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A Tyr-W-MIF-1 analog containing D-Pro^2 discriminates among antinociception in mice mediated by different classes of μ-opioid receptors

Daisuke Nakayama [a], Chizuko Watanabe [a], Hiroyuki Watanabe [a], Hirokazu Mizoguchi [a], Tsukasa Sakurada [b], Shinobu Sakurada [a,*]

[a] *Department of Physiology and Anatomy, Tohoku Pharmaceutical University, 4-4-1 Komatsushima, Aoba-ku, Sendai 981-8558, Japan*
[b] *Department of Biochemistry, Daiichi College of Pharmaceutical Sciences, Fukuoka 815-8511, Japan*

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Abstract

The antagonism by 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) analog, of 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 with the mouse tail-flick test. D-Pro^2-Tyr-W-MIF-1 (0.5–3 nmol) given intracerebroventricularly (i.c.v.) had no effect on the thermal nociceptive threshold. High doses of D-Pro^2-Tyr-W-MIF-1 (4–16 nmol) administered i.c.v. produced antinociception with a low intrinsic activity of about 30% of the maximal possible effect. D-Pro^2-Tyr-W-MIF-1 (0.25–2 nmol) co-administered i.c.v. showed a dose-dependent attenuation of the antinociception induced by Tyr-W-MIF-1 or DAMGO without affecting endomorphin-2-induced antinociception. A 0.5 nmol dose of D-Pro^2-Tyr-W-MIF-1 significantly attenuated Tyr-W-MIF-1-induced antinociception but not DAMGO- or endomorphin-1-induced antinociception. The highest dose (2 nmol) of D-Pro^2-Tyr-W-MIF-1 almost completely attenuated Tyr-W-MIF-1-induced antinociception. However, that dose of D-Pro^2-Tyr-W-MIF-1 significantly but not completely 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.c.v. 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 that 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 a selective antagonist for the μ_2-opioid receptor in the mouse brain. 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, which both show a preference for the μ_2-opioid receptor in the brain.

Keywords: D-Pro^2-Tyr-W-MIF-1; Tyr-W-MIF-1; Antinociception; Opioid receptor; Supraspinal

1. Introduction

Tyr-Pro-Trp-Gly-NH_2 (Tyr-W-MIF-1) was isolated from human 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, 1993; Hackler 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 classical property of an agonistic for μ- or κ-opioid receptors

* Corresponding author. Tel.: +81 22 727 0124; fax: +81 22 727 0125.
E-mail address: s-sakura@tohoku-pharm.ac.jp (S. Sakurada).

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(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), suggesting that Tyr-W-MIF-1-induced antinociception is mediated through the spinal or supraspinal μ_2-opioid receptors. However, the characterization of μ_2-opioid receptor-mediated antinociception has been limited because a selective antagonist for the μ_2-opioid receptor has not been 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., 2002b). This evidence suggests that synthetic opioid peptides in which the L-Pro^2 of their parent peptide has been replaced with D-Pro^2 would be antagonists against their parent peptide. Based on the above hypothesis, we have now 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 was to characterize the antagonistic properties of D-Pro^2-Tyr-W-MIF-1 against the supraspinal 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. In addition, D-Pro^2-Tyr-W-MIF-1, an analog of Tyr-W-MIF-1, was designed to examine whether it produces antinociception after i.c.v. administration.

2. Materials and 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 animals used in the following experiments.

2.1. 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.

2.2. Drugs

The drugs used were Tyr-W-MIF-1 (Bachem, San Carlos, CA, USA), [D-Ala^2,$NMePhe^4$,Gly(ol)^5]enkephalin (DAMGO; Sigma Chemical Co., St. Louis, MO, USA), endomorphin-1 (Tocris Cookson, Bristol, UK), endomorphin-2 (Tocris Cookson), D-Pro^2-Tyr-W-MIF-1 (synthesized in our laboratory), D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2 (CTOP; Bachem), and naloxonazine dihydrochloride (Sigma Chemical Co.). 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 $CaCl_2$ per 1000 ml.

2.3. Administration procedures

The i.c.v. administration was performed according to the procedure described by Haley and McCormick (1957) using a 10-μl Hamilton microsyringe with a 29-gauge needle. The injection site was 1 mm lateral to the bregma at a depth of 3.5 mm. The injection volume was 2 μl.

2.4. Assessment of antinociceptive responses

The antinociceptive response was assessed with the thermal tail-flick 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 stimulus was applied from underneath the glass floor toward the tail. The light beam focused on the ventral surface of the tail, and the latency for the tail-flick response to the noxious radiant heat stimulus was measured. The intensity of the noxious radiant heat stimulus was adjusted so that the pre-drug latency for the tail-flick response was 2.5–3.5 s. The antinociceptive effect was expressed as a percent of the maximal 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 tail-flick response, respectively. To prevent tissue damage to the tail, the noxious radiant heat stimulus was terminated automatically if the mouse did not flick its tail within 10 s. The measurement of the tail-flick latency was performed by only one individual who was uninformed about the drug treatment for each mouse.

