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Research Letter

Superior Analgesic Effect of H-Dmt-D-Arg-Phe-Lys-NH_2 ([Dmt^1]DALDA), a Multifunctional Opioid Peptide, Compared to Morphine in a Rat Model of Neuropathic Pain

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H-Dmt-D-Arg-Phe-Lys-NH_2 ([Dmt^1]DALDA) is a synthetic tetrapeptide with extraordinary selectivity for the mu-opioid receptor and is an extremely potent analgesic. [Dmt^1]DALDA is unusual in the way that the greater part of its analgesic potency appears to be produced by its actions in the spinal cord. Furthermore, [Dmt^1]DALDA inhibits norepinephrine re-uptake and is a mitochondria-targeted antioxidant. Such characteristics may make [Dmt^1]DALDA particularly effective against neuropathic pain. The present study was designed to compare the effects of [Dmt^1]DALDA and morphine on thermal hyperalgesia in an experimental neuropathic pain model. Neuropathic pain was induced in rats by surgical ligation of the L5 spinal nerve, and thermal pain thresholds were assessed by latencies of paw withdrawal to radiant heat. The increase in paw withdrawal latency was greater after the administration of [Dmt^1]DALDA than that of morphine in neuropathic rats at doses that were equianalgesic in naïve animals. We conclude that [Dmt^1]DALDA is more effective than morphine against thermal hyperalgesia in this experimental model of neuropathic pain.

Key words: [Dmt^1]DALDA, hyperalgesia, morphine, neuropathic pain, opioid

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H-Dmt-D-Arg-Phe-Lys-NH_2 ([Dmt^1]DALDA; Dmt = 2′,6′-dimethyltyrosine) is a synthetic tetrapeptide with extraordinary selectivity for the mu-opioid receptor. Compared to the prototypical mu-opioid, morphine, the affinity of [Dmt^1]DALDA for the mu-opioid receptor is sevenfold greater and its potency in the *in vitro* functional assay (GPI) is 20-fold greater (1). In addition, [Dmt^1]DALDA has some very interesting characteristics. As an analgesic, it is very potent especially after spinal administration with a potency 3000 times that of spinal morphine in an acute pain assay, which cannot be explained by its affinity and potency at the mu receptor (2). Furthermore, even after systemic administration [Dmt^1]DALDA appears to act predominantly in the spinal cord to produce analgesia, while morphine acts both in the brain and in the spinal cord (3). [Dmt^1]DALDA inhibits norepinephrine re-uptake (2), and it is also a mitochondria-targeted antioxidant (4).

With such characteristics, [Dmt^1]DALDA may be superior as an opioid analgesic especially in neuropathic pain states. Firstly, the propensity of [Dmt^1]DALDA to act in the spinal cord (3) may be advantageous because the spinal cord is a major site in the mechanisms of neuropathic pain (5). Secondly, norepinephrine reuptake inhibitors have been shown to be effective in neuropathic pain (6). Thirdly, mitochondrial reactive oxygen species (ROS) in spinal cord dorsal horn neurons have been suggested to play a role in the mechanisms of neuropathic pain, and antioxidants are effective against allodynia/hyperalgesia of neuropathic pain (7–9). Thus, the effect of [Dmt^1]DALDA as a mitochondrial antioxidant may also add to its effect in neuropathic pain.

This study was designed to compare the effects of systemic [Dmt^1]DALDA and morphine on thermal hyperalgesia of experimental neuropathic pain in rats. Neuropathic pain in the hind limb was produced by tight ligation of the right L5 spinal nerve (10), and thermal pain threshold was evaluated by the Hargreaves (11) paw withdrawal test.

Methods and Materials

Experiments adhered to the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the United States

National Institutes of Health and were approved by the Institutional Animal Use Committee of Chiba University Graduate School of Medicine.

