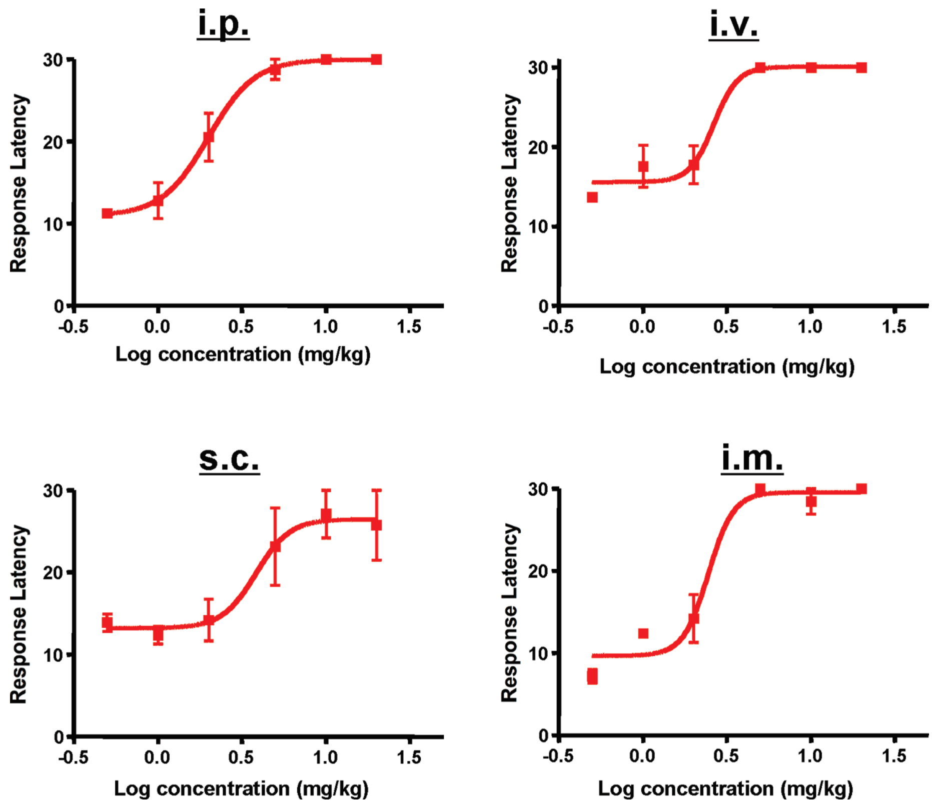

Figure 3. Effect of route of administration of **1** in the hot plate model. Rats were injected ip, iv, sc, or im with various concentrations of **1** and then assessed in the hot plate assay after 60 min. EC_{50} values are as follows: ip = 2.00 mg/kg (95% confidence levels of 1.543 and 2.583 mg/kg); iv = 2.64 mg/kg (95% confidence levels of 1.261 and 5.546 mg/kg); sc = 3.84 mg/kg (95% confidence levels of 1.567 and 9.392 mg/kg); im = 2.45 mg/kg (95% confidence levels of 1.376 and 4.369 mg/kg). n = 3 for all doses. Individual points indicate the mean ± SEM.

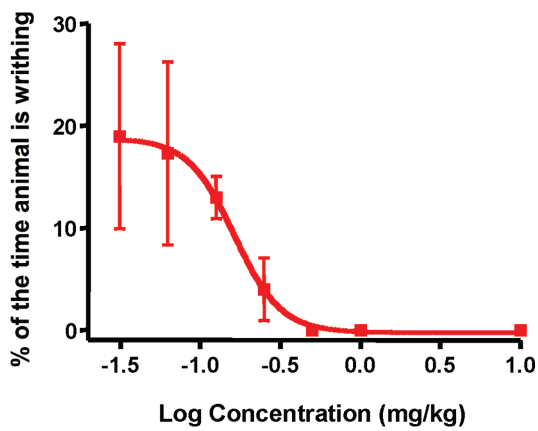

Figure 4. Dose response of **1** in the writhing model. Rats received varying concentrations of **1** iv before analysis in the writhing model. EC_{50} = 0.16 mg/kg, with 95% confidence levels of 0.072 69 and 0.3669 mg/kg; n = 3 for all doses. Individual points indicate the mean ± SEM.