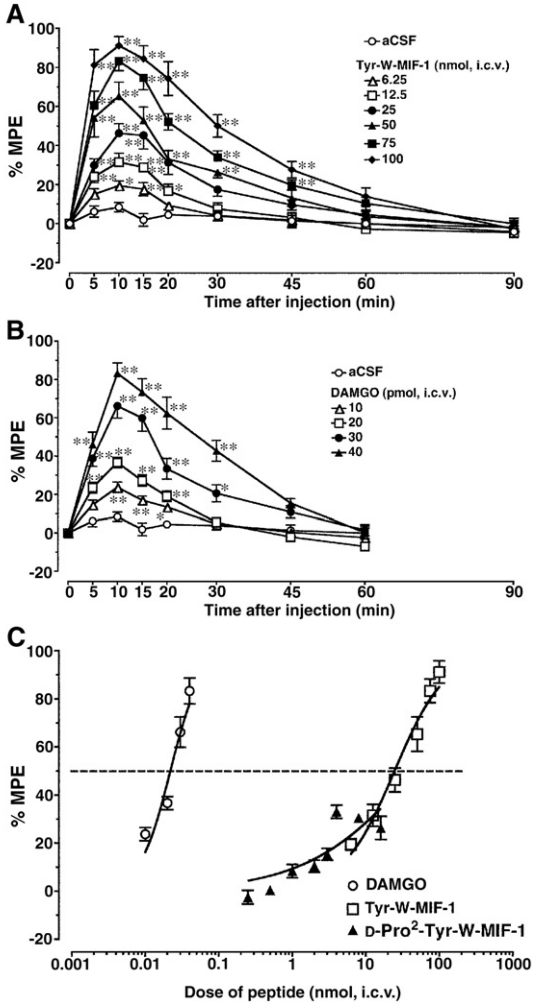

Fig. 1. Antinociceptive effects induced by i.c.v. Tyr-W-MIF-1, DAMGO and D-Pro^2-Tyr-W-MIF-1 in the mouse tail-flick test. Time courses of antinociception induced by i.c.v. administration of Tyr-W-MIF-1 (A) and DAMGO (B). Groups of mice were treated with i.c.v. various doses of Tyr-W-MIF-1 (6.25–100 nmol) and DAMGO (10–40 pmol) or aCSF, and antinociception induced by Tyr-W-MIF-1 and DAMGO was measured. 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. (A) The F values of the two-way ANOVA for Tyr-W-MIF-1 (6.25, 12.5, 25, 50, 75, and 100 nmol) in comparison with aCSF are $F[1,180]=14.00$ ($P<0.01$), $F[1,180]=43.15$ ($P<0.01$), $F[1,180]=99.44$ ($P<0.01$), $F[1,180]=154.6$ ($P<0.01$), $F[1,180]=403.8$ ($P<0.01$), and $F[1,180]=427.9$ ($P<0.01$), respectively. *, $P<0.05$ and **, $P<0.01$ *versus* aCSF. (B) The F values of the two-way ANOVA for DAMGO (10, 20, 30, and 40 pmol) in comparison with aCSF are $F[1,144]=26.29$ ($P<0.01$), $F[1,144]=61.56$ ($P<0.01$), $F[1,144]=193.4$ ($P<0.01$), and $F[1,144]=314.2$ ($P<0.01$), respectively. *, $P<0.05$ and **, $P<0.01$ *versus* aCSF. (C) Antinociception induced by various i.c.v. doses of Tyr-W-MIF-1, DAMGO and D-Pro^2-Tyr-W-MIF-1 injected at the peak effect time. Groups of mice were treated with various i.c.v. doses of Tyr-W-MIF-1 (6.25–100 nmol), DAMGO (10–40 pmol) and D-Pro^2-Tyr-W-MIF-1 (0.25–16 nmol) and the antinociception induced by Tyr-W-MIF-1, DAMGO and D-Pro^2-Tyr-W-MIF-1 were measured 10 min after the treatment. Each value represents the mean±S.E.M. for 10 mice. For the statistical significance of the differences between groups, the entire curves were compared using the F-test, according to the instructions provided with GraphPad Prism. The F values for DAMGO against Tyr-W-MIF-1 and D-Pro^2-Tyr-W-MIF-1 is 144.96 ($P<0.01$) and 38.85 ($P<0.01$), respectively.

2.5. 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 the Dunnett's test or the 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, USA). 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.