Animals

Male SD rats, 7 weeks old at the time of drug testing, were used in the experiments. Rats were caged in groups of three prior to surgery and individually after surgery with free access to food and water and were maintained on a regular light-dark cycle.

Paw withdrawal test

Thermal pain threshold of the hind paw was assessed by measuring paw withdrawal latencies (PWLs) (seconds) to radiant heat stimulation (Analgesiometer; IITC, Woodland Hills, CA, USA). Rats were placed in a clear plastic testing chamber on a glass floor, and radiant heat was applied to the hind paws from underneath. The intensity of the radiant heat was adjusted so that the PWL of the hind paws of normal rats fell in the range of 10 ± 2 seconds. Cutoff latency was set at 20 seconds. Paw withdrawal latencies of each paw was determined as a mean of three measurements per paw.

Neuropathic pain model

Nerve injury was produced by a surgical ligation of the L5 spinal nerve described by Kim and Chung (10). Each rat was anesthetized with sevoflurane (2–3%) and was placed in a prone position. Right paraspinal muscles were separated from spinous processes at the L5–L6 level and retracted. The right L6 transverse process was carefully removed with small forceps to visualize the L4 and L5 spinal nerves. The L5 spinal nerve was tightly ligated with an 8-0 silk ligature proximal to the confluence of the spinal nerves and distal to the dorsal root ganglion. Paw withdrawal latencies were determined prior to and at 7 days after ligation procedure to observe the development of thermal hyperalgesia. In control animals, the same procedure up to the visualization of the spinal nerves was performed, but the right L5 nerve was not ligated.

Drug testing and data analysis

[Dmt[1]]DALDA was synthesized by methods described elsewhere (1). Morphine hydrochloride was obtained from Takeda Pharmaceuticals, Osaka, Japan. Drugs were dissolved in saline and were delivered subcutaneously (s.c.) at a volume of 0.1 mL per 100 g rat weight. First, dose–response studies were performed in naïve rats to determine equianalgesic doses of morphine and [Dmt[1]]DALDA in the paw withdrawal test. Paw withdrawal latencies were determined prior to (baseline latency) and 30 or 120 min after the administration of morphine or [Dmt[1]]DALDA, respectively (response latency). The timing of testing was at peak effects of the compounds determined in previous studies (3). Maximum possible effect (%MPE) was calculated by the following equation.

$$\%\text{MPE} = \frac{(\text{response latency} - \text{baseline latency})}{(\text{cut-off latency} - \text{baseline latency})} \times 100$$

A value of 20 (seconds) was used for the cutoff latency value. Dose–response curves were constructed using the GraphPad Prism computer software (version 5) (GraphPad Software, Inc., La Jolla, CA, USA). Drug testing was performed in neuropathic and control rats 7 days after surgery. ED_{30} and ED_{90} doses determined from dose–response curves of morphine and [Dmt[1]]DALDA were tested. Paw withdrawal latencies of the right hind paws were determined prior to and 30 or 120 min after the administration of morphine or [Dmt[1]]DALDA, respectively. Normal saline was given as vehicle control and tested 30 min after administration. Data were analyzed using the two-way analysis of variance for repeated measures followed by the Student–Newman–Keuls' test. A p value <0.05 was considered significant.

Results and Discussions

Morphine (2, 3, 4.5, 6 mg/kg) and [Dmt[1]]DALDA (0.3, 0.5, 0.7 mg/kg) showed dose-dependent analgesic effects in the paw withdrawal test in naïve rats (Figure 1). ED_{30} and ED_{90} doses determined from the dose–response curves were 2.5 and 5.4 mg/kg for morphine, and 0.37 and 0.71 mg/kg for [Dmt[1]]DALDA, respectively.

Spinal nerve ligation resulted in a significant reduction in PWL from 10.10 ± 0.17 to 8.01 ± 0.17 (mean ± SEM) seconds in the right hind paw at day 7 postligation, demonstrating the development of thermal hyperalgesia (Figure 2). No significant change in PWL was observed in the right hind paw of control rats.

