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Title

A Tyr-W-MIF-1 Analogue Containing D-Pro[2] Acts as a Selective μ_2-Opioid Receptor Antagonist in the Mouse

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ACSF	artificial cerebrospinal fluid
ANOVA	analysis of variance
CTOP	D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH$_2$
DAMGO	[D-Ala^2,NMePhe^4,Gly(ol)^5]enkephalin
D-Pro^2-endomorphin-1	Tyr-D-Pro-Trp-Phe-NH$_2$
D-Pro^2-endomorphin-2	Tyr-D-Pro-Phe-Phe-NH$_2$
D-Pro^2-Tyr-W-MIF-1	Tyr-D-Pro-Trp-Gly-NH$_2$
Endomorphin-1	Tyr-Pro-Trp-Phe-NH$_2$
Endomorphin-2	Tyr-Pro-Phe-Phe-NH$_2$
i.t.	intrathecal/intrathecally
MIF-1	melanocyte-stimulating hormone-release inhibiting factor-1
95% CI	95% confidence interval
% MPE	percent of maximum possible effect
Tyr-W-MIF-1	Tyr-Pro-Trp-Gly-NH$_2$

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Abstract

The antagonistic properties of Tyr-D-Pro-Trp-Gly-NH$_2$ (D-Pro^2-Tyr-W-MIF-1), a Tyr-Pro-Trp-Gly-NH$_2$ (Tyr-W-MIF-1) analogue, on the antinociception induced by the μ-opioid receptor agonists Tyr-W-MIF-1, [D-Ala^2,NMePhe^4,Gly(ol)^5]enkephalin (DAMGO), Tyr-Pro-Trp-Phe-NH$_2$ (endomorphin-1), and Tyr-Pro-Phe-Phe-NH$_2$ (endomorphin-2) was studied in the mouse paw-withdrawal test. D-Pro^2-Tyr-W-MIF-1 injected intrathecally (i.t.) had no apparent effect on the thermal nociceptive threshold. D-Pro^2-Tyr-W-MIF-1 (0.1-0.4 nmol) co-administered i.t. showed a dose-dependent attenuation of the antinociception induced by Tyr-W-MIF-1 without affecting endomorphin- or DAMGO-induced antinociception. However, higher doses of D-Pro^2-Tyr-W-MIF-1 (0.8-1.2 nmol) significantly attenuated endomorphin-1- or DAMGO-induced antinociception, whereas the antinociception induced by endomorphin-2 was still not affected by D-Pro^2-Tyr-W-MIF-1. Pretreatment i.t. with various doses of naloxonazine, a μ$_1$-opioid receptor antagonist, attenuated the antinociception induced by Tyr-W-MIF-1, endomorphin-1, endomorphin-2, or DAMGO. Judging from the ID$_{50}$ values for naloxonazine against the antinociception induced by the μ-opioid receptor agonists, the antinociceptive effect of Tyr-W-MIF-1 is extremely less sensitive to naloxonazine than those of endomorphin-1 or DAMGO. In contrast, endomorphin-2-induced antinociception is extremely sensitive to naloxonazine. The present results clearly suggest that D-Pro^2-Tyr-W-MIF-1 is the selective antagonist to be identified for the μ$_2$-opioid receptor in the mouse spinal cord. D-Pro^2-Tyr-W-MIF-1 may also discriminate between Tyr-W-MIF-1-induced antinociception and the antinociception induced by endomorphin-1 or DAMGO, all of which show a preference for the μ$_2$-opioid receptor in the spinal cord.

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Introduction

Tyr-Pro-Trp-Gly-NH$_2$ (Tyr-W-MIF-1) has been isolated from human the cerebral cortex

(Erchegyi et al., 1992) and bovine hypothalamus (Hackler et al., 1993), and named for its

structural similarity to the melanocyte-stimulating hormone-release inhibiting factor-1 (MIF-1)

family of brain peptides (Reed et al., 1994). Tyr-W-MIF-1 has a high affinity for μ-opioid

receptors (Erchegyi et al., 1992; Hackler et al., 1993; Erchegyi et al., 1993) and its own specific

non-opioid receptors in the brain (Zadina et al., 1990), without any appreciable affinities for δ-

and κ-opioid receptors (Zadina et al., 1994a,b). Tyr-W-MIF-1 has been reported to show a

prolonged and naloxone-reversible antinociception after both intracerebroventricular (i.c.v.) and

intrathecal (i.t.) administration (Gergen et al., 1996a,b). Tyr-W-MIF-1 also showed a potent

inhibition of the electrically-elicited contraction of the guinea pig ileum, a property of an

agonistic for μ- or κ-opioid receptors (Erchegyi et al., 1992, 1993). The Tyr-W-MIF-1-induced

inhibition of the contractions was eliminated by the selective μ-opioid receptor antagonist D-

Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH$_2$ (CTOP), but not by the selective κ-opioid receptor

antagonist nor-binaltorphimine (Erchegyi et al., 1992). This evidence clearly suggests that Tyr-

W-MIF-1 is a potent agonist for μ-opioid receptors.

The μ-opioid receptor has been divided into μ$_1$- and μ$_2$-opioid receptors based on their

sensitivity to the μ-opioid receptor antagonist naloxonazine, which irreversibly binds to μ$_1$-

opioid receptors (Hahn et al., 1982; Ling et al., 1986). In fact, the antinociception mediated by

the spinal or supraspinal μ-opioid receptors can be divided into naloxonazine (35 mg/kg, s.c.)-

sensitive (μ$_1$-opioid receptor-mediated) antinociception and naloxonazine-insensitive (μ$_2$-opioid

receptor-mediated) antinociception (Sakurada et al., 1999; Sato et al., 1999). The

antinociception induced by Tyr-W-MIF-1 was significantly attenuated by pretreatment with β-

funaltrexamine, but not by naloxonazine (Zadina et al., 1993; Gergen et al., 1996a,b), indicating

that Tyr-W-MIF-1-induced antinociception may be mediated through the spinal or supraspinal

μ_2-opioid receptors. However, the extensive characterization of μ_2-opioid receptor-mediated

antinociception has been limited because a selective antagonist for the μ_2-opioid receptor was

not available.

The antinociception induced by the endogenous μ-opioid receptor agonists Tyr-Pro-Trp-

Phe-NH_2 (endomorphin-1) and Tyr-Pro-Phe-Phe-NH_2 (endomorphin-2) is considered to be

mediated by the spinal μ_2- and μ_1-opioid receptors, respectively. This contention is supported by

the evidence that the antinociception induced by i.t. administration of endomorphin-2, but not

endomorphin-1, is suppressed by the pretreatment with the μ_1-opioid receptor antagonist

naloxonazine (Sakurada et al., 1999, 2000a). We recently found that Tyr-D-Pro-Trp-Phe-NH_2 (D-

Pro^2-endomorphin-1) and Tyr-D-Pro-Phe-Phe-NH_2 (D-Pro^2-endomorphin-2), in which the L-Pro^2

of endomorphin-1 and endomorphin-2 has been replaced with D-Pro^2, selectively attenuated the

antinociception induced by endomorphin-1 and endomorphin-2, respectively (Sakurada et al.,

2002). This evidence suggests the possibility that the synthetic peptides, which have replaced

the L-Pro^2 of their parent peptide with D-Pro^2, are antagonists against their parent peptide. Based

on the above hypothesis, in the present study, we newly synthesized D-Pro^2-Tyr-W-MIF-1 as a

possible and primary antagonist for the Tyr-W-MIF-1 binding site, probably the μ_2-opioid

receptor.