After injection of rats with 1 mg/kg **1**, blood levels were assessed at increasing time points by mass spectrometry. As shown in Figure 11, the initial spike in concentration expected immediately following injection was observed. Surprisingly, however, these levels rapidly decreased and returned to baseline by 60 min despite the fact that the PD analgesic response remained near maximal for at least 180 min. One possible explanation for the discrepancy between the PK and PD is that a hydrolysis product or an unidentified metabolite of **1** passes through the BBB to activate the NTRs and maintain the sustained analgesia well after **1** is degraded. To test this possibility, the kinetics of NTR-1 binding in the plasma of rats after injection of **1** were examined and are overlaid on the data in Figure 11. As shown, very quickly (1 min) after iv injection there is a large increase in NTR-1 binding that begins to rapidly decrease by 15 min. After 60 min, levels have returned to concentrations not significantly different from nontreated animals. Notably, the kinetic profile of receptor binding mirrored almost exactly the level of parent compound measured by mass spectrometry. Indeed, calculated half-lives ($T_{1/2}$) were 0.407 and 0.438 h, respectively.

To further ensure that metabolites of **1** were not responsible for the demonstrated PD response, the various possible hydrolytic metabolites of this compound were synthesized and the 5-mers and 4-mers assayed in the hot plate model. Table 2 lists these possible metabolites, while Figure 12 shows the results of that analysis. In response to treatment with **1** response latency rapidly increased to maximum (30 s) and remained high during the course of the experiment. In contrast, no increase in response latency was seen with any of the possible metabolites. There was an early increase with **4** that did not approach maximal and rapidly returned to baseline; this is probably a spurious result. Because removal of one or two amino acids from either end completely abolished activity, further analysis of the 3- and 2-mers was deemed unnecessary. These results strongly argue that hydrolytic metabolites are not responsible for the long lasting PD effect observed with the parent compound.

Discussion

Neurotensin (NT) has been shown to be an effective endogenous pain modulator[27] and as such is an excellent

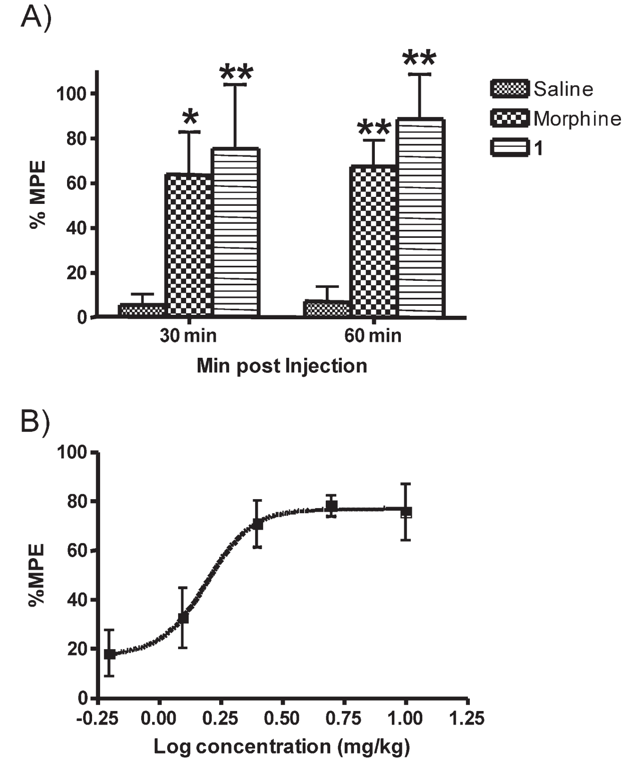

Figure 5. Effects of **1** in the tail flick model. (A) Typical PD response elicited by **1**, morphine, and saline. Rats were dosed ip with **1** at 10 mg/kg, morphine at 5 mg/kg, or sterile saline and analyzed in the tail flick assay at 30 and 60 min; $n = 6$ for each treatment. Individual points indicate the mean ± SEM. Single asterisks denote values significantly different from saline, with $p < 0.05$, whereas double asterisks indicate $p < 0.01$. (B) Dose response of **1**. Rats were injected ip with various doses of **1** and assessed in the tail flick model 30 min later. The EC_{50} was 1.57 mg/kg, with 95% confidence levels of 0.967 and 2.552 mg/kg; $n = 6$ for all doses. Individual points indicate the mean ± SEM.