Drug testing in control rats showed similar increases in PWL produced by morphine at 2.5 mg/kg and [Dmt[1]]DALDA at 0.37 mg/kg (Figure 2A), and by morphine at 5.4 mg/kg and [Dmt[1]]DALDA at 0.71 mg/kg (Figure 2B), confirming that the ED_{30} as well as ED_{90} doses of morphine and [Dmt[1]]DALDA were equianalgesic in the paw withdrawal test. In the hyperalgesic paw of the neuropathic rat, morphine and [Dmt[1]]DALDA both increased PWL, but the

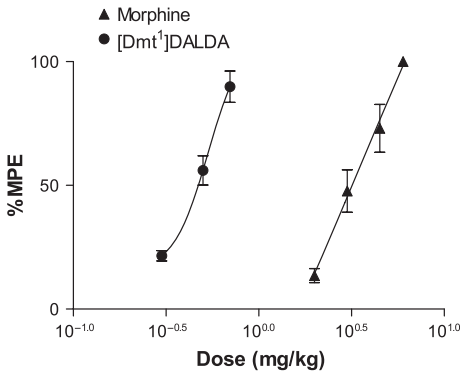

Figure 1: Dose–response curves of the effects of morphine and [Dmt[1]]DALDA in the paw withdrawal test of the rat. Radiant heat was applied to the right hind paws of naïve rats, and paw withdrawal latencies were measured. Paw withdrawal latency of each paw was determined as a mean of three measurements per paw. Paw withdrawal latencies were determined prior to (baseline latencies) and 30 or 120 min after subcutaneous administration of morphine or [Dmt[1]]DALDA, respectively (response latencies). The number of animals tested for each dose was 6. Data are shown as maximum possible effect (%MPE) (see text).

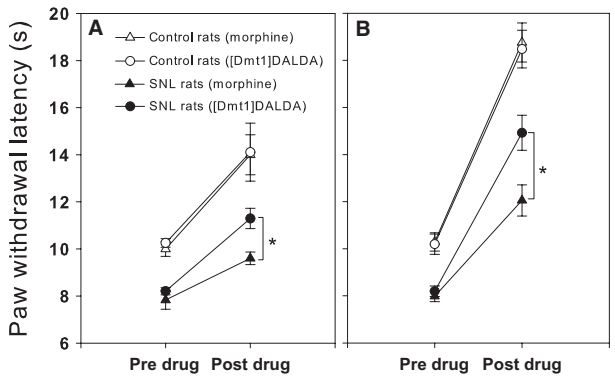

Figure 2: The effects of morphine and [Dmt[1]]DALDA at (A) ED_{30} and (B) ED_{90} doses on paw withdrawal latencies (PWLs) in spinal nerve ligated (SNL) and control rats. Radiant heat was applied to the right hind paw, and PWLs were measured. Paw withdrawal latency of each paw was determined as a mean of three measurements per paw. Paw withdrawal latencies were determined prior to (predrug) and 30 or 120 min after subcutaneous administration of morphine or [Dmt[1]]DALDA, respectively (postdrug). The number of animals in each group was 8–9. *Significantly different between treatment groups ($p < 0.05$).

increase was significantly greater in rats that were given [Dmt[1]]DALDA compared to those given equianalgesic doses of morphine (Figure 2A,B). [Dmt[1]]DALDA was just as effective in neuropathic rats as in control rats, whereas morphine was less effective. The administration of saline control had no effects in both control and neuropathic rats (data not shown).