The purpose of the present study is now to characterize the antagonistic properties of D-

Pro^2-Tyr-W-MIF-1 against the spinal antinociception induced by four distinct μ-opioid receptor

agonists, Tyr-W-MIF-1, [D-Ala^2,NMePhe^4,Gly(ol)^5]enkephalin (DAMGO), endomorphin-1, and

endomorphin-2.

Methods

All experiments were approved by and conformed to the guidelines of the Committee of Animal Experiments at Tohoku Pharmaceutical University. Every effort was made to minimize the number of animals and any suffering to the animal used in the following experiments.

Animals. Male ddY mice weighing 22-25 g (SLC, Hamamatsu, Japan) were housed in a light- and temperature-controlled room (light on at 09:00 and off at 21:00; 23°C). Food and water were available *ad libitum*. Animals were used only once.

Assessment of Antinociceptive Response. The antinociceptive response was assessed with the thermal paw-withdrawal test, using an automated tail-flick unit (BM kiki, Tokyo, Japan). Mice were adapted to the testing environment for at least 1 h before any stimulation. Each animal was restrained with a soft cloth to reduce visual stimuli, and the light beam as a noxious radiant heat stimulation was applied from underneath the glass floor toward the hind paw. The light beam focused on the plantar surface of the hind paw, and the latency for the paw-withdrawal response against the noxious radiant heat stimulation was measured. The intensity of the noxious radiant heat stimulation was adjusted so that the pre-drug latency for the paw-withdrawal response was 2.5-3.5 s. The antinociceptive effect was expressed as percent of the maximum possible effect (% MPE), which was calculated with the following equation: $[(T_1-T_0)/(10-T_0)] \times 100$, where T_0 and T_1 are the pre-drug and post-drug latencies for the paw-withdrawal response, respectively. To prevent tissue damage in paw, the noxious radiant heat stimulation was terminated automatically if the mouse did not lift the paw within 10 s. The measurement of the paw-withdrawal latency was performed by only one individual who was uninformed for drug treatment for each mouse.

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Intrathecal Administration. The i.t. administration was performed according to the procedure described by Hylden and Wilcox (1980) using a 10-μl Hamilton microsyringe with a 29-gauge needle. The injection volume was 2 μl.

Drugs. Drugs used were Tyr-W-MIF-1 (Bachem, San Carlos, CA); DAMGO (Sigma, St. Louis, MO); endomorphin-1 (Tocris Cookson, Bristol, UK); endomorphin-2 (Tocris Cookson); deltorphin II (Bachem); (-)-U-50,488 hydrochloride (Tocris Cookson); D-Pro^2-Tyr-W-MIF-1 (synthesized in our laboratory); β-funaltrexamine hydrochloride (Tocris Cookson); naloxonazine dihydrochloride (RBI, Natick, MA); naltrindole hydrochloride (Tocris Cookson); and nor-binaltorphimine dihydrochloride (Tocris Cookson). All drugs were dissolved in sterile artificial cerebrospinal fluid (ACSF) containing 7.4 g of NaCl, 0.19 g of KCl, 0.19 g of MgCl$_2$, and 0.14 g of CaCl$_2$ 1000 ml^{-1}.

Statistical Analysis. The data are expressed as the mean ± S.E.M. The statistical significance of the differences between groups was assessed with a one-way analysis of variance (ANOVA) followed by either Dunnett's test or Newman-Keuls's test, or a two-way ANOVA followed by Bonferroni's test. The ED$_{50}$, ID$_{50}$, and hill slope values with their 95% confidence intervals were calculated with a computer-associated curve-fitting program (GraphPad Prism, GraphPad Software, Inc., San Diego, CA). For the statistical significance of differences between groups, the entire curves were compared using the F-test, according to the instruction provided with GraphPad Prism.

Results

The Antinociception Induced by Tyr-W-MIF-1. Groups of mice were treated with ACSF or various i.t. doses of Tyr-W-MIF-1 (2.0-16 nmol), and the antinociception was measured 5, 10, 15, 20, 30, and 45 min after the treatment. As shown in Fig. 1a and 1b, Tyr-W-MIF-1 given i.t. produced a marked dose-dependent antinociception. The antinociception induced by Tyr-W-MIF-1 developed rapidly, reached its peak at 10 min, and then gradually disappeared by 30 min after the treatment (Fig. 1a). The ED_{50} value of Tyr-W-MIF-1 for antinociception at the peak time was 5.89 (95% CI: 4.42-7.85) nmol (Fig. 1b). At the peak time, 16 nmol of Tyr-W-MIF-1 produced approximately an 80% MPE.

Effects of β-Funaltrexamine, nor-Binaltorphimine and Naltrindole on the Antinociception Induced by Tyr-W-MIF-1. Groups of mice were pretreated i.t. with the μ-opioid receptor antagonist β-funaltrexamine (4.0 nmol), the κ-opioid receptor antagonist nor-binaltorphimine (4.0 nmol) or ACSF 24 h before, or with the δ-opioid receptor antagonist naltrindole (0.033 nmol) or ACSF 5 min before the i.t. administration of Tyr-W-MIF-1 (16 nmol), and the antinociception induced by Tyr-W-MIF-1 was measured 10 min after the treatment. The antinociception induced by i.t. administration of Tyr-W-MIF-1 was almost eliminated by i.t. pretreatment with β-funaltrexamine, while i.t. pretreatment with nor-binaltorphimine or naltrindole failed to affect the Tyr-W-MIF-1-induced antinociception (Table 1). The same pretreatment with either nor-binaltorphimine or naltrindole completely attenuated the antinociception induced by i.t. administration of either the κ-opioid receptor agonist U-

50,488H or the δ-opioid receptor agonist deltorphin II, respectively (data not shown).