3. Results

3.1. Time-course and dose-response relations for i.c.v. administration of Tyr-W-MIF-1, D-Pro^2-Tyr-W-MIF-1 and DAMGO on tail-flick responses

Groups of mice were treated i.c.v. with aCSF or various doses of Tyr-W-MIF-1 (6.25–100 nmol), D-Pro^2-Tyr-W-MIF-1 (0.25–16 nmol) and DAMGO (10–40 pmol), and the antinociception was measured 5, 10, 15, 20, 30, 45, 60, and 90 min after treatment. The i.c.v. injection of Tyr-W-MIF-1 or DAMGO produced a dose-dependent antinociception (Fig. 1A and B). The antinociception induced by Tyr-W-MIF-1 or DAMGO developed rapidly, reached its peak at 10 min, and then gradually disappeared by 60 min after the treatment. The ED_{50} values of Tyr-W-MIF-1 and DAMGO for antinociception at the peak time were 24.7 (95% CI, 18.93–32.12) nmol and 22.09 (2.16–225.80) pmol, respectively (Fig. 1C). At the peak time, 75 nmol of Tyr-W-MIF-1 and 40 pmol of DAMGO produced about 80% MPE.

D-Pro^2-Tyr-W-MIF-1 (0.25–16 nmol) given i.c.v. produced an apparent dose-dependent antinociception (Fig. 1C). However, at the three highest doses of D-Pro^2-Tyr-W-MIF-1 (4, 8 and

Table 1
Effect of CTOP on the antinociception induced by DAMGO, endomorphin-1, endomorphin-2, or Tyr-W-MIF-1 in the mouse tail-flick test

Treatment	%MPE
DAMGO (40 pmol)+aCSF	83.31±5.37
DAMGO+CTOP (100 pmol)	13.29±2.85[a]
Endomorphin-1 (40 nmol)+aCSF	84.62±4.32
Endomorphin-1 (40 nmol)+CTOP (100 pmol)	16.86±2.27[a]
Endomorphin-2 (40 nmol)+aCSF	85.30±5.52
Endomorphin-2 (40 nmol)+CTOP (100 pmol)	15.46±2.64[a]
Tyr-W-MIF-1 (75 nmol)+aCSF	83.28±4.92
Tyr-W-MIF-1 (75 nmol)+CTOP (100 pmol)	8.15±2.31[a]

Groups of mice were co-administered i.c.v. CTOP with DAMGO, endomorphin-1, endomorphin-2, or Tyr-W-MIF-1, and the antinociception induced by DAMGO, endomorphin-1, endomorphin-2, and Tyr-W-MIF-1 was measured 10, 5, 5, and 10 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 Neuman–Keuls test. The F value of the one-way ANOVA was $F[7,72]=89.82$ ($P<0.01$). [a]$P<0.01$ *versus* each agonist plus aCSF.

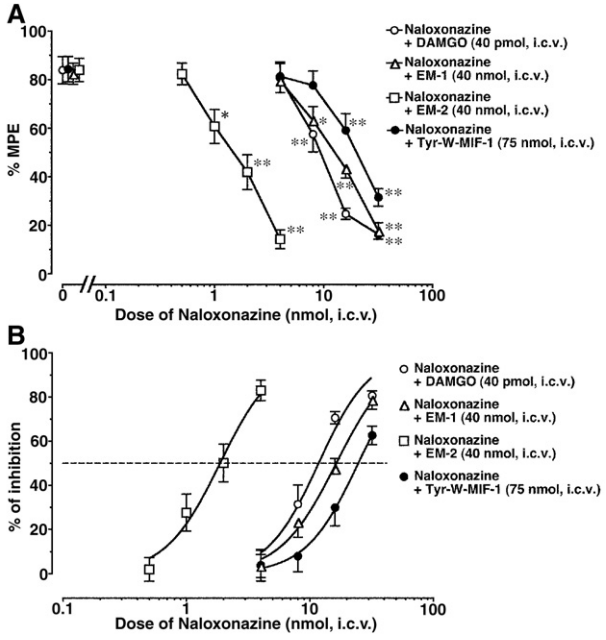

Fig. 2. Effect of naloxonazine on the antinociception induced by i.c.v. injection of Tyr-W-MIF-1, DAMGO, endomorphin-1 (EM-1), or endomorphin-2 (EM-2) in the mouse tail-flick test. Groups of mice were pretreated with various i.c.v. doses of naloxonazine (0.5–32 nmol) or aCSF 24 h before the i.c.v. administration of Tyr-W-MIF-1 (75 nmol), DAMGO (40 pmol), EM-1 (40 nmol), or EM-2 (40 nmol). The antinociception induced by i.c.v. administration of Tyr-W-MIF-1, DAMGO, EM-1, and EM-2 were measured 10, 10, 5, and 5 min after the treatment, respectively. Both the antinociceptive effects (%MPE) of the agonists (A) and the inhibitory effect (percentage) of naloxonazine (B) against the agonists are represented as 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 are $F[4,45]=15.26$ ($P<0.01$), $F[4,45]=37.76$ ($P<0.01$), $F[4,45]=38.06$ ($P<0.01$), and $F[4,45]=26.90$ ($P<0.01$), respectively. *, $P<0.05$ and **, $P<0.01$ *versus* groups treated with aCSF instead of naloxonazine. (B) 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 are 18.42 ($P<0.01$), 32.47 ($P<0.01$) and 184.9 ($P<0.01$), respectively. The F values for DAMGO against EM-1 and EM-2 are 3.29 (N.S.) and 42.82 ($P<0.01$), respectively. The F value for EM-1 against EM-2 is 111.87 ($P<0.01$).