In the present study, we showed that [Dmt[1]]DALDA was more effective than morphine in increasing thermal pain threshold in thermal hyperalgesia of neuropathic pain produced by spinal nerve ligation in rats. Studies using Chinese hamster ovary (CHO) cells expressing the human mu-opioid receptor (hMOR) (12) or rat brain membranes for the [35S]GTPgammaS assay (13) showed that morphine has an intrinsic efficacy (*e*) of 86% and 85%, respectively, relative to [D-Ala2, N-MePhe4, Gly-ol]-enkephalin (DAMGO) (*e* = 100%). Further two studies have shown that [Dmt[1]]DALDA also has slightly reduced efficacy at the MOR as compared to DAMGO (*e* = 100%): a study using calf striatal- or whole mouse brain-membranes (*e* = 75–85%) (14), and a study using CHO expressing hMOR (*e* = 91%) (15). Taken together, these results indicate that [Dmt[1]]DALDA and morphine have comparable efficacies (75–91%, which are slightly reduced as compared to DAMGO (*e* = 100%). Thus, the superior effectiveness shown in this study cannot be explained by the difference in efficacies between the two opioids.

Neuropathic pain involves multiple spinal mechanisms (5), and [Dmt[1]]DALDA is a potent mu-receptor selective opioid peptide with several unusual properties that might make it superior to morphine for neuropathic pain. [Dmt[1]]DALDA is especially potent after spinal administration, with potency 3000 times that of spinal morphine, while its potency is only 30 times that of morphine after systemic administration in acute pain testing (2). This finding suggests that there are specific spinal analgesic mechanisms that may be unique to [Dmt[1]]DALDA that produces its potent spinal analgesic effects. Furthermore, the observation in our prior study that systemic administration of [Dmt[1]]DALDA was more potent in the tail flick test than the hot plate test, while morphine was equally effective in the two tests also supports the theory that [Dmt[1]]DALDA has a propensity to act in the spinal cord (3). The specific spinal mechanism involved in the potent action of [Dmt[1]]DALDA, at least in part, may likely be its ability to inhibit norepinephrine reuptake. [Dmt[1]]DALDA has a norepinephrine reuptake–inhibiting potency that is more than 100-fold higher than morphine (2). A synergistic interaction between the mu-opioid receptor and norepinephrine receptor in the spinal cord has long been recognized (16,17), and [Dmt[1]]DALDA's potent spinal analgesic effect may be produced by such interaction. Furthermore, it has been shown that norepinephrine reuptake inhibitors by themselves are effective in neuropathic pain (6).

In addition, ROS are involved in the development and maintenance of neuropathic pain, and large doses of free radical scavengers have been shown to reduce neuropathic pain (7). Recent studies suggest that mitochondria are the primary source of ROS in spinal cord neurons. Mitochondrial superoxide was increased 60–100% in dorsal horn neurons of rats following L5 spinal nerve ligation (8). A subsequent study showed that intrathecal injection of inhibitors of the mitochondrial electron transport complexes produced increase in mitochondrial superoxide in the dorsal horn and long-lasting mechanical hyperalgesia (9). Although free radical scavengers have been shown to reduce spinal cord ROS and neuropathic pain, extremely high doses are required (9). This is probably due to the poor delivery of most antioxidants to mitochondria. [Dmt[1]]DALDA selectively targets and concentrates ∼1000-fold on the inner mitochondrial membrane and is therefore extremely potent in reducing mitochondrial oxidative stress (4,18). As a result, the effective dose of [Dmt[1]]DALDA (∼0.7 mg/kg) is 100- to 300-fold less than the dose of 4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl (TEMPOL) used in other neuropathic pain models (9,19).

In summary, [Dmt[1]]DALDA was more effective than morphine in increasing thermal pain threshold in thermal hyperalgesia of experimental neuropathic pain in the rat. The superior effect may be due to the multiple functions of [Dmt[1]]DALDA that likely enhance its opioid action in neuropathic pain states.

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Conflict of Interest Disclosure

The peptide described in this article is licensed for commercial research and development to Stealth Peptides Inc, a clinical stage biopharmaceutical company, in which Hazel H. Szeto and Peter W. Schiller have financial interests.

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