Systemically and Topically Active Antinociceptive Neurotensin Compounds

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Received December 28, 2009; accepted May 17, 2010

ABSTRACT

Neurotensin is a neurotransmitter/modulator with a wide range of actions. Using a series of 10 stable analogs, we have examined neurotensin antinociception in mice. By incorporating (2S)-2-amino-3-(1*H*-4-indoyl)propanoic acid (L-neoTrp), a series of neurotensin analogs have been synthesized that are stable in serum and are systemically active in vivo. When administered in mice, they all were antinociceptive in the radiant heat tail-flick assay. Time-action curves revealed a peak effect at 30 min and a duration of action ranging from 2 to 4 h. Dose-response curves revealed that two compounds were partial agonists with

maximal responses below 75%, whereas all of the remaining compounds displayed a full response. Overall, the compounds were quite potent, with ED₅₀ values similar to those of opioids. At peak effect, the ED₅₀ values ranged from 0.91 to 9.7 mg/kg s.c. Two of the analogs were active topically. Together, these studies support the potential of neurotensin analogs as analgesics. They are active systemically and by using them topically, it may be possible to avoid problematic side effects, such as hypothermia and hypotension.

Introduction

Neurotensin has long been appreciated for a wide range of actions, the most prominent of which are hypothermia, hypotension, and antinociception (Tyler-McMahon et al., 2000a; Boules et al., 2006). These actions are mediated through neurotensin receptors (Remaury et al., 2002; Maeno et al., 2004; Dobner, 2006). The analgesic actions of neurotensin are readily dissociated from the opioids based upon their insensitivity to the highly opioid selective antagonist naloxone, ruling out an opioid mechanism. This lack of involvement of opioid systems would be advantageous clinically due to the potential of a reduced abuse liability and the utility of combining neurotensin receptor drugs with other classes of analgesic agents. Although there is general agreement that antinociceptive neurotensin analogs act through neurotensin receptors, it is still uncertain whether these effects are mediated through NTS1, NTS2, or a combination (Boules et al., 2006; Dobner, 2006; Roussy et al., 2008).

Targeting neurotensin receptors with drugs has been dif-

ficult due to the problems with centrally acting peptides and the general lack of availability of nonpeptide neurotensin agonists. The activity of neurotensin resides in the C-terminal six peptides corresponding to NT(8-13), with NT(9-13) also showing some activity in various paradigms (Kitabgi et al., 1977; Keegan et al., 1994). Despite the ability to truncate the peptide and retain activity, it is still not an effective agent systemically. To overcome the challenge inherent in developing centrally active peptides that retain activity when given systemically, many neurotensin analogs have been generated. One series of compounds using L-neoTrp (Fauq et al., 1998) has proven valuable due to both pharmacokinetic stability and blood-brain barrier penetration capability (Tyler et al., 1999; Tyler-McMahon et al., 2000b; Katz et al., 2001; Remaury et al., 2002). To understand further the antinociceptive actions of these compounds, we have now examined the activity of a series of these analogs in mice both systemically and topically.

Materials and Methods

Male Crl:CD-1 (ICR) BR mice (20–25 g) were purchased from Charles River Laboratories (Kingston, NY) and were housed in a 12:12-h light/dark cycle temperature-controlled room with food and water freely available. The neurotensin peptides were synthesized using solid-phase techniques, purified by high-performance liquid

This study was supported in part by the National Institutes of Health National Institute on Drug Abuse [Grants DA07242, DA2165, DA00220] (to G.W.P.) and an unrestricted research grant from Sarentis Therapeutics Corp. Article, publication date, and citation information can be found at <http://jpet.aspetjournals.org>. doi:10.1124/jpet.109.165282.

ABBREVIATIONS: NT, neurotensin; L-neoTrp, (2S)-2-amino-3-(1*H*-4-indoyl)propanoic acid; DMSO, dimethyl sulfoxide.

chromatography, and converted to the acetate salt (Mimotopes, Clayton, VIC, Australia). Doses are given as the weight of the acetate salt. Compounds for systemic administration were dissolved in 0.9% saline and administered subcutaneously. Topical dosing used DMSO solutions.

Antinociception. Antinociception was determined using the radiant heat tail-flick assay (Ling and Pasternak, 1983; Ling et al., 1983). In this assay, baseline latencies were determined for each animal using a focused beam of light on the ventral part of the tail (Ugo Basile, Comerio, Italy) and typically ranged between 2 and 3 s. Responses can be assessed either quantally or through graded responses (D'Amour and Smith, 1941; Le Bars et al., 2001). For the systemic studies, we used quantal assessments whereby antinociception was defined as a doubling or greater of baseline values for each subject. Maximal tail-flick latencies of 10 s were used to minimize any tissue damage. Results analyzed quantally were similar to those using graded responses with regard to both the ED₅₀ values and the maximal responses. All experimental protocols were approved by our Institutional Animal Care and Use Committee, and all procedures were in compliance with the Care and Use of Laboratory Animals.