16 nmol), there was a ceiling effect (about 30% MPE), *i.e.*, an increase in the dose did not lead to a greater effect. Small doses of D-Pro^2-Tyr-W-MIF-1 (0.25–2.0 nmol) did not show any significant antinociception.

3.2. Effect of CTOP on the antinociception induced by μ-opioid receptor agonists

Groups of mice were co-administered i.c.v. the μ-opioid receptor antagonist CTOP (100 pmol) or aCSF with equipotent doses of Tyr-W-MIF-1 (75 nmol), DAMGO (40 pmol), endomorphin-1 (40 nmol), or endomorphin-2 (40 nmol). The antinociception induced by Tyr-W-MIF-1, DAMGO, endomorphin-1, or endomorphin-2 was measured 10 min, 10 min, 5 min, and 5 min after the treatment, respectively, at the peak effect. The antinociception induced by i.c.v. administered Tyr-W-MIF-1, DAMGO, endomorphin-1, or endomorphin-2 was completely blocked by i.c.v. co-administration of CTOP (Table 1).

3.3. Effect of naloxonazine on the antinociception induced by μ-opioid receptor agonists

Groups of mice were pretreated i.c.v. with various doses of the μ_1-opioid receptor antagonist naloxonazine (0.5–32 nmol) or aCSF 24 h before the i.c.v. administration of equipotent doses of Tyr-W-MIF-1 (75 nmol), DAMGO (40 pmol), endomorphin-1 (40 nmol), or endomorphin-2 (40 nmol). The antinociception induced by i.c.v. administration of Tyr-W-MIF-1, DAMGO, endomorphin-1, and endomorphin-2 was measured 10 min, 10 min, 5 min, and 5 min after the treatment, respectively, at the peak effect. 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 4.0 nmol completely antagonized the antinociceptive effect induced by endomorphin-2, without affecting the antinociception induced by endomorphin-1, DAMGO or Tyr-W-MIF-1. Higher dose (8.0 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 (32 nmol), which completely eliminated the antinociception induced by endomorphin-1 or DAMGO. The ID_{50} value for naloxonazine against the antinociception induced by endomorphin-2 (1.87 nmol) was extremely smaller than those against the antinociception induced by DAMGO (11.78 nmol), endomorphin-1 (16.37 nmol) and Tyr-W-MIF-1 (24.68 nmol) (Fig. 2B, Table 2). The dose-response curves for inhibition by naloxonazine against DAMGO- and endomorphin-1-induced

Table 2
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 tail-flick test

Treatment	ID_{50} (nmol)	Hill slope
Naloxonazine		
DAMGO (40 pmol)	11.78 (7.24–19.18)	2.09 (0.10–4.08)
Endomorphin-1 (40 nmol)	16.37 (13.31–20.12)	1.90 (1.16–2.64)
Endomorphin-2 (40 nmol)	1.87 (1.31–2.67)	1.96 (0.63–3.30)
Tyr-W-MIF-1 (75 nmol)	24.68 (22.59–26.96)	2.03 (1.64–2.42)
D-Pro^2-Tyr-W-MIF-1		
DAMGO (40 pmol)	2.29 (2.15–2.45)	2.97 (2.28–3.66)
Endomorphin-1 (40 nmol)	1.89 (0.65–5.48)	1.29 (−0.65 to 3.22)
Endomorphin-2 (40 nmol)	NA	NA
Tyr-W-MIF-1 (75 nmol)	0.86 (0.64–1.17)	1.96 (0.83–3.09)

Groups of mice were pretreated with various i.c.v. doses of naloxonazine (0.5–32 nmol) 24 h before or co-administered various i.c.v. doses of D-Pro^2-Tyr-W-MIF-1 (0.25–2 nmol) with DAMGO (40 pmol), endomorphin-1 (40 nmol), endomorphin-2 (40 nmol), or Tyr-W-MIF-1 (75 nmol), and the antinociception induced by DAMGO, endomorphin-1, endomorphin-2, and Tyr-W-MIF-1 was measured 10, 5, 5, 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.