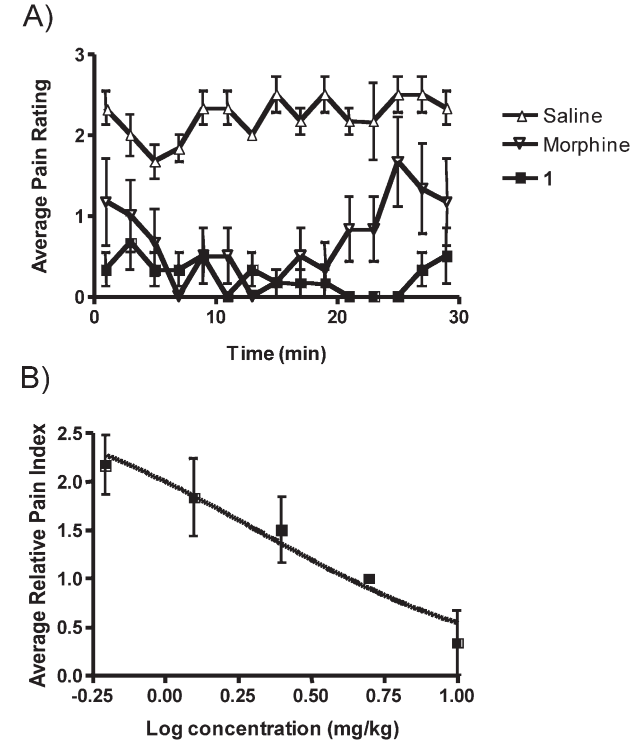

Figure 6. Effect of **1** in the formalin model. (A) Typical PD response elicited by **1**, morphine, and saline. Rats were dosed ip with **1** at 10 mg/kg, morphine at 5 mg/kg, or sterile saline and analyzed in the formalin assay for 30 min; $n = 6$ for each treatment. Individual points indicate the mean ± SEM. (B) Dose response of **1**. Rats were injected ip with various doses of **1** and assessed in the tail flick model 30 min later. The EC_{50} was 2.06 mg/kg, with 95% confidence levels of 1.27 and 3.30 mg/kg; $n = 6$ for all doses. Individual points indicate the mean ± SEM.

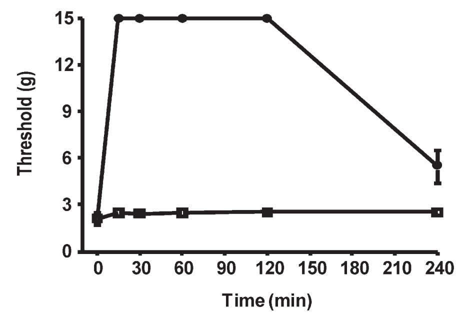

Figure 7. Effect of **1** in the Chung model. Rats were given an ip dose of 10 mg/kg **1** or saline and analyzed in the Chung assay for 30 min; $n = 5$ for both treatments. Individual points indicate the mean ± SEM.

candidate for pharmaceutical applications. However, this peptide and its fully active fragment NT(8−13) suffer from several druggability issues including deliverability, stability, selectivity, and efficacy. When derivatives are designed to address these issues, several elements of the peptide must be retained in order not to adversely degrade its activity. In particular, the positively charged side chains at the N-terminus[2,28] and the free carboxylate at position 13 are necessary for significant activity. Loss of potency also is noted when Arg^8 is changed from L- to D-.[28,29]

We have previously developed a derivative of NT(8−13), **2**, that retained the structural features necessary for activity but included a modified N-terminus and Arg^8 residue and substituted *t*Leu for Ile.[12] This compound had strong antipsychotic but weak analgesic activity. In this study a series of 43 additional analogues were synthesized that feature further modification of the N-terminus and Arg residue (Table 1). As anticipated, this resulted in compounds with a wide variety of activities in the hot plate model of acute pain.

A few generalizations can be made on the structure−function relationships of the current series of NT(8−13) derivatives. In the Arg^8 position, nearly all Orn substituted derivatives (ABS209−ABS212 (**1**) and ABS234−ABS236) possessed significantly increased activity (five of eight analogues reached 100% MPE, while two others were over 80%). The effect of the Orn substitution has been similarly reported for a class of NT(8−13)-based antipsychotic drugs.[20] In addition, analogues containing Arg^8 plus a methyl addition (ABS214 and ABS238) or hLys alone (**2** and ABS225) performed quite well. In these cases activity was not affected by a change in the N-terminus group. These results indicate that smaller side chains at Arg^8 are more effective analgesics. Overall, however, it was surprising that broader structure−activity relationships did not result from the data, which may indicate a complex cross-talk between binding and activating NTR-1 and NTR-2 to promote the observed physiological responses.

Data from the hot plate model enabled identification of **1** as the best lead candidate for further characterization. This molecule demonstrated a potent antinociceptive response in