Effect of Naloxonazine on the Antinociception Induced by μ-Opioid Receptor

Agonists. Groups of mice were pretreated i.t. with various doses of the μ_1-opioid receptor antagonist naloxonazine (1.4-44.2 nmol) or ACSF 24 h before the i.t. administration of equipotent doses of Tyr-W-MIF-1 (16 nmol), DAMGO (20 pmol), endomorphin-1 (5.0 nmol), or endomorphin-2 (5.0 nmol). The antinociception induced by i.t. administration of Tyr-W-MIF-1, DAMGO, endomorphin-1, and endomorphin-2 were measured 10 min, 5 min, 5 min, and 5 min after the treatment, respectively, as peak effects. The pretreatment with naloxonazine attenuated the antinociception induced by these four distinct μ-opioid receptor agonists in a dose-dependent manner (Fig. 2a). However, naloxonazine at a dose of 5.5 nmol only significantly antagonized the antinociceptive effect induced by endomorphin-2, without affecting the antinociception induced by endomorphin-1, DAMGO and Tyr-W-MIF-1. Higher doses (11.1 or 22.1 nmol) of naloxonazine significantly attenuated endomorphin-1- and DAMGO-induced antinociception, but still had no effect against the antinociception induced by Tyr-W-MIF-1. The Tyr-W-MIF-1-induced antinociception was significantly, but not completely, attenuated by the pretreatment with a much higher dose of naloxonazine (44.2 nmol), which completely eliminated the antinociception induced by endomorphin-1 and DAMGO. The ID_{50} values for naloxonazine against the antinociception induced by endomorphin-2, DAMGO, endomorphin-1, and Tyr-W-MIF-1 were 4.58 (95% CI: 3.82-5.49), 13.66 (95% CI: 10.35-18.04), 15.79 (95% CI: 14.99-16.63), and 35.34 (95% CI: 33.69-37.07) nmol, respectively (Fig. 2b, Table 2). The dose-response curves for inhibition by naloxonazine against DAMGO- and endomorphin-1-induced antinociception were statistically distinct from those against endomorphin-2- and Tyr-W-MIF-1-

induced antinociception.

Effect of D-Pro^2-Tyr-W-MIF-1 on the Antinociception Induced by μ-Opioid

Receptor Agonists. Groups of mice were co-administered various i.t. doses of D-Pro^2-Tyr-W-MIF-1 (0.025-1.2 nmol) with equipotent doses of Tyr-W-MIF-1 (16 nmol), DAMGO (20 pmol), endomorphin-1, (5.0 nmol) or endomorphin-2 (5.0 nmol), and the antinociception induced by Tyr-W-MIF-1, DAMGO, endomorphin-1, and endomorphin-2 were measured 10 min, 5 min, 5 min, and 5 min after the treatment, respectively, as peak effects. D-Pro^2-Tyr-W-MIF-1 at any of the doses used did not show any antinociceptive or hyperalgesic effect by itself at 5 or 10 min after the treatment. Co-administered D-Pro^2-Tyr-W-MIF-1 dose-dependently attenuated the antinociception induced by Tyr-W-MIF-1 (Fig. 3a). The ID$_{50}$ value for D-Pro^2-Tyr-W-MIF-1 against Tyr-W-MIF-1-induced antinociception was 0.21 (95% CI: 0.15-0.28) nmol (Fig. 3b, Table 2). In contrast, co-administered D-Pro^2-Tyr-W-MIF-1 at a dose of 0.4 nmol, which almost completely attenuated Tyr-W-MIF-1-induced antinociception, did not affect the antinociception induced by DAMGO and endomorphin-1, whereas higher doses of D-Pro^2-Tyr-W-MIF-1 (0.8-1.2 nmol) significantly attenuated the antinociception induced by either DAMGO or endomorphin-1. The ID$_{50}$ values for D-Pro^2-Tyr-W-MIF-1 against DAMGO- and endomorphin-1-induced antinociception were 0.98 (95% CI: 0.82-1.18) and 0.75 (95% CI: 0.63-0.89) nmol, respectively (Fig. 3b, Table 2). The dose-response curves for inhibition by D-Pro^2-Tyr-W-MIF-1 against DAMGO- and endomorphin-1-induced antinociception were statistically distinct from that against Tyr-W-MIF-1-induced antinociception. On the other hand, the antinociception induced by endomorphin-2 was not affected by co-administration of D-Pro^2-Tyr-W-MIF-1 at any of the doses used (Fig. 3a and 3b, Table 2).

Effect of D-Pro^2-Tyr-W-MIF-1 on the Antinociception Induced by U-50,488H and Deltorphin II. Groups of mice were co-administered various i.t. doses of D-Pro^2-Tyr-W-MIF-1 (0.025-1.2 nmol) with equipotent doses of the κ-opioid receptor agonist U-50,488H (30 nmol) or δ-opioid receptor agonist deltorphin II (4 nmol), and the antinociception induced by U-50,488H and deltorphin II were measured 10 min and 5 min after the treatment, respectively. As shown in Table 3, co-administered D-Pro^2-Tyr-W-MIF-1 at any of the doses used failed to affect the antinociception induced by either U-50,488H or deltorphin II.

Discussion

Tyr-W-MIF-1 induced a dose-dependent antinociception in the paw-withdrawal test after spinal administration. The effects of Tyr-W-MIF-1-induced antinociception occurred at 5-10 min after the i.t. injection and disappeared at 30 min after the injection, whereas those of endomorphin-1, which is structurally similar to Tyr-W-MIF-1 with a Phe in position 4, occurred within 1 min after i.t. injection and were reduced at 15 min. The time-course of antinociception with Tyr-W-MIF-1 is similar to that with DAMGO (Sakurada et al., 2001).

We used a variety of i.t. doses of naloxonazine to determine the sensitivity to antagonists of the μ-opioid receptor subclasses involved in the antinociceptive responses to Tyr-W-MIF-1. There is biochemical and pharmacological evidence supporting the existence of μ-opioid receptor subclasses which are localized in the spinal and supraspinal structures involved in the modulation of nociception (Wolozin and Pasternak, 1981; Moskowitz and Goodman, 1985). At least two μ-opioid receptor subclasses have been proposed: μ_1- and μ_2-opioid receptors. β-Funaltrexamine irreversibly antagonizes both μ_1- and μ_2-opioid receptors and inhibits both supraspinal and spinal antinociception, whereas naloxonazine selectively antagonizes the μ_1-opioid receptors and inhibits supraspinal antinociception. Recent behavioral pharmacological studies suggest the presence of μ_1-opioid receptors sensitive to naloxonazine in spinal sites as assayed with the formalin, hot-plate, tail-pressure, and tail-flick tests (Sakurada et al., 1999; Sato et al., 1999; Sakurada et al., 2000b). Autoradiographic studies show that μ_1- and μ_2-opioid receptor subclasses are localized in the spinal and supraspinal structures involved in the modulation of nociception (Moskowitz et al., 1985). The difference in μ_1 and μ_2 binding could

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be due to induced differences in the receptor conformation.