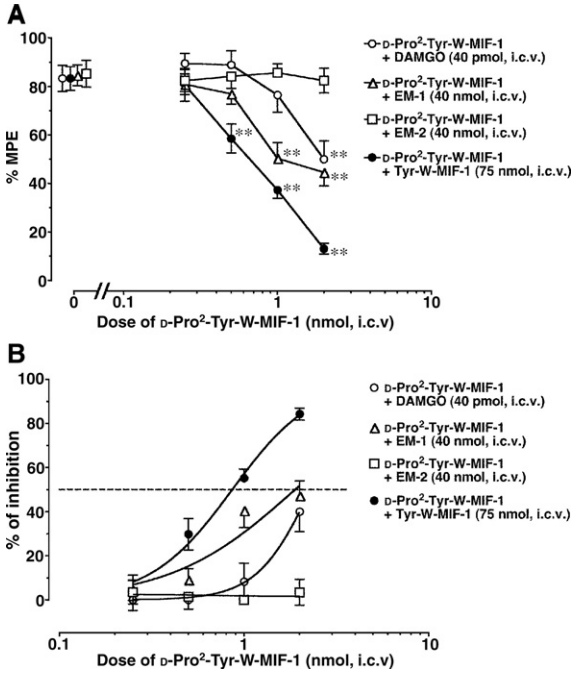

Fig. 3. Effect of D-Pro^2-Tyr-W-MIF-1 on the antinociception induced by i.c.v. administration of DAMGO, endomorphin-1 (EM-1), endomorphin-2 (EM-2), and Tyr-W-MIF-1 in the mouse tail-flick test. Groups of mice were co-administered various doses of D-Pro^2-Tyr-W-MIF-1 (0.25–2 nmol) with Tyr-W-MIF-1 (75 nmol), DAMGO (40 pmol), EM-1 (40 nmol), or EM-2 (40 nmol), and the antinociception induced by Tyr-W-MIF-1, DAMGO, EM-1, and EM-2 was measured 10, 10, 5, and 5 min after the treatment, respectively. Both the antinociceptive effects (%MPE) of the agonists (A) and the inhibitory effect (percentage) of D-Pro^2-Tyr-W-MIF-1 against the agonists (B) are represented as 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 are $F[4,45]=36.38$ ($P<0.01$), $F[4,45]=7.11$ ($P<0.01$), $F[4,45]=13.36$ ($P<0.01$), and $F[4,45]=0.10$ (N.S.), respectively. **, $P<0.01$ *versus* agonist alone. (B) 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 106.42 ($P<0.01$) and 8.56 ($P<0.05$), respectively. The F value for DAMGO against EM-1 was 5.85 (N.S.).

antinociception were statistically distinct from those against endomorphin-2- and Tyr-W-MIF-1-induced antinociception.

3.4. Effect of D-Pro^2-Tyr-W-MIF-1 on the antinociception induced by μ-opioid receptor agonists

Groups of mice were co-administered i.c.v. various doses of D-Pro^2-Tyr-W-MIF-1 (0.25–2.0 nmol) with equipotent doses of Tyr-W-MIF-1 (75 nmol), DAMGO (40 pmol), endomorphin-1 (40 nmol), or endomorphin-2 (40 nmol), and the antinociception induced by Tyr-W-MIF-1, DAMGO, endomorphin-1, or endomorphin-2 was measured 10 min, 10 min, 5 min, and 5 min after the treatment, respectively, at the peak effect. D-Pro^2-Tyr-W-MIF-1 at the doses (0.25, 0.5, 1.0, and 2.0) co-administered 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). In contrast, co-administered D-Pro^2-Tyr-W-MIF-1 at a dose of 0.5 nmol, which significantly attenuated Tyr-W-MIF-1-induced antinociception, did not affect the antinociception induced by DAMGO or endomorphin-1, whereas higher doses of D-Pro^2-Tyr-W-MIF-1 (1–2 nmol) significantly attenuated the antinociception induced by either DAMGO or endomorphin-1. The ID_{50} value for D-Pro^2-Tyr-W-MIF-1 against Tyr-W-MIF-1-induced antinociception (0.86 nmol) was smaller than those against DAMGO-induced antinociception (2.29 nmol) and endomorphin-1-induced antinociception (1.89 nmol) (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 B, Table 2).

4. Discussion

Tyr-W-MIF-1 induced a dose-dependent antinociception with the tail-flick test after supraspinal administration. The Tyr-W-MIF-1-induced antinociception occurred at 5–15 min after the i.c.v. injection and disappeared by 60 min after the injection, whereas that of endomorphin-1, which is structurally similar to Tyr-W-MIF-1 with a Phe in position 4, occurred within 5 min after i.c.v. injection and was reduced at 20 min (Sakurada et al., 2000a). The time course of antinociception with Tyr-W-MIF-1 is similar to that with DAMGO (Sakurada et al., 2002a).

We used a variety of i.c.v. 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 that 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 and supraspinal sites as assayed with the formalin, hot-plate, tail-pressure, and tail-flick tests (Sakurada et al., 1999, 2000b, 2002a,b; Sato et al., 1999). 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 and Goodman, 1985).

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 i.t. 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 i.t. 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). However, the i.c.v. dose of naloxonazine required to selectively block μ_1-opioid receptors has not yet been determined.

