Novel 2',6'-Dimethyl-L-Tyrosine-Containing Pyrazinone Opioid Mimetic µ-Agonists with Potent Antinociceptive Activity in Mice

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ABSTRACT

Novel bioactive opioid mimetic agonists containing 2',6'-dimethyl-L-tyrosine (Dmt) and a pyrazinone ring interact with µ- and δ-opioid receptors. Compound 1 [3-(4'-Dmt-aminobutyryl)-6-(3'-Dmt-aminopropyl)-5-methyl-2(1H)pyrazinone] exhibited high µ-opioid receptor affinity and selectivity (Ki,µ = 0.021 nM and Kδ/Kµ = 1.519, respectively), and agonist activity on guinea pig ileum (IC50 = 1.7 nM) with weaker δ-activity on mouse vas deferens (IC50 = 25.8 nM). Other compounds (2-4) had µ-opioid receptor affinities and selectivities 2- to 5-fold and 4- to 7-fold less than 1, respectively. Intracerebroventricular administration of 1 in mice exhibited potent naloxone reversible antinociception (65 to 71 times greater than morphine) in both tail-flick (TF) and hot-plate (HP) tests. Distinct opioid antagonists had differential effects on antinociception: naltrindole (δ-antagonist) partially blocked antinociception in the TF, but it was ineffective in the HP test, whereas β-funaltrexamine (irreversible antagonist, µ1/µ2-subtypes) but not naloxonazine (µ1-subtype) inhibited TF test antinociception, yet both blocked antinociception in the HP test. Our data indicated that 1 acted through µ- and δ-opioid receptors to produce spinal antinociception, although primarily through the µ2-receptor subtype; however, the µ1-receptor subtype dominates supraspinally. Subcutaneous and oral administration indicated that 1 crossed gastrointestinal and blood-brain barriers to produce central nervous system-mediated antinociception. Furthermore, daily s.c. dosing of mice with 1 for 1 week developed tolerance in a similar manner to that of morphine in TF and HP tests, implicating that 1 also acts through a similar mechanism analogous to morphine at µ-opioid receptors.

Since the discovery of the endogenous opioid pentapeptides, [Met5]- and [Leu5]-enkephalin (Hughes et al., 1975), many peptide and nonpeptide compounds were synthesized in a search for greater receptor selectivity, stability, and potent bioactivity. Although all known endogenous and exogenous µ-, δ-, and κ-opioid receptor peptides have distinct structural diversity, they share a common message domain, which is considered important for recognition by opioid receptors. This message region consists of a tyrosine residue, an N-terminal amino group, and a spacer (D-Ala, D-Met, Pro, or Gly-Gly) between the tyrosine and second aromatic ring, usually Phe, or Trp in the endomorphins (Zadina et al., 1997). The address domain, which is responsible for the biological response, contains the second aromatic ring and residues C-terminal thereafter. Message and address domains of opioid peptides represent distinct starting points for the design and development of novel opioid mimetics. For example, studies on opioid peptides showed that the substitution of the N-terminal tyrosine by 2',6'-dimethyl-L-tyrosine (Dmt) dramatically increased receptor affinity in numerous peptides and enhanced their antinociceptive effect (Chandrukar et al., 1992; Hansen et al., 1992; Pitzele et al., 1994; Guerrini et al., 1996; Sasaki et al., 1999; Schiller et al., 2000; Bryant et al., 2003). Moreover, Dmt played a key role in the formation of the Dmt-Tic pharmacophore family of potent δ-opioid receptor antagonists (Salvadori et al., 1997; Bryant et al., 1998, 1999), which were transformed into potent δ-opioid receptor agonists by subtle changes in the C-terminal link to a third aromatic nucleus (Balboni et al., 2002). Recently, we reported that symmetric opioid mimetic substances, which

AABBREVIATIONS: Dmt, 2',6'-dimethyl-L-tyrosine; TF, tail-flick; HP, hot-plate; DPDPE, [D-Pen2,D-Pen5]-enkephalin; DAMGO, [D-Ala2,N-Me-Phe4,Gly5-ol]-enkephalin; GPI, guinea pig ileum; MVD, mouse vas deferens; NTI, naltrindole hydrochloride; NAZ, naloxonazine; β-FNA, β-funaltrexamine; HPLC, high-performance liquid chromatography; Boc, tert-butylxycarbonyl; TFL, tail-flick latency; MED, minimum effective dose; AUC, area under the curve; ICI-154,129, N,N-diallyl-Tyr-Gly-ψ-(CH2)-Phe-Leu-OH.
contain two identical Dmt residues separated by simple unbranched alkyl chain, are able to serve as both the message and address domains for binding to \(\mu\)-opioid receptors (Okada et al., 2003). The cyclization of dipeptidyl chloromethyl ketones yielded pyrazinone derivatives, which can be easily inserted into an opioid peptide sequence (Okada et al., 1998, 1999), leading to our synthesis of a new series of opoidimimetic substances containing Dmt.

As we report herein, these Dmt-containing pyrazinone opioid mimetic compounds exhibit high affinity and high selectivity for the \(\mu\)-opioid receptor in rat brain membrane preparations. Furthermore, they were biologically potent toward the \(\mu\)-opioid receptor in functional guinea pig ileum biosays and general had considerably weaker to insignificant activity for the \(\delta\)-opioid receptor based on the mouse vas deferens bioassay. In this study, we focused on an examination of their in vivo activity using TF and HP tests as the analgesic endpoints in mice for their centrally mediated activity (intracerebroventricular administration) and systemic injections (subcutaneous and per oral) in comparison with the effects of morphine. To determine the specificity of those interactions, we used a series of specific opiate antagonists, \(\beta\)-funaltrexamine to differentiate between \(\mu_\text{I}\) and \(\mu_\text{II}\)-receptor subtypes, and the \(\mu_\text{III}\)-opioid receptor antagonist naloxonazine, in addition to the \(\delta\)-opioid antagonist naltrindole.

## Materials and Methods

### Animals

Male Swiss-Webster mice (20–25 g; Taconic Farms, Germantown, NY) were used. Animals were housed in plastic cages and maintained on a 12-h light/dark cycle with free access to food and water. Guinea pigs were housed for several days before use. All experiments with animals were carried out according to protocols approved by and on file with the National Institute of Environmental Health Sciences Animal Care and Use Committee.

### Opioid Receptor Competitive Binding Assays

The receptor binding affinities were determined under equilibrium conditions (2.5 h at 22°C) using rat brain membranes. The synaptosomal preparation (P2) was preincubated to remove endogenous opioid ligands in a solution containing 0.1 M NaCl, 0.4 mM GDP, 50 mM HEPES, pH 7.5, and 50 \(\mu\)g/ml soybean trypsin inhibitor for 60 min at room temperature (22