It is noteworthy that both the s.c. 35 mg/kg dose and the i.t. 5.5 nmol/mouse dose of

naloxonazine are reasonable doses to selectively block μ_1-opioid receptors in mice (Ling et al.,

1986; Sakurada et al., 2000a). Recent studies have shown that the antinociceptive response to

DAMGO is not blocked by pretreatment with naloxonazine at a dose of 35 mg/kg s.c. or 5.5

nmol/mouse i.t., whereas higher doses of naloxonazine (52.5, 65.6 or 78.8 mg/kg s.c. or 11.1

nmol/mouse i.t.) significantly attenuated DAMGO-induced antinociception (Sakurada et al.,

2000a), indicating that naloxonazine at high doses loses much of its selectivity for μ_1-opioid

receptors (Sakurada et al., 2000a). The antinociception induced by i.t. administration of Tyr-W-

MIF-1 was significantly attenuated by pretreatment with β-funaltrexamine, whereas the

antinociceptive activity was not antagonized by pretreatment with a reasonable i.t. dose of

naloxonazine, i.e., 5.5 nmol/mouse. The present results with naloxonazine on Tyr-W-MIF-1-

induced antinociception are in agreement with those of Gergen et al. (1996b). This result

suggests that the antinociception with Tyr-W-MIF-1 is mediated through μ_2-opioid receptors,

since higher doses of i.t. naloxonazine attenuated the antinociception with Tyr-W-MIF-1 (Fig. 2a

and 2b). Furthermore, i.t. pretreatment with the κ-opioid receptor antagonist nor-binaltorphimine

or the δ-opioid receptor antagonist naltrindole did not attenuate the antinociception induced by

i.t. administration of Tyr-W-MIF-1 (Table 1). These results strongly support the previous reports

that the antinociception induced by i.t. administration of Tyr-W-MIF-1 was mediated by

stimulation of the μ_2-opioid receptor at the spinal cord level (Gergen et al., 1996b).

Unexpectedly, higher doses of naloxonazine (11.1 or 22.1 nmol/mouse i.t.), which suppressed

the antinociception with DAMGO, did not significantly inhibit the Tyr-W-MIF-1-induced

antinociception. Even the highest dose of naloxonazine (44.2 nmol/mouse, i.t.) did not completely antagonize the antinociception with Tyr-W-MIF-1.

Two new endogenous opioid peptides, endomorphin-1 (Tyr-Pro-Trp-Phe-NH$_2$) and endomorphin-2 (Tyr-Pro-Phe-Phe-NH$_2$) have been found to be highly selective for μ-opioid receptors (Zadina et al., 1997). Both endomorphin-1 and endomorphin-2 significantly increase nociceptive thresholds after both i.t. and i.c.v. administration, and these effects are antagonized by the μ-opioid receptor selective antagonists naloxone and β-funaltrexamine. However, more recent results indicate that different subclasses of μ-opioid receptors may be involved in the antinociceptive effects induced by endomorphin-1 and endomorphin-2. The antinociception induced by endomorphin-1 is blocked by the μ$_1$- and μ$_2$-opioid receptor antagonist β-funaltrexamine, but not by the selective μ$_1$-opioid receptor antagonist naloxonazine, whereas the antinociception induced by endomorphin-2 is blocked by both β-funaltrexamine and naloxonazine (Tseng et al., 2000; Sakurada et al., 2002).

A μ$_1$-opioid receptor antagonist, naloxonazine, was more effective in blocking the antinociceptive effects in mice induced by endomorphin-2 than by endomorphin-1 (Sakurada et al., 1999). A reasonable dose of naloxonazine, 35 mg/kg (s.c.) or 5.5 nmol/mouse (i.t.), to obtain a relative μ$_1$-opioid receptor selectivity (Ling et al., 1986), did not attenuate the antinociceptive effects induced by i.t. administration of endomorphin-1 (Sakurada et al., 2000a) or Tyr-W-MIF-1 (Gergen et al., 1996b) but did attenuate the antinociception due to endomorphin-2, suggesting that endomorphin-1 acts as a μ$_2$-opioid receptor agonist and endomorphin-2 acts as a μ$_1$-opioid receptor agonist at the spinal site. Thus, based on antagonism by naloxonazine, endomorphin-1, but not endomorphin-2, has behavioral and pharmacological similarities to Tyr-W-MIF-1.

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We have demonstrated that D-Pro^2-endomorphin-1 and D-Pro^2-endomorphin-2, analogs of the endomorphins (Sakurada et al., 2002; Hung et al., 2002), are opioid receptor antagonists which selectively block the antinociception induced by endomorphin-1 and endomorphin-2, respectively, in the spinal cord. Furthermore, D-Pro^2-endomorphin-2 attenuated the antinociception induced by i.t. administration of endomorphin-2 or the selective μ_1-opioid receptor agonist Tyr-D-Arg-Phe-β-Ala (Sakurada et al., 2000b) but not that induced by DAMGO or endomorphin-1 (Sakurada et al., 2002; Hung et al., 2002), indicating that D-Pro^2-endomorphin-2 is an antagonist which selectively blocks the antinociception induced by μ_1-opioid receptor agonists in the spinal cord. D-Pro^2-endomorphin-2 acts as a selective μ_1-opioid receptor antagonist like naloxonazine. On the other hand, D-Pro^2-endomorphin-1 attenuated the antinociception induced by i.t.-administered endomorphin-1 and DAMGO but not endomorphin-2, suggesting that D-Pro^2-endomorphin-1 may act as a selective μ_2-opioid receptor antagonist (Sakurada et al., 2002).

We found in the present study that D-Pro^2-Tyr-W-MIF-1, an analogue of Tyr-W-MIF-1 containing D-Pro in position 2, inhibited the antinociception induced by Tyr-W-MIF-1, endomorphin-1, and DAMGO, but not endomorphin-2, deltorphin II, or U-50,488H, in a dose-dependent manner. The present results clearly suggest that D-Pro^2-Tyr-W-MIF-1 is a selective antagonist for μ_2-opioid receptor. Interestingly, the antinociception of Tyr-W-MIF-1 was significantly attenuated at the doses of 0.1-0.4 nmol (Fig. 3a and 3b), doses which did not affect endomorphin-1-, endomorphin-2- or DAMGO-induced antinociception (Fig. 3a and 3b). A higher dose (0.8 nmol) of D-Pro^2-Tyr-W-MIF-1 significantly attenuated the antinociception with endomorphin-1 or DAMGO without affecting the antinociception with endomorphin-2, deltorphin II, or U-50,488H (Fig. 3a and 3b, Table 3). The finding that the antinociception

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induced by Tyr-W-MIF-1 can be antagonized by D-Pro^2-Tyr-W-MIF-1 at doses which are

inactive against endomorphin-1 and DAMGO indicates that it could be used to distinguish the

different antinociceptive mechanism within the μ_2-opioid receptor agonists. We previously

reported that D-Pro^2-endomorphin-1 shows the antagonistic property for μ_2-opioid receptor. Like

a D-Pro^2-Tyr-W-MIF-1, the i.t. co-administration of D-Pro^2-endomorphin-1 significantly

attenuated the antinociception induced by endomorphin-1, DAMGO, and Tyr-W-MIF-1

(Sakurada et al., 2002; unpublished observation). However, the antagonistic property of D-Pro^2-

endomorphin-1 against Tyr-W-MIF-1 is characteristically similar to those against endomorphin-