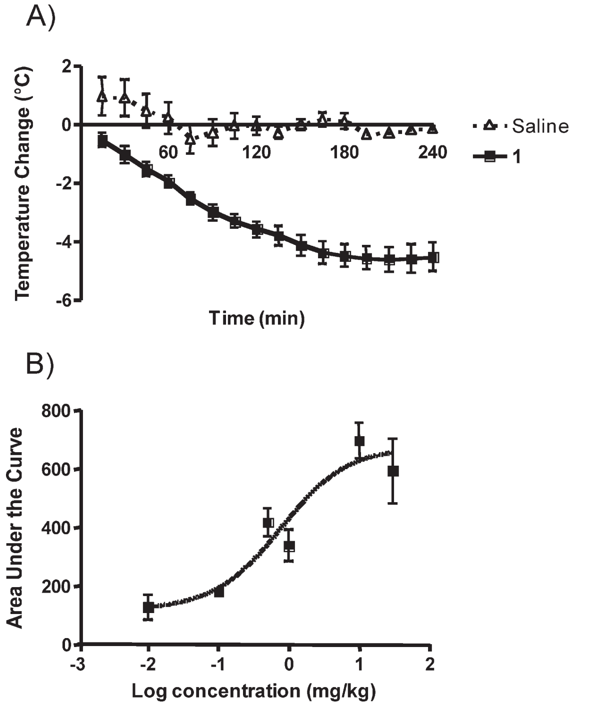

Figure 8. Effect of **1** in the hypothermia assay. (A) Typical PD response elicited by **1** and saline. Rats were dosed iv with **1** at 10 mg/kg or sterile saline and analyzed in the hypothermia assay for 4 h; $n \geq 4$ for each treatment. Individual points indicate the mean ± SEM. (B) Dose response of **1**. Rats were injected iv with various doses of **1** and assessed in the hypothermia assay. The total area under the various PD curves was calculated and used to generate the dose−response curve. EC_{50} = 0.78 mg/kg with 95% confidence levels of 0.190 and 3.237 mg/kg; n = 3 for all doses. Individual points indicate the mean ± SEM.

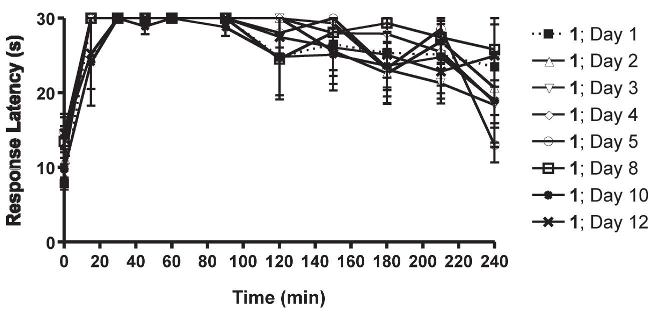

Figure 9. Evaluation of tolerance development in response to repeated daily doses of **1**. Six rats were separated into two groups of three, and both groups were injected iv with 10 mg/kg **1** per day for 12 days consecutively. Each set was allowed at least 48 h between consecutive evaluations in the hot plate model. Thus, any given data point represents the mean ± SEM of three rats (one set), although overall a total of six rats were analyzed for the development of tolerance.

four additional rat analgesic models. The EC_{50} values for three of the four iv analgesic tests were quite similar (approximately 2.0 mg/kg), validating the use of the hot plate model as an initial screening tool. A significantly lower EC_{50} was detected in the acetic acid writhing assay that may be a result of the enhanced sensitivity of this assay relative to the others.[23,24]

This EC_{50} did not differ because of route of administration, suggesting it is freely permeable throughout the periphery. Repeated administration of **1** for 12 straight days did not

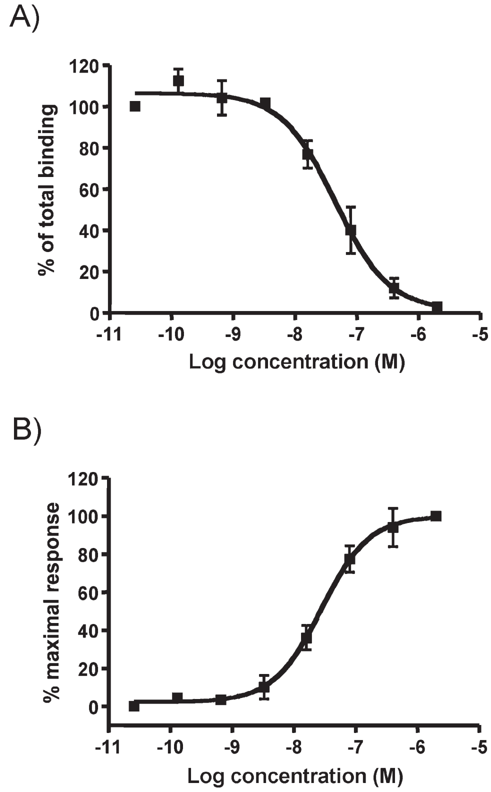

Figure 10. Evaluation of **1** binding and activation of rat NTR-1. (A) Radioligand competition assays with [^{125}I]NT were performed and the data normalized to percent of maximal binding. Individual experiments were measured in triplicate, and each experiment was performed a minimum of three independent times. The data points represent the mean ± SEM of all experiments. The K_i was 23.31 nM with 95% confidence levels of 14.01 and 38.79 nM. (B) Calcium release assays were performed on the fluorometric imaging plate reader (FLIPR, Molecular Devices Corp.). Individual points indicate the mean ± SEM of at least three independent measurements. The EC_{50} was 27.88 nM with 95% confidence levels of 17.62 and 44.10 nM.