Intracerebroventricularly administered naloxonazine did not block the antinociceptive effects of DAMGO, endomorphin-1 or Tyr-W-MIF-1 at doses of 1.0–4 nmol. On the contrary, naloxonazine at a dose of 4 nmol completely antagonized the antinociception of endomorphin-2. Even a low dose of naloxonazine (1 nmol) significantly attenuated the antinociception with endomorphin-2. These results suggest that a reasonable dose of i.c.v. administered naloxonazine to block the μ_1-opioid receptor selectively in mice would be 1.0–4 nmol/mouse.

The antinociception induced by i.c.v. administration of Tyr-W-MIF-1 was significantly attenuated by co-administration of CTOP, whereas the antinociceptive activity was not antagonized by pretreatment with a reasonable i.c.v. dose of naloxonazine, *i.e.*, 4.0 nmol/mouse. The present results with naloxonazine on Tyr-W-MIF-1-induced antinociception are in agreement with those of Gergen et al. (1996b). These results suggest that the antinociception with Tyr-W-MIF-1 is mediated through μ_2-opioid receptors, even though higher doses of i.c.v. naloxonazine attenuated the antinociception with Tyr-W-MIF-1 (Fig. 2A and B). A higher dose of naloxonazine (8 nmol/mouse, i.c.v.), which suppressed the antinociception with DAMGO or endomorphin-1, did not significantly inhibit the Tyr-W-MIF-1-induced antinociception. Even the highest dose of naloxonazine (32 nmol/mouse, i.c.v.) did not completely antagonize the antinociception with Tyr-W-MIF-1.

The 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., 2001).

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.), to obtain a relative μ_1-opioid receptor selectivity (Ling et al., 1986) did not attenuate the antinociceptive effects induced by i.c.v. administration of endomorphin-1 (Sakurada et al., 2002a) 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.

We have demonstrated that D-Pro^2-endomorphin-1 and D-Pro^2-endomorphin-2, analogs of the endomorphins (Sakurada et al., 2002b; Hung et al., 2002), and D-Pro^2-Tyr-W-MIF-1, an analog of Tyr-W-MIF-1 (Watanabe et al., 2005), act as opioid receptor antagonists that selectively block the antinociception induced by endomorphin-1, endomorphin-2 and Tyr-W-MIF-1, respectively, in the spinal cord. Furthermore, D-Pro^2-endomorphin-2 attenuated the antinociception induced by i.t. administration of endomorphin-2 but not that induced by DAMGO (Mizoguchi et al., 2006), endomorphin-1 (Sakurada et al., 2002b; Hung et al., 2002) or Tyr-W-MIF-1 (unpublished observations), indicating that D-Pro^2-endomorphin-2 is an antagonist that 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 by endomorphin-2, suggesting that D-Pro^2-endomorphin-1 acts as a selective μ_2-opioid receptor antagonist (Sakurada et al., 2002b). More recently, we have reported that the antinociception induced by i.t. administered Tyr-D-Arg-Phe-sarcosine is significantly attenuated by co-administration of D-Pro^2-endomorphin-2, whereas co-administered D-Pro^2-endomorphin-1 or D-Pro^2-Tyr-W-MIF-1 does not affect the antinociceptive effect of Tyr-D-Arg-Phe-sarcosine. On the contrary, the antinociceptive effect of i.t. administered DAMGO is significantly attenuated by the co-administration of D-Pro^2-endomorphin-1 or D-Pro^2-Tyr-W-MIF-1, but not D-Pro^2-endomorphin-2. These results suggest that the antinociception induced by Tyr-D-Arg-Phe-sarcosine like endomorphin-2, is mediated through μ_1-opioid receptors, but not through μ_2-opioid receptor (Mizoguchi et al., 2006). However, it has not yet been determined whether the three D-Pro^2-analogs, D-Pro^2-endomorphin-1, D-Pro^2-endomorphin-2 and D-Pro^2-Tyr-W-MIF-1, act as μ_2- or μ_1-opioid receptor antagonists at the supraspinal level.

We found in the present study that i.c.v. co-administration of D-Pro^2-Tyr-W-MIF-1 inhibited the antinociception induced by Tyr-W-MIF-1, endomorphin-1 and DAMGO, but not by endomorphin-2, in a dose-dependent manner. The present results clearly suggest that D-Pro^2-Tyr-W-MIF-1 is a selective antagonist for the μ_2-opioid receptor. Interestingly, the antinociception of Tyr-W-MIF-1 was significantly attenuated by D-Pro^2-Tyr-W-MIF-1 at a dose of 0.5 nmol, a dose that did not affect endomorphin-1-, endomorphin-2- or DAMGO-induced antinociception (Fig. 3A and B). A higher