1 and DAMGO, suggesting that unlike D-Pro^2-Tyr-W-MIF-1, D-Pro^2-endomorphin-1 can not

discriminate the antinociception induced by these μ_2-opioid receptor agonists. The present study

is the first to show that D-Pro^2-Tyr-W-MIF-1 can also distinguish the actions of different peptidic

μ_2-opioid receptor agonists. D-Pro^2-Tyr-W-MIF-1 selectively blocked the antinociception of Tyr-

W-MIF-1 far more effectively than that of endomorphin-1 and DAMGO, while the

antinociception induced by endomorphin-2 was not reduced by co-administered with D-Pro^2-

Tyr-W-MIF-1 (Fig. 3a and 3b). The differential antagonistic sensitivity of D-Pro^2-Tyr-W-MIF-1

on inhibition of the thermal nociceptive response by μ_2-opioid receptor agonists led us to

speculate that the μ_2-opioid receptors could be subdivided into a subclass of the μ_2-opioid

receptor which is relatively insensitive to D-Pro^2-Tyr-W-MIF-1 and a subclass of the μ_2-opioid

receptor which is extremely sensitive to D-Pro^2-Tyr-W-MIF-1, whereas D-Pro^2-endomorphin-1

failed to separate the different subclasses of μ_2-opioid receptors.

D-Pro^2-Tyr-W-MIF-1 selectively blocked the antinociceptive effect of i.t. administration

of Tyr-W-MIF-1, whereas the antinociceptive effect of DAMGO or endomorphin-1, which are

insensitive to naloxonazine, was not inhibited at same dose at which the antinociception caused

by Tyr-W-MIF-1 was eliminated. These results also indicate that D-Pro^2-Tyr-W-MIF-1 may be a

useful tool to discriminate between the antinociceptive effects of μ_2-opioid receptor agonists

which act via the different subclasses of the μ_2-opioid receptor.

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Legends for figures

Fig. 1. Tyr-W-MIF-1-induced antinociception in the mouse paw-withdrawal test. (a) Time course

of antinociception induced by i.t. administration of Tyr-W-MIF-1. Groups of mice were treated

with i.t. various doses of Tyr-W-MIF-1 (2.0-16 nmol) or ACSF, and the antinociception induced

by Tyr-W-MIF-1 was measured 5, 10, 15, 20 30, and 45 min after the treatment. Each value

represents the mean ± S.E.M. for 10 mice. The statistical significance of the differences between

groups was assessed with a two-way ANOVA followed by the Bonferroni's test. The F value of

the two-way ANOVA for Tyr-W-MIF-1 (2.0, 4.0, 8.0, and 16 nmol) in comparison with ACSF

were $F_{[1,126]} = 6.932$ ($p < 0.01$), $F_{[1,126]} = 39.17$ ($p < 0.001$), $F_{[1,126]} = 68.17$ ($p < 0.001$),

and $F_{[1,126]} = 135.1$ ($p < 0.001$), respectively. $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ vs.

ACSF. (b) The antinociception induced by various i.t. doses of Tyr-W-MIF-1 injected at peak

effect time. Groups of mice were treated with various i.t. doses of Tyr-W-MIF-1 (2.0-16 nmol),

and the antinociception induced by Tyr-W-MIF-1 was measured 10 min after the treatment. Each

value represents the mean ± S.E.M. for 10 mice. The ED_{50} value with its 95% confidence

interval was calculated with the computer-assisted curve-fitting program GraphPad Prism.

Fig. 2. Effect of naloxonazine on the antinociception induced by i.t. injection of Tyr-W-MIF-1,

DAMGO, endomorphin-1 (EM-1), or endomorphin-2 (EM-2) in the mouse paw-withdrawal test.

Groups of mice were pretreated with various i.t. doses of naloxonazine (1.4-44.2 nmol) or ACSF

24 h before the i.t. administration of Tyr-W-MIF-1 (16 nmol), DAMGO (20 pmol), EM-1 (5.0

nmol), or EM-2 (5.0 nmol). The antinociception induced by i.t. administration of Tyr-W-MIF-1,

DAMGO, EM-1, and EM-2 were measured 10 min, 5 min, 5 min, and 5 min after the treatment,

respectively. Both (a) the antinociceptive effects (% MPE) of the agonists and (b) the inhibitory

effect (%) of naloxonazine against the agonists are represented the mean ± S.E.M. for 10 mice.

(a) The statistical significance of the differences between groups was assessed with a one-way

ANOVA followed by the Dunnett's test. The F values of the one-way ANOVA for Tyr-W-MIF-1,

DAMGO, EM-1, and EM-2 were $F[4,45] = 11.55$ ($p < 0.001$), $F[4,45] = 18.07$ ($p < 0.001$),

$F[4,45] = 19.97$ ($p < 0.001$), and $F[5,54] = 12.85$ ($p < 0.001$), respectively. *$p < 0.05$ and ***p

< 0.001 vs. ACSF. (b) The ID_{50} values with their 95% confidence intervals were calculated with

the computer-assisted curve-fitting program GraphPad Prism. For the statistical significance of

the differences between groups, the entire curves were compared using the F-test, according to

the instruction provided with GraphPad Prism. The F values for Tyr-W-MIF-1 against DAMGO,

EM-1 and EM-2 were 121.1 ($p < 0.001$), 1278 ($p < 0.001$) and 607.5 ($p < 0.001$), respectively.

The F values for EM-2 against DAMGO and EM-1 were 70.35 ($p < 0.001$) and 209.9 ($p <$

0.001), respectively.

Fig. 3. Effect of D-Pro^2-Tyr-W-MIF-1 on the antinociception induced by i.t. administration of

DAMGO, endomorphin-1 (EM-1), endomorphin-2 (EM-2), and Tyr-W-MIF-1 in the mouse

paw-withdrawal test. Groups of mice were co-administered various i.t. doses of D-Pro^2-Tyr-W-

MIF-1 (0.025-1.2 nmol) with Tyr-W-MIF-1 (16 nmol), DAMGO (20 pmol), EM-1 (5.0 nmol), or

EM-2 (5.0 nmol), and the antinociception induced by Tyr-W-MIF-1, DAMGO, EM-1 and EM-2

were measured 10 min, 5 min, 5 min, and 5 min after the treatment, respectively. Both (a) the

antinociceptive effects (% MPE) of the agonists and (b) the inhibitory effect (%) of D-Pro^2-Tyr-

W-MIF-1 against the agonists are represented the mean ± S.E.M. for 10 mice. (a) The statistical

significance of the differences between groups was assessed with a one-way ANOVA followed

by the Dunnett's test. The F values of the one-way ANOVA for Tyr-W-MIF-1, DAMGO, EM-1, and EM-2 were $F_{[7,72]} = 16.06$ ($p < 0.001$), $F_{[7,72]} = 9.222$ ($p < 0.001$), $F_{[7,72]} = 9.478$ ($p < 0.001$), and $F_{[7.72]} = 0.9996$ (ns), respectively. *$p < 0.05$ and ***$p < 0.001$ vs. agonist alone.