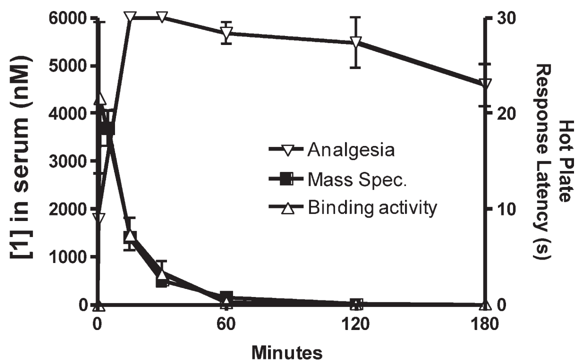

Figure 11. PK analysis of **1** levels and receptor binding activity overlaid on PD information from the hot plate model. Rats were injected with 1 mg/kg **1**. At each time point of 1 (or 5), 15, 30, 60, 120, 180, and 240 min, three rats were assessed using the hot plate model. Thus, individual points represent the mean ± SEM of three independently assessed rats. Animals were immediately sacrificed after analysis, and plasma was isolated and analyzed in the NTR-1 radiolabel competition assay. Thus, individual points represent the mean ± SEM of three independently assessed rats. Plasma concentrations were calculated from a standard curve prepared in plasma from untreated rats. Plasma levels of **1** were also assessed by MS in a separate experiment performed by Covance, Inc. Individual points represent the mean ± SEM of at least three independently assessed rats.

result in the development of significant tolerance to the drugs even though previous studies with other NT(8−13) analogues found tolerance after a single dose.[16]

Analysis of the PK of this drug surprisingly revealed that it is rapidly cleared from the circulation (< 60 min), despite the fact that the analgesic response is maintained for nearly 4 h. This possibly explains the absence of tolerance that is generally due to desensitization following long-term exposure of drugs. The observed dichotomy between the PK and PD cannot be explained by a previously unrecognized metabolite of the analogue because NTR-1 binding decreased at an identical rate to levels of parent compound measured by LC/MS. Moreover, none of the possible 5-mer or 4-mer hydrolytic metabolites of **1** possessed significant endogenous activity. Thus, the mechanisms of this PK/PD dichotomy remain undefined although it could be explained by selective uptake and distribution between the blood and the brain and/or compound binding to the central NTRs manifesting long-lived molecular changes that sustain the analgesic activity.

The discovery and development of peptide drugs provide the most direct route for exploiting genomic and proteomic insights into the treatment of disease. Peptides can act as agonists to positively affect biological processes, with the potential to repair damaged, injured, or undeveloped tissue. Peptide therapeutics represent an extremely powerful approach that can reduce the time and risk of bringing a drug to market. Compared to synthetic small molecules, drugs based on native peptides have the potential for shorter and more reliable development cycles because their physiological activity is well-defined prior to development and they are much less likely to cause unexpected toxicities. We conclude that **1** is an exciting new candidate for potential development as a broad-spectrum, first-in class analgesic.

Table 2. The 5-mer and 4-mer Hydrolytic Fragments of **1**

compd	structure						*m*/*e* (obsd/calcd)
	Length: 5-mers						
3	Me3-Orn	Arg	Pro	Tyr	*t*Leu		703.52/704.88
4		Arg	Pro	Tyr	*t*Leu	Leu	661.47/660.80
	Length: 4-mers						
5	Me3-Orn	Arg	Pro	Tyr			590.38/5912.70
6		Arg	Pro	Tyr	*t*Leu		548.30/547.61
7			Pro	Tyr	*t*Leu	Leu	nd

Experimental Section

Peptide Synthesis. Appropriately protected non-natural amino acid analogues of Arg and Lys were synthesized as described previously by our laboratory[30−35] and others[36] for incorporation at the 8 position of NT(8−13) via Merrifield solid-phase synthesis (Figure 1). All analogues were determined to be greater than 95% enantiomerically pure. Peptides were prepared by manual solid-phase peptide synthesis on Wang resin (Fmoc chemistry), purified by reverse-phase HPLC, and isolated and studied as the trifluoroacetate salts.[19] Peptide identity was determined by MALDI-TOF MS on a Voyager DE-STR system