Topical Antinociception. Topical antinociceptive assays were determined as described previously by using the radiant heat tail-flick assay (Kolesnikov et al., 2000). In this paradigm, after determining the baseline latency, approximately 3 cm of the distal tail of the mouse is immersed in a solution of NT compound in DMSO for the indicated time (1–3 min) and withdrawn, and analgesia was tested immediately over the region of the tail exposed to the drug. A local effect of the drug was confirmed by also testing a more proximal site that was not exposed to the drug. Local effects were defined by responses limited to the distal, drug-exposed region and not seen on the nonexposed proximal region. In this paradigm, DMSO alone has no effect on tail-flick latencies.

Data Analysis. When using quantal data, individual comparisons were made using the Fisher's exact test. Graded responses were compared using analysis of variance. ED₅₀ values were determined by nonlinear regression analysis (Prism; GraphPad Software Inc., La Jolla, CA).

Results

Systemic Antinociception. Neurotensin itself is not active systemically, presumably due to a combination of its rapid degradation and difficulty traversing the blood-brain barrier. Incorporation of L-neoTrp into a series of neurotensin analogs led to compounds (Table 1) that were exceedingly stable in plasma *in vitro* for hours (E. Richelson, unpublished data). Several of these agents have been examined in a variety of behavioral assays and were found to be active (Boules et al., 2003; Fantegrossi et al., 2005; Boules et al., 2006), but they have not been examined systematically in tests of nociception. Therefore, we embarked upon studies to compare the actions of a series of these stabilized neurotensin analogs in

TABLE 1
Structures of neurotensin analogs

NT Analog	Structure
NT66	D-Lys-L-Arg-L-Pro-L-neoTrp-tertLeu-L-Leu
NT67	D-Lys-L-Arg-L-Pro-L-neoTrp-L-Ile-L-Leu
NT69L	N-MeArg-L-Lys-L-Pro-L-neoTrp-tertLeu-L-Leu
NT71	N-MeArg-DAB-L-Pro-L-neoTrp-tertLeu-L-Leu
NT72	D-Lys-L-Pro-L-neoTrp-tertLeu-L-Leu
NT73	D-Lys-L-Pro-L-neoTrp-L-Ile-L-Leu
NT74	DAB-L-Pro-L-neoTrp-tertLeu-L-Leu
NT76	L-Arg-D-Orn-L-neoTrp-L-Ile-L-Leu
NT77	L-Arg-D-Orn-L-Pro-L-neoTrp-tertLeu-L-Leu
NT78	N-MeArg-D-Orn-L-Pro-L-neoTrp-tertLeu-L-Leu

Fig. 1. Time course of analgesia with NT analogs. A–C, the indicated NT compounds were administered subcutaneously, and analgesia was assessed in the radiant heat tail-flick assay as described under *Materials and Methods*. All analogs were administered at 5 mg/kg, with the exception of NT71 (10 mg/kg) and NT72, NT76 and NT77 (1.5 mg/kg). Results are the mean of two independent replications ($n = 10$ for each). The peak effects of all the drugs were significantly increased ($p < 0.002$), as determined by the Fisher's exact test.

a thermal assay of nociception, the radiant heat tail-flick assay.

First, by use of a fixed dose, we defined the peak effect through time-action curves (Fig. 1). Peak effects were observed at 30 min. Because equianalgesic doses were not used, comparison of actual durations among the drugs must be done cautiously. The duration of action of the drugs was similar to that seen with traditional opioids such as morphine. Most of the NT analogs were active for 2 h. Although the antinociceptive response of NT72 persisted longer, it also had a higher peak effect, which might have led to its slightly longer duration of action.

Dose-response studies confirmed the activity of all the drugs in the radiant heat tail-flick assay after subcutaneous administration (Fig. 2). However, the drug potencies varied by 10-fold among the NT analogs (Table 2). NT69L, NT72, and NT78 were all equipotent, with ED₅₀ values under 1

Fig. 2. A–C, analgesic dose-response curves for NT analogs in mice. All NT compounds were administered subcutaneously at the indicated doses. Results are the means of three independent replications ($n = 10$ for each) and are displayed as the means $\pm$ S.E.M., with the exception of NT66, which was replicated twice. Error bars not visible for the others were smaller than the symbol. Antinociception was assessed at peak effect at 30 min.