(b) The ID_{50} values with their 95% confidence intervals were calculated with the computer-assisted curve-fitting program GraphPad Prism. For the statistical significance of the differences between groups, the entire curves were compared using the F-test, according to the instruction provided with GraphPad Prism. The F values for Tyr-W-MIF-1 against DAMGO and EM-1 were 68.52 ($p < 0.001$) and 45.53 ($p < 0.001$), respectively.

TABLE 1

Effects of β-funaltrexamine, nor-binaltorphimine and naltrindole on the antinociception induced

by Tyr-W-MIF-1 in the mouse paw-withdrawal test

Treatment	% MPE
Tyr-W-MIF-1 (16 nmol)	
+ ACSF (24 h)	79.55 ± 6.17
Tyr-W-MIF-1 (16 nmol)	
+ β-funaltrexamine (4.0 nmol, 24 h)	16.84 ± 4.06***
Tyr-W-MIF-1 (16 nmol)	
+ nor-binaltorphimine (4.0 nmol, 24 h)	81.11 ± 6.81
Tyr-W-MIF-1 (16 nmol)	
+ ACSF (5 min)	81.49 ± 8.26
Tyr-W-MIF-1 (16 nmol)	
+ naltrindole (0.033 nmol, 5 min)	79.06 ± 7.37

Groups of mice were pretreated i.t. with μ-opioid receptor antagonist β-funaltrexamine (4.0

nmol), κ-opioid receptor antagonist nor-binaltorphimine (4.0 nmol) or ACSF 24 h before, or

with δ-opioid receptor antagonist naltrindole (0.033 nmol) or ACSF 5 min before the i.t.

administration of Tyr-W-MIF-1 (16 nmol), and the antinociception induced by Tyr-W-MIF-1 was

measured 10 min after the treatment. Each value represents the mean $\pm$ S.E.M. for 10 mice. The

statistical significance of the differences between groups was assessed with a one-way ANOVA

followed by the Newman-Keuls's test. The F value of the one-way ANOVA was $F[4,45] = 18.05$

$(p < 0.001)$. ***$p < 0.001$ vs. ACSF (24-h pretreatment).

TABLE 2

The inhibiting effects of naloxonazine or D-Pro^2-Tyr-W-MIF-1 on the antinociception induced

by DAMGO, endomorphin-1, endomorphin-2, and Tyr-W-MIF-1 in the mouse paw-withdrawal

test

	Naloxonazine	
Treatment	ID$_{50}$ (nmol)	Hill slope
DAMGO (20 pmol)	13.66 (10.35-18.04)	1.77 (0.90-2.64)
Endomorphin-1 (5.0 nmol)	15.79 (14.99-16.63)	1.73 (1.58-1.89)
Endomorphin-2 (5.0 nmol)	4.58 (3.82-5.49)	1.10 (0.85-1.35)
Tyr-W-MIF-1 (16 nmol)	35.34 (33.69-37.07)	2.52 (2.21-2.83)
	D-Pro^2-Tyr-W-MIF-1	
Treatment	ID$_{50}$ (nmol)	Hill slope
DAMGO (20 pmol)	0.98 (0.82-1.18)	1.64 (1.05-2.23)
Endomorphin-1 (5.0 nmol)	0.75 (0.63-0.89)	1.79 (1.17-2.40)
Endomorphin-2 (5.0 nmol)	NA	NA
Tyr-W-MIF-1 (16 nmol)	0.21 (0.15-0.28)	1.16 (0.76-1.55)

Groups of mice were pretreated various i.t. doses of naloxonazine (1.4-44.2 nmol) 24 h prior or

co-administered various i.t. doses of D-Pro^2-Tyr-W-MIF-1 (0.025-1.2 nmol) with DAMGO (20

pmol), endomoprhin-1 (5.0 nmol), endomoprhin-2 (5.0 nmol), or Tyr-W-MIF-1 (16 nmol), and

the antinociception induced by DAMGO, endomorphin-1, endomorphin-2 and Tyr-W-MIF-1

were measured 5 min, 5 min, 5 min, and 10 min after the treatment, respectively. The ID$_{50}$

values and hill slope values with their 95% confidence intervals were calculated with the

computer-assisted curve-fitting program GraphPad Prism. NA: not available.

TABLE 3

Effect of D-Pro^2-Tyr-W-MIF-1 on U-50,488H- and deltorphin II-induced antinociception in the

mouse paw-withdrawal test

Treatment	% MPE
U-50,488H (30 nmol)	83.33 ± 6.15
U-50,488H (30 nmol)	
+ D-Pro^2-Tyr-W-MIF-1 (0.025 nmol)	83.82 ± 5.05
U-50,488H (30 nmol)	
+ D-Pro^2-Tyr-W-MIF-1 (0.05 nmol)	88.59 ± 3.54
U-50,488H (30 nmol)	
+ D-Pro^2-Tyr-W-MIF-1 (0.1 nmol)	88.99 ± 4.22
U-50,488H (30 nmol)	
+ D-Pro^2-Tyr-W-MIF-1 (0.2 nmol)	86.47 ± 5.18
U-50,488H (30 nmol)	
+ D-Pro^2-Tyr-W-MIF-1 (0.4 nmol)	87.63 ± 3.76
U-50,488H (30 nmol)	
+ D-Pro^2-Tyr-W-MIF-1 (0.8 nmol)	86.79 ± 4.88
U-50,488H (30 nmol)	
+ D-Pro^2-Tyr-W-MIF-1 (1.2 nmol)	86.49 ± 4.86
Deltorphin II (4 nmol)	78.20 ± 7.57
Deltorphin II (4 nmol)	
+ D-Pro^2-Tyr-W-MIF-1 (0.025 nmol)	85.37 ± 5.82

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Deltorphin II (4 nmol)

  + D-Pro^2-Tyr-W-MIF-1 (0.05 nmol)   84.24 ± 5.87

Deltorphin II (4 nmol)

  + D-Pro^2-Tyr-W-MIF-1 (0.1 nmol)   87.54 ± 4.29

Deltorphin II (4 nmol)

  + D-Pro^2-Tyr-W-MIF-1 (0.2 nmol)   85.00 ± 5.88

Deltorphin II (4 nmol)

  + D-Pro^2-Tyr-W-MIF-1 (0.4 nmol)   88.03 ± 4.40

Deltorphin II (4 nmol)

  + D-Pro^2-Tyr-W-MIF-1 (0.8 nmol)   87.78 ± 5.50

Deltorphin II (4 nmol)