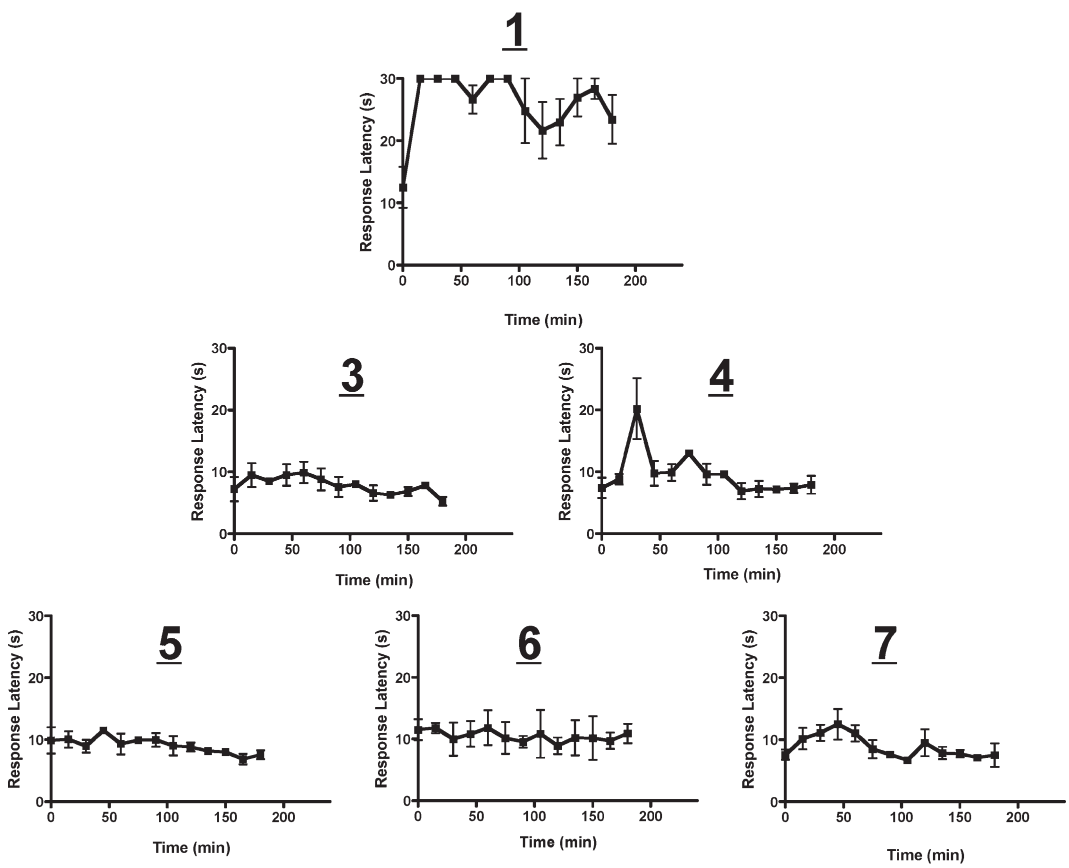

Figure 12. Evaluation of all potential 5- and 4-mer hydrolysis products of **1** in the hot plate model. All possible 5- and 4-mer products that might result from proteolysis from the N-terminal, the C-terminal, or both were synthesized and injected into rats iv at 12.5 mmol/kg. The change in concentration from mg/kg was necessary to ensure equimolar levels, given the differences in molecular weights. Each graph is the individual PD response curve for that hydrolysis product, and each point on the graphs represents the mean ± SEM of at least three rats independently assessed at that time point.

4117 mass spectrometer (Applied Biosystems, Foster City, CA), and the peptides were determined to be greater than 95% pure (MS data provided in Table 1).

Animals. All animal work was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at the Medical University of South Carolina. Animal work at the University of California and Covance were reviewed and approved by their institutions' IACUCs. All experimental protocols were performed in accordance with the guidelines set forth in the NIH Guide for the Care and Use of Laboratory Animals, published by the Public Health Service. All protocols were performed on male Sprague−Dawley rats (Harlan, Prattville AL, 240−280 g), which were housed in an AAALAC-approved colony room maintained at a constant temperature and humidity. Animals (two per cage) were kept on a 12 h light−dark cycle with ad libitum access to food and water. Because many of the assays are susceptible to learning phenomena, which results in progressive decrease of the physiological response or behavior to be monitored, rats were used no more than three times per analgesic assay. Only experimentally naive rats were used in the formalin test.

Pain Models. Hot Plate Model. The hot plate model evaluates central pain attenuation in a rodent using an acute thermal stimulus known to be mediated through central processing pathways.[37−39] In this assay the rat is treated with compound and, at a series of time points thereafter, assessed on a hot plate analgesia meter (Columbus Instruments, Columbus, OH), essentially a flat surface maintained at 53.0 ± 0.2 °C. After the rat is placed on the hot surface, the time until the rat lifts, nibbles, or shakes one of its hind paws is recorded, which is known as the response latency. To avoid tissue damage, animals were not allowed to remain on the hot plate longer than 30 s. The individual experiments were scored as the percent of maximal possible effect (%MPE) calculated using the following equation: %MPE = [(postdrug latency − predrug latency)/(cutoff predrug latency)] × 100. The dose−response curves and ED_{50} values were generated using GraphPad Prism.