dose (1.0 nmol) of D-Pro^2-Tyr-W-MIF-1 significantly attenuated the antinociception with endomorphin-1 or Tyr-W-MIF-1 without affecting the antinociception with endomorphin-2 or DAMGO (Fig. 3A and B). The highest dose (2 nmol) of D-Pro^2-Tyr-W-MIF-1, which significantly but not completely suppressed the antinociception with endomorphin-1 or DAMGO, almost completely antagonized the antinociception with Tyr-W-MIF-1. 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 (Fig. 3A and B). The finding that the antinociception induced by Tyr-W-MIF-1 can be antagonized by D-Pro^2-Tyr-W-MIF-1 at a dose of 0.5 nmol, which is inactive against endomorphin-1 and DAMGO, indicates that it could be used to distinguish between different antinociceptive mechanisms within the μ_2-opioid receptor class. D-Pro^2-Tyr-W-MIF-1 selectively blocked the antinociception of Tyr-W-MIF-1 more effectively than that of endomorphin-1 and DAMGO, while the antinociception induced by endomorphin-2 was not reduced by co-administration with D-Pro^2-Tyr-W-MIF-1 (Fig. 3A and B). The differential antagonistic sensitivity of D-Pro^2-Tyr-W-MIF-1 for inhibition of the antinociception by μ_2-opioid receptor agonists led us to speculate that the μ_2-opioid receptors could be subdivided into two subclasses of the μ_2-opioid receptor. One subclass is less sensitive to D-Pro^2-Tyr-W-MIF-1, and the other is extremely sensitive to D-Pro^2-Tyr-W-MIF-1.

D-Pro^2-Tyr-W-MIF-1 selectively blocked the antinociceptive effect of i.c.v. administration of Tyr-W-MIF-1, whereas the antinociceptive effect of DAMGO or endomorphin-1 was not inhibited by D-Pro^2-Tyr-W-MIF-1 at the low dose at which the antinociception caused by Tyr-W-MIF-1 was completely 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 that act *via* the different subclasses of the μ_2-opioid receptor.

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References

Erchegyi, J., Kastin, A.J., Zadina, J.E., 1992. Isolation of a novel tetrapeptide with opiate and antiopiate activity from human brain cortex. Tyr-Pro-Trp-Gly-NH$_2$ (Tyr-W-MIF-1). Peptides 13, 623–631.

Erchegyi, J., Zadina, J.E., Qiu, X.D., Kersh, D.C., Ge, L.J., Brown, M.M., Kastin, A.J., 1993. Structure–activity relationships of analogs of the endogenous brain peptides Tyr-MIF-1 and Tyr-W-MIF-1. Pept. Res. 6, 31–38.

Gergen, K.A., Zadina, J.E., Kastin, A.J., Paul, D., 1996a. Intrathecal Tyr-W-MIF-1 produces potent, naloxone-reversible analgesia modulated by α_2-adrenoceptors. Eur. J. Pharmacol. 298, 235–239.

Gergen, K.A., Zadina, J.E., Paul, D., 1996b. Analgesic effects of Tyr-W-MIF-1: a mixed μ_2-opioid receptor agonist/μ_1-opioid receptor antagonist. Eur. J. Pharmacol. 316, 33–38.

Hackler, L., Kastin, A.J., Erchegyi, J., Zadina, J.E., 1993. Isolation of Tyr-W-MIF-1 from bovine hypothalami. Neuropeptides 24, 159–164.

Hahn, E.F., Carroll-Buatti, M., Pasternak, G.W., 1982. Irreversible opiate agonist and antagonists: the 14-hydroxydihydromorphine azines. J. Neurosci. 2, 572–576.

Haley, T.G., McCormick, W.G., 1957. Pharmacological effects produced by intracerebral injections of drugs in the conscious mouse. Br. J. Pharmacol. 12, 12–15.

Hung, K.C., Wu, H.E., Mizoguchi, H., Sakurada, S., Okayama, T., Fujimura, T., Murayama, K., Sakurada, T., Fujimoto, J.M., Tseng, L.F., 2002. D-Pro^2-Endomorphin-1 and D-Pro^2-endomorphin-2, respectively, attenuate the antinociception induced by endomorphin-1 and endomorphin-2 given intrathecally in the mouse. J. Pharmacol. Exp. Ther. 303, 874–879.

Ling, G.S.F., Simantov, R., Clark, J.A., Pasternak, G.W., 1986. Naloxonazine actions in vivo. Eur. J. Pharmacol. 129, 33–38.

Mizoguchi, H., Nakayama, D., Watanabe, H., Ito, K., Sakurada, W., Sawai, T., Fujimura, T., Sato, T., Sakurada, T., Sakurada, S., 2006. Involvement of spinal μ_1-opioid receptors on the Tyr-D-Arg-Phe-sarcosine-induced antinociception. Eur. J. Pharmacol. 540, 67–72.

Moskowitz, A.S., Goodman, R.R., 1985. Autoradiographic distribution of Mu_1 and Mu_2 opioid binding in the mouse central nervous system. Brain Res. 360, 117–129.

Reed, G.W., Olson, G.A., Olson, R.D., 1994. The Tyr-MIF-1 family of peptides. Neurosci. Biobehav. Rev. 18, 519–525.