TABLE 2

Analgesic activity of NT analogs in mice

ED₅₀ values were determined by nonlinear regression of at least three independent replications of the dose-response curves and are presented with 95% confidence limits (CL). Analysis yielded a maximal response of 100% with all drugs except NT69L (74%) and NT78 (55%).

NT Analog	ED ₅₀	95% CL
	mg/kg s.c.	
NT66	2.5	1.5, 4.3
NT67	1.6	1.0, 2.5
NT69L	0.96	0.16, 5.6
NT71	3.5	2.3, 5.3
NT72	0.94	0.66, 1.4
NT73	9.7	6.4, 14.7
NT74	3.4	2.7, 4.3
NT76	1.9	1.2, 2.9
NT77	2.8	1.8, 4.2
NT78	0.91	0.56, 1.5

mg/kg s.c. NT73 was the least active, with an ED₅₀ value of 9.7 mg/kg. With the exception of NT69L and NT78, all other NT analogs elicited a full response, as shown by their curves and confirmed by nonlinear regression analysis. Both NT78 and NT69L seemed to be partial agonists, with their dose-response curves revealing maximal antinociceptive responses of approximately 55% for NT78 and 74% for NT69L. It is interesting to note that NT78 was among the most potent of the drug analogs despite its partial agonist activity.

Topical Antinociception. Neurotensin and its analogs have several actions other than analgesia, including hypotension and hypothermia, which might interfere with their utility as systemic analgesics. By limiting the site of action locally in the periphery, nontherapeutic centrally mediated side effects may be avoided, an approach previously demonstrated by the topical analgesia seen with opioids (Kolesnikov and Pasternak, 1999a; Kolesnikov et al., 2000). Therefore, we examined the topical activity of several of the NT compounds in the mouse.

The 5-mer NT72 was one of the most potent analogs when given systemically; therefore, our studies focused on this compound. As stated above, the technique involved immersion of the distal portion of the tail into a DMSO solution containing the specific compound immediately before testing the drug-exposed portion of the tail in the radiant heat tail-flick assay. This approach has worked very well for a range of opioids. To ensure that the activity of the drug was due to a local effect, an additional control included testing a more proximal portion of the tail that had not been exposed to the drug in the same animal. If the activity were due to systemic absorption of the compound, the proximal region should show the same effects as the distal region.

Immersion of the tail into DMSO solutions of NT72 at either 2 or 5 mg/ml elicited a reproducible antinociceptive response. The response was progressively greater with a longer duration of immersion in the drug solution, although it showed evidence of plateauing between 2 and 3 min (Fig. 3). In this study, there was no statistical difference for NT72 between the doses, implying that we were at maximal response. In control studies, DMSO solutions alone had no effect on tail-flick latencies in this assay, establishing the specificity of

Fig. 3. Exposure time course for topical NT72. The distal portion of the tails of mice was exposed to NT72 at either 2 or 5 mg/ml in DMSO for the indicated time. The tails were then immediately withdrawn and tested using the radiant heat tail-flick assay, with the light beam focused upon the region of the tail exposed to the drug. Results are the means $\pm$ S.E.M. of replicates. Error bars not visible were smaller than the symbol. Two-way analysis of variance of the results were highly significant ($p < 0.001$), with the differences restricted across time. No significant differences were seen between the two concentrations of drug.

the response and confirming prior observations on the inactivity of the vehicle alone (Kolesnikov and Pasternak, 1999a; Kolesnikov et al., 2000). In this model, immersion of the tail into opioid solutions for periods longer than 3 min led to some systemic absorption. Thus, we limited our exposures to 3 min to avoid this absorption.

When we extended the study to include several NT analogs, we were surprised to see that only NT71 and NT72 displayed major activity topically (Fig. 4). When examined quantally, both drugs elicited a response of 50% or greater. None of the other drugs achieved a doubling of the baseline value. More subtle effects can be assessed using graded responses from the same data set. With both compounds, the latency of the distal tail exposed to both NT71 and NT72 was significantly prolonged ($p < 0.001$) compared with baseline. The proximal region of the tail unexposed to drug did not show a significant prolongation. NT76 also revealed a statistically significant increase in latency distally compared with baseline ($p < 0.05$), but this elevation was quite modest and of questionable relevance in a clinical situation. In contrast, neither NT67 nor NT69L displayed any topical antinociceptive activity in the distal, exposed tail compared with baseline, although there was a small increase with NT67 compared with the proximal tail value.