Acetic Acid Writhing Model. This protocol is a well-established model of analgesia.[40−42] Rats are administered drug iv and then rested for 20 min before ip injection with 2 mL/kg of 3% acetic acid. Animals were then placed in a 10 in. by 10 in. chamber and allowed to rest an additional 10 min. For the next 20 min, rats are observed every 20 s. At each point individual rats are scored on whether they are writhing or not. Writhing is defined as a constriction of the abdominal area, often with extension of the hind legs. At the end of the observation period the percent of the time the animal was observed to be writhing was calculated. The dose−response curves and ED_{50} values were generated using GraphPad Prism.

Tail Flick Model. This model evaluates phasic pain, involving less surface area stimulation than the hot plate model.[43] This protocol was performed by placing rats in individual Plexiglas containment and immersing the distal 3 cm of the rat's tail in a hot (49 ± 1 °C) water bath. The elapsed time was measured from insertion in the water to tail withdrawal. The cutoff latency for tail withdrawal was 10 s, at which time the animal's tail was removed from the water bath. Assays were scored as percent MPEs over time as discussed above (hot plate model). The dose−response curves and ED_{50} values were generated using GraphPad Prism.

Formalin Model. The formalin model was performed according to the methods of Dubuisson and Dennis.[44] As opposed to the procedures described above, this evaluates behavioral changes rather than changes in response latency and is considered a model of chronic pain. The rats were administered either control or test compound. Thirty minutes later, 50 μL of a 5% formalin solution in sterile saline was injected intradermally into the right forepaw. The animal was placed in a clear Plexiglas cage, and the animal's posture was graded at 1 min intervals on a scale from 0 to 3 (0 = normal posture, 1 = paw on ground but not supporting rat, 2 = paw raised, and 3 = paw being licked, nibbled, or shaken) for a period of 30 min (60 min after drug application). An average pain response was calculated for a minimum of six animals and plotted over time. The dose−response curves and ED_{50} values were generated using GraphPad Prism.

Chung Model. The Chung model for neuropathic pain was performed in the laboratories of Tony Yaksh (University of California, San Diego). To assess tactile thresholds, rats are placed in a clear plastic, wire mesh-bottomed cage divided into individual compartments. Animals are allowed to acclimate, and then baseline thresholds are assessed prior to drug treatment. To assess the 50% mechanical threshold for paw withdrawal, von Frey hairs are applied to the plantar midhind paw, avoiding the tori (footpads). The eight von Frey hairs used are designated by log(10 × force required to bend hair, mg) and range from 0.4 to 15.1 g (numbers 3.61−5.18). Each hair is pressed perpendicularly against the paw with sufficient force to cause slight bending and held for approximately 6−8 s. A positive response is noted if the paw is sharply withdrawn. Flinching immediately upon removal of the hair is also considered a positive response. Absence of a response ("−") is cause to present the next consecutive stronger stimulus. A positive response ("+") is cause to present the next weaker stimulus. Stimuli are presented successively until either six data points are collected or the maximum or minimum stimulus is reached. If a minimum stimulus is reached and positive responses still occurred, the threshold is assigned an arbitrary minimum value of 0.25 g. If a maximum stimulus is presented and no response occurred, a maximum threshold value of 15 g is assigned. If a change in response occurs, either "−" to "+" or "+" to "−", causing a change in the direction of stimulus presentation from descending to ascending or vice versa, four additional data points are collected subsequent to the change. The resulting pattern of responses are tabulated and the 50% response threshold is computed using the formula

$$\log(\text{threshold, mg} \times 10) = X_f + kr$$

where X_f is the value of the last von Frey hair applied, k is the correction factor based on pattern of responses (from calibration table), and r is the mean distance in log units between stimuli. On the basis of observations on normal, unoperated rats and sham-operated rats, the cutoff of a 15.1 g hair is selected as the upper limit for testing.[45]

Hypothermia Assay. Hypothermia assays were performed as previously described.[46] Briefly, rats were placed in Plas-Labs plastic restraining apparatuses (250−500 g model) and rectal probes (Physitemp, RET-2) lubricated with mineral oil and inserted in the animals' rectums. Probes were connected to a microprobe thermometer (Physitemp, BAT-12) equipped with a switchbox (Physitemp, SWT-5). Rats were allowed to equilibrate in the restraining apparatus for 15 min before initial temperatures were recorded. For injections, peptides were dissolved in saline and injected into the tail vein. Subsequent temperature measurements were taken every 15 min for 4 h. The area under the curve, the resulting dose−response curve, and the EC_{50} values were generated using GraphPad Prism software.

Tolerance to the Hot Plate Model. For tolerance experiments, six rats were separated into two groups of three. Both groups were injected iv with 10 mg/kg **1** per day for 12 days consecutively. To avoid learning behaviors, each set was allowed at least 48 h between consecutive evaluations in the hot plate model as described above. Thus, any given data point represents the mean ± SEM of three rats (one set), although overall a total of six rats were analyzed for the development of tolerance.