Sakurada, S., Zadina, J.E., Kastin, A.J., Katsuyama, S., Fujimura, T., Murayama, K., Yuki, M., Ueda, H., Sakurada, T., 1999. Differential involvement of μ-opioid receptor subtypes in endomorphin-1- and -2-induced antinociception. Eur. J. Pharmacol. 372, 25–30.

Sakurada, S., Hayashi, T., Yuhki, M., Fujimura, T., Murayama, K., Yonezawa, A., Sakurada, C., Takeshita, M., Zadina, J.E., Kastin, A.J., Sakurada, T., 2000a. Differential antagonism of endomorphin-1 and endomorphin-2 spinal antinociception by naloxonazine and 3-methoxynaltrexone. Brain Res. 881, 1–8.

Sakurada, S., Takeda, S., Sato, T., Hayashi, T., Yuki, M., Kutsuwa, M., Tan-No, K., Sakurada, C., Kisara, K., Sakurada, T., 2000b. Selective antagonism by naloxonazine of antinociception by Tyr-D-Arg-Phe-β-Ala, a novel dermorphin analogue with high affinity at μ-opioid receptors. Eur. J. Pharmacol. 395, 107–112.

Sakurada, S., Hayashi, T., Yuhki, M., Orito, T., Zadina, J.E., Kastin, A.J., Fujimura, T., Murayama, K., Sakurada, C., Sakurada, T., Narita, M., Suzuki, T., Tan-no, K., Tseng, L.F., 2001. Differential antinociceptive effects induced by intrathecally administered endomorphin-1 and endomorphin-2 in the mouse. Eur. J. Pharmacol. 427, 203–210.

Sakurada, S., Hayashi, T., Yuhki, M., Fujimura, T., Murayama, K., Yonezawa, A., Sakurada, C., Takeshita, M., Sato, T., Zadina, J., Kastin, A.J., Sakurada, T., 2002a. Differential antagonism of endomorphin-1 and endomorphin-2 supraspinal antinociception by naloxonazine and 3-methylnaltrxone. Peptides 23, 895–901.

Sakurada, S., Watanabe, H., Hayashi, T., Yuhki, M., Fujimura, T., Murayama, K., Sakurada, C., Sakurada, T., 2002b. Endomorphin analogues containing D-Pro^2 discriminate different μ-opioid receptor mediated antinociception in mice. Br. J. Pharmacol. 137, 1143–1146.

Sato, T., Sakurada, S., Takahashi, N., Sakurada, T., Tan-No, K., Wako, K., Kisara, K., 1999. Contribution of spinal μ_1-opioid receptors to morphine-induced antinociception. Eur. J. Pharmacol. 369, 183–187.

Tseng, L.F., Narita, M., Suganuma, C., Mizoguchi, H., Ohsawa, M., Nagase, H., Kampine, J.P., 2000. Differential antinociceptive effects of endomorphin-1 and endomorphin-2 in the mouse. J. Pharmacol. Exp. Ther. 292, 576–583.

Watanabe, H., Nakayama, D., Ito, K., Watanabe, C., Mizoguchi, H., Fujimura, T., Murayama, K., Kawamura, S., Sato, T., Sakurada, C., Sakurada, T., Sakurada, S., 2005. A Tyr-W-MIF-1 analog containing D-Pro^2 acts as a selective μ_2-opioid receptor antagonist in the mouse. J. Pharmacol. Exp. Ther. 312, 1075–1081.

Wolozin, B.L., Pasternak, G.W., 1981. Classification of multiple morphine and enkephalin binding sites in the central nervous system. Proc. Natl. Acad. Sci. U. S. A. 78, 6181–6185.

Zadina, J.E., Kastin, A.J., Ge, L.J., Brantl, V., 1990. Hemorphins, cytochrophins and human beta-casomorphins bind to antiopiate (Tyr-MIF-1) as well as opiate binding sites in rat brain. Life Sci. 47, PL25–PL30.

Zadina, J.E., Kastin, A.J., Kenigs, V., Bruno, C., Hackler, L., 1993. Prolonged analgesia after intracerebroventricular Tyr-W-MIF-1 (Tyr-Pro-Trp-Gly-NH_2). Neurosci. Lett. 155, 220–222.

Zadina, J.E., Kastin, A.J., Ge, L.J., Hackler, L., 1994a. Mu, delta, and kappa opiate receptor binding of Tyr-MIF-1 and of Tyr-W-MIF-1, its active fragments, and two potent analogs. Life Sci. 55, PL461–PL466.

Zadina, J.E., Kastin, A.J., Hackler, L., Ge, L.J., Kenigs, V., Gergen, K.A., Messenger, K.A., Chang, S.L., 1994b. Opiate receptor binding, guinea pig ileum activity, and prolonged analgesia induced by the brain peptide Tyr-W-MIF-1 and two potent analogs. Regul. Pept. 54, 341–342.

Zadina, J.E., Hackler, L., Ge, L.J., Kastin, A.J., 1997. A potent and selective endogenous agonist for the μ-opiate receptor. Nature 386, 499–502.