Discussion

Pain remains a major issue in a vast array of medical conditions. Although many effective drugs are available, they

all have limitations. The availability of a new class of drug would have many advantages. Neurotensin has long been known to have many actions, including analgesia (Clineschmidt and McGuffin, 1977; Nemeroff et al., 1979; Boules et al., 2006; Dobner, 2006). Clearly, these early reports documented an antinociceptive response. Distinguishing between analgesic and anesthetic actions can often be difficult in animal models. However, there is evidence supporting an analgesic response. The distribution of NT receptors within the central nervous system includes regions known to be important in pain modulation, including the dorsal horn of the spinal cord and the periaqueductal gray, with a pathway to the nucleus raphe magnus (Dobner, 2006). Microinjection of NT into the rostroventral medulla elicited a strong analgesic response (Fang et al., 1987). Neurotensin also lowers body temperature, which can influence results in many of the nociceptive assays. However, there is evidence suggesting a dissociation of hypothermia and analgesia in two different rat strains, implying distinct mechanisms (Bauco and Rompré, 2003). Regardless, these analogs are clearly antinociceptive.

Neurotensin itself has variable activity systemically, presumably due to its rapid degradation and its difficulty traversing the blood-brain barrier. NT69L has been demonstrated previously to have analgesic actions in rodents and in primates (Boules et al., 2003; Fantegrossi et al., 2005). The current study extends these observations to a series of stabilized neurotensin derivatives in the radiant heat tail-flick assay.

All of the compounds were analgesic with similar onsets and durations of action, with the exception of NT72 analgesia that persisted slightly longer than the others. The peak effect after subcutaneous administration was 30 min, a time point used in all the dose-response studies. Although they all were active, their potencies varied by independent factors. Evidence to date suggests that the analogs are stable in plasma but that does not address issues with metabolism and other pharmacokinetic parameters. Many peptides have difficulty traversing the blood-brain barrier, and it is not clear whether this is important in the current study. In mice, NT78 and NT69L both appear to have ceiling effects, implying that they are partial agonists in this assay.

Neurotensin has several distinct central nervous system actions that might be undesirable in an analgesic, such as hypotension and hypothermia. Ultimately, it may be possible to avoid these actions through the design of receptor subtype-selective agents. However, currently these compounds are not available. Alternatively, selectivity of action can be achieved by limiting the site of action of a drug, as seen with topical approaches. Previous studies in our laboratory have documented the ability of topical μ -, δ -, and κ -opioids to elicit analgesia locally (Kolesnikov et al., 1992, 1996a,b; Kolesnikov and Pasternak, 1999a,b,c; King et al., 2001). Likewise, two NT analogs, NT71 and NT72, were active topically. The activity was dependent upon the time of exposure, and under the conditions used, there was no evidence of significant systemic absorption because the activity of the drugs was limited to the distal portion of the tail. Why this topical activity was limited to these two agents is not clear. This might reflect inherent receptor mechanistic differences among the compounds that are only evident in the peripheral nerves or they may be due to physicochemical properties of

Fig. 4. Specificity of topical NT analog analgesia. Groups of mice ($n = 7-10$) had their tails immersed in the indicated NT analog solution at 5 mg/ml in DMSO for 3 min. The tails were immediately withdrawn and tested distally in the region exposed to the drug and proximally over a region of the tail that was not exposed to the drug. Results were analyzed both quantally (A) or by using graded responses (B). Quantal responses were analyzed using Fisher's exact test. Graded responses are the means $\pm$ S.E.M. and were analyzed using analysis of variance. None of the values proximally on the tail in a region not exposed to drug were statistically different from baseline values. The distal values for NT71 and NT72 were highly statistically different from baseline ($p < 0.001$), whereas NT76 was also increased, although to a lesser extent ($p < 0.05$).

the drugs that influence their ability penetrate the skin, which is necessary to show activity in this assay.

There also was no simple correlation between systemic potency and topical activity. NT72 and NT71 showed equivalent responses topically, whereas NT71 was significantly less active systemically. Although the inactivity of topical NT69L might be due to the limited efficacy of a partial agonist, this would not explain the inactivity of NT67 and the limited actions of NT76, which were both fully efficacious and more potent systemically than NT71. Together, these observations raise interesting questions regarding the topical responses, perhaps relating to the penetration of the peptides into the skin or other pharmacokinetic issues by using topical approaches.

Neurotensin offers a new approach in the management of pain that has the potential of expanding our ability to treat many of the intractable pain problems currently seen in clinical medicine. The development of stable neurotensin analogs capable of traversing the blood-brain barrier with systemic activity may therefore prove valuable. The ability to use a topical approach to limit side effects further enhances the potential utility of these agents, as does their ability to synergize with other agents such as morphine (J. E. Matulonis, D. Barbut, and G.W. Pasternak, unpublished observations).

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