NTR-1 and NTR-2 Binding Assays. Binding to Rat NTR-1. NTR binding assays were performed as previously described.[19] LTK cells expressing rat NTR were propagated in using standard cell culture techniques. For the assay, cells were scraped off the plates, washed, and resuspended in binding buffer (20 mM Hepes (pH 7.4), 50 mM NaCl, 1.5 mM $CaCl_2$, 0.1 mg/mL L-lysine, 1% BSA, 0.01% NaN_3) at a concentration of 2 × 10^6 cells/mL. Samples for the determination of K_D were prepared by resuspending compound in binding buffer at various concentrations. For PK analysis, animals were sacrificed by CO_2 asphyxiation at the appropriate times and blood was harvested into a prechilled heparin-coated tube by heart puncture. Tubes were then centrifuged (500*g*) to pellet cells, and the resulting plasma was immediately aliquoted and frozen at −70 °C for subsequent analysis. For generation of a standard curve, compound was resuspended and diluted in plasma isolated from untreated rats.

To perform the assay, 50 μL of sample was combined in a 1.5 mL microcentrifuge tube with 50 μL of cells (100 000 cells) and 50 μL containing 0.0375 pmol of $[^{125}I]$NT (approximately 100 000 CPM, diluted in binding buffer). The mixture was allowed to incubate for 60 min at room temperature to come to equilibrium. Following incubation, 1 mL of ice cold binding buffer was added, the cells were pelleted, and the supernatants were aspirated. Cells were washed again in 1 mL of ice cold binding buffer, and the tip of the tubes containing the pelleted cells were cut off and counted in a γ counter.

Binding to Human NTR-1 and NTR-2. These studies were performed essentially as described previously.[19] Briefly, cells transfected with either the hNTR-1 or hNTR-2 plasmids were homogenized and prepared as previously described.[47] An amount of 10−50 μg of membranes was incubated for 20 min (25 °C) in 250 μL of binding buffer (50 mM Tris-HCl, pH 7.5, containing 0.1% bovine serum albumin) with 0.4 nM $[^{125}I]$Tyr(3)-NT (2000 Ci/mmol) and various concentrations of unlabeled **1**. After incubation solutions were filtered through cellulose acetate filters and the filters rinsed twice with 3 mL of ice cold binding buffer and counted in a Packard γ-counter. Nonspecific binding was determined in the presence of 1 μM unlabeled NT and represented less than 5% of the total binding.

Calcium Release Assay. NTR-1 is a G-protein-coupled receptor that associates with Gq/11 to stimulate calcium release from the ER. This release was measured using a Calcium No Wash Plus assay kit from DiscoveRx Inc. (Fremont, CA). LTK cells expressing NTR1 were plated at 20 000 cells/well in 100 μL in a 96-well black, clear-bottom plate and incubated overnight. Media were then removed by inverting the plate, and then 100 μL/well of dye reagent was added (prepared as recommended by the manufacturer). Plates were then incubated 1 h at 37 °C before being analyzed for calcium release in response to various concentrations of compound (prepared in binding buffer) using a fluorometric imaging plate reader (FLIPR, Molecular Devices Corp.) that automatically injects various doses of compound prepared in the binding buffer used above (20 mM Hepes (pH 7.4), 50 mM NaCl, 1.5 mM $CaCl_2$, 0.1 mg/mL L-lysine, 1% BSA, 0.01% NaN_3). Maximal response was determined by treatment with 1 μM ionomycin, whereas background was determined by treatment with binding buffer alone. Responses were calculated as the percent of the maximal response after subtraction of background. EC_{50} values were determined using GraphPad Prism software.

PK Analysis by Liquid Chromatography/Mass Spectrometry (LC/MS). PK analysis by LC/MS/MS was performed by Covance Laboratories Inc. (Madison, WI). Briefly, rats were injected iv via the tail vein with 1 mg/kg **1**. Then blood samples (approximately 0.75 mL) were collected from two or three animals per time point from a jugular vein via syringe and needle and transferred into tubes containing sodium heparin anticoagulant maintained on wet ice. Samples were centrifuged, and plasma was placed into 96-well plates, quick-frozen on dry ice, and stored at −70 °C until use. Samples were then extracted and subjected to HPLC/MS analysis using Covance proprietary methods. The maximum concentration (C_{max}) in plasma and the time to reach maximum concentration (T_{max}) were obtained by visual inspection of the raw data. PK parameters calculated included half-life ($t_{1/2}$), area under the concentration−time curve from time 0 to the last time point (AUC_{0-t}), and area under the concentration−time curve from 0 to infinity ($AUC_{0-\infty}$). PK parameters were calculated by using WinNonlin Professional edition (Pharsight Corp., version 5.2).

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