  + D-Pro^2-Tyr-W-MIF-1 (1.2 nmol)   88.07 ± 4.86

Groups of mice were co-administered various i.t. doses of D-Pro^2-Tyr-W-MIF-1 (0.025-1.2 nmol) and 30 nmol of U-50,488H or 4 nmol of deltorphin II, and the antinociception induced by U-50,488H or deltorphin II was measured 10 or 5 min after the treatment, respectively. Each value represents the mean ± S.E.M. for 10 mice. The statistical significance of the differences between groups was assessed with a one-way ANOVA followed by the Dunnett's test.
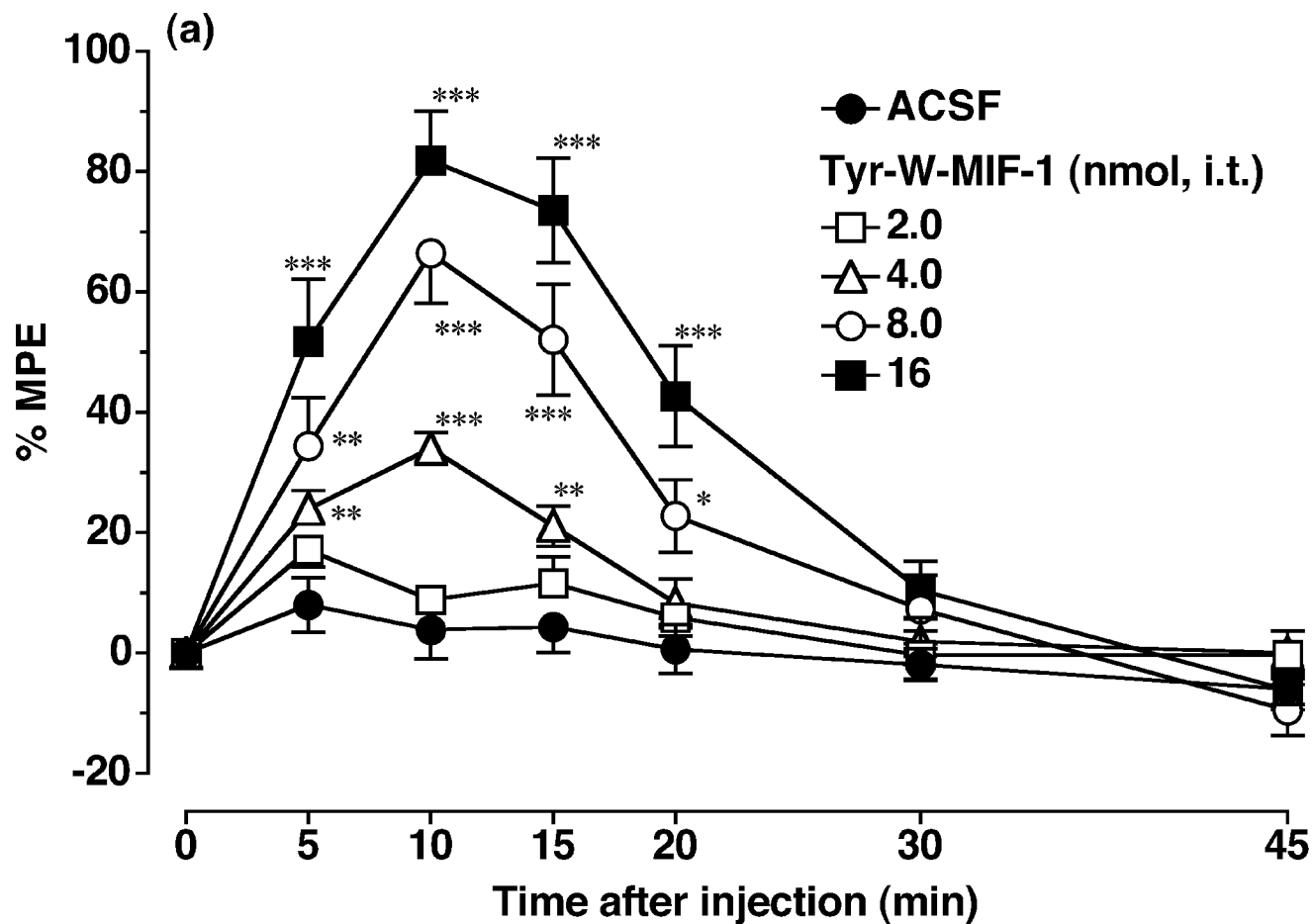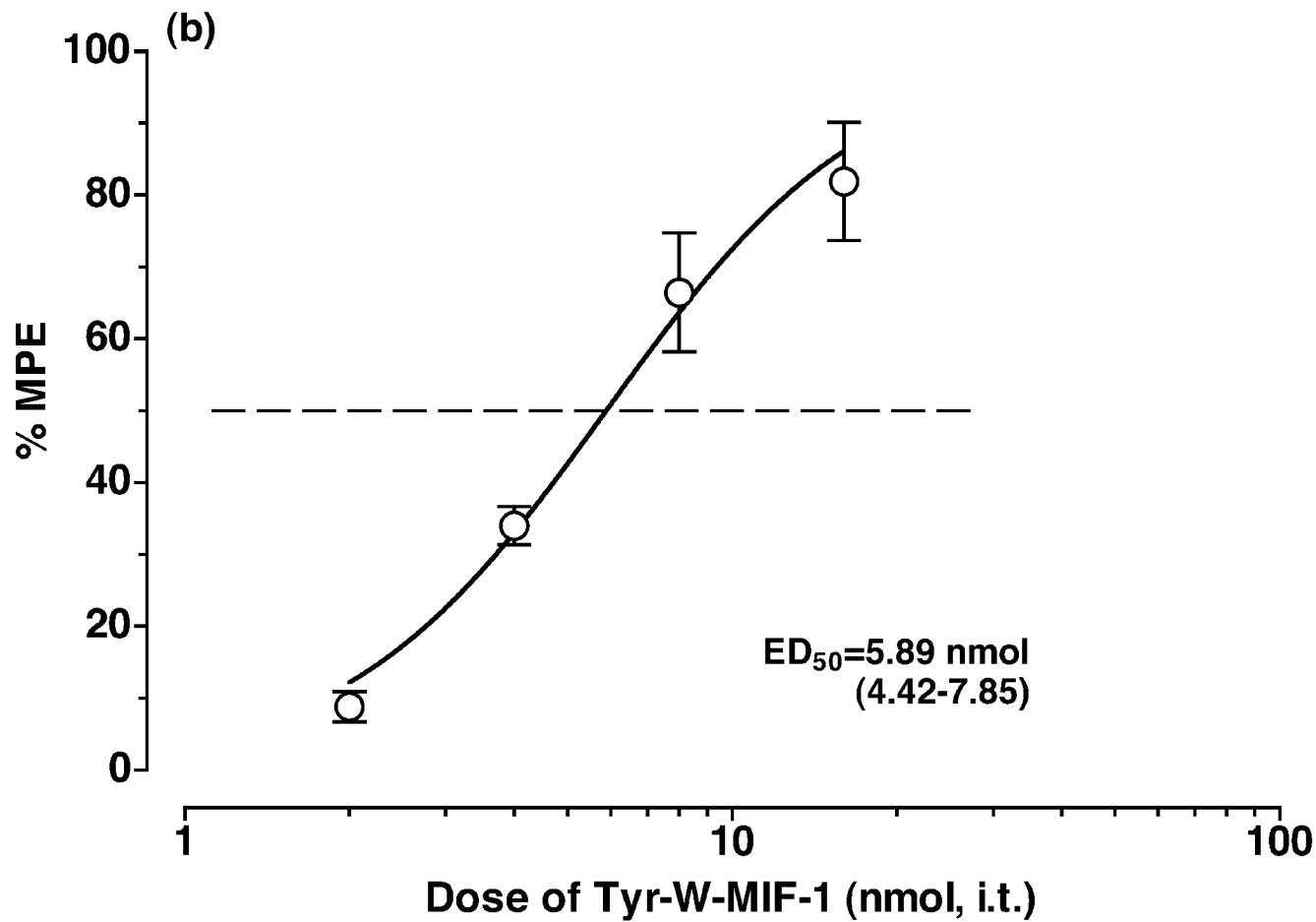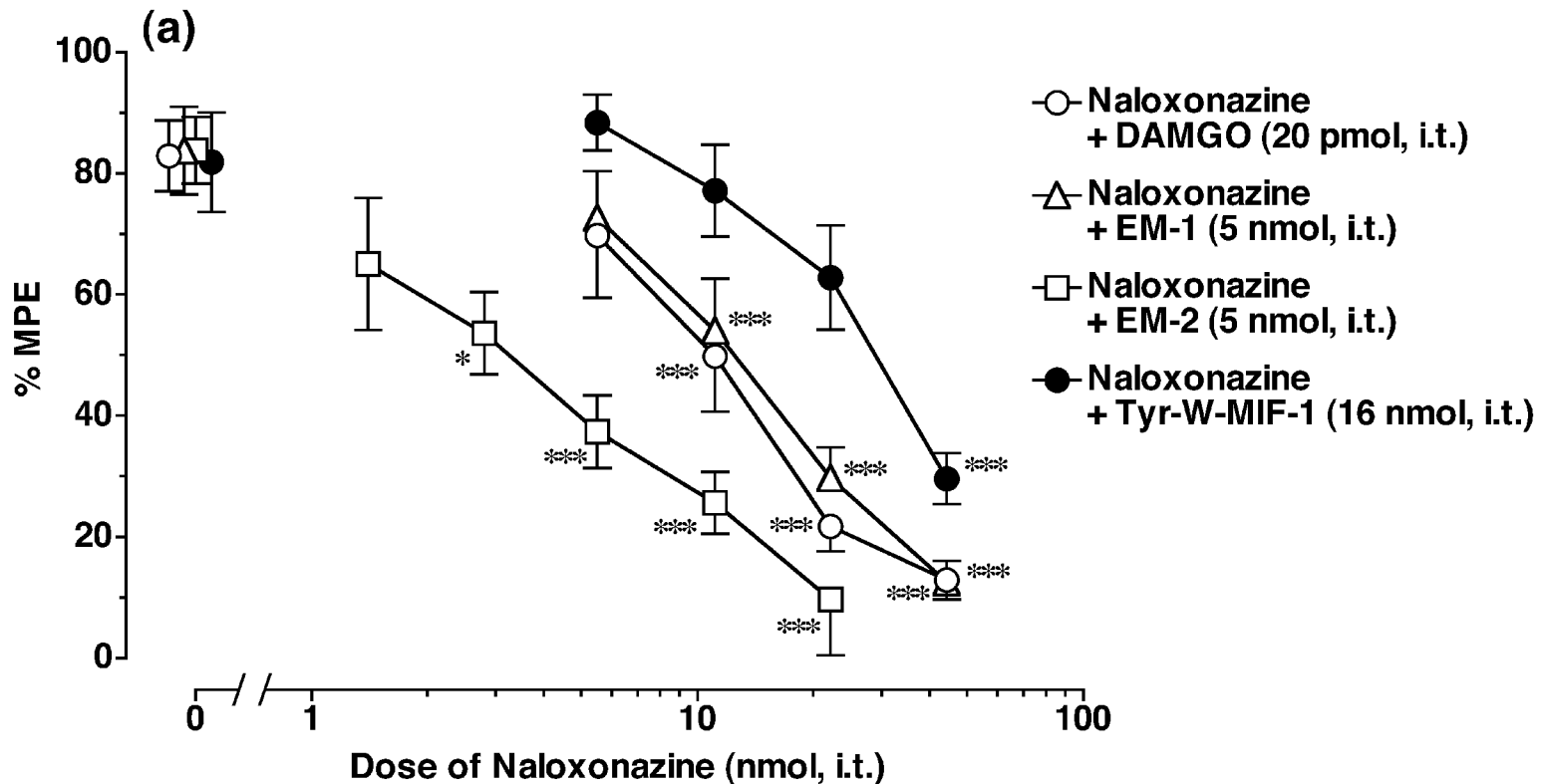
Fig. 1a

Fig. 1b

(b)

Figure axes: y-axis labeled "% MPE" ranging from 0 to 100; x-axis labeled "Dose of Tyr-W-MIF-1 (nmol, i.t.)" ranging from 1 to 100.

$ED_{50}=5.89$ nmol
(4.42-7.85)

Fig. 2 a

Figure shows a graph with Y-axis labeled "% MPE" (0 to 100) and X-axis labeled "Dose of Naloxonazine (nmol, i.t.)" (0, 1, 10, 100).

Legend:
- Naloxonazine + DAMGO (20 pmol, i.t.)
- Naloxonazine + EM-1 (5 nmol, i.t.)
- Naloxonazine + EM-2 (5 nmol, i.t.)
- Naloxonazine + Tyr-W-MIF-1 (16 nmol, i.t.)

Fig. 2b

(b)

Y-axis: % of inhibition (0 to 100)

X-axis: Dose of Naloxonazine (nmol, i.t.) (1 to 100)

Legend:
- ○ Naloxonazine + DAMGO (20 pmol, i.t.)
- △ Naloxonazine + EM-1 (5 nmol, i.t.)
- □ Naloxonazine + EM-2 (5 nmol, i.t.)
- ● Naloxonazine + Tyr-W-MIF-1 (16 nmol, i.t.)

Fig. 3a

Fig. 3b

(b)

% of inhibition

Dose of D-Pro²-Tyr-W-MIF-1 (nmol, i.t.)

○ D-Pro²-Tyr-W-MIF-1 + DAMGO (20 pmol, i.t.)

△ D-Pro²-Tyr-W-MIF-1 + EM-1 (5 nmol, i.t.)

□ D-Pro²-Tyr-W-MIF-1 + EM-2 (5 nmol, i.t.)

● D-Pro²-Tyr-W-MIF-1 + Tyr-W-MIF-1 (16 nmol, i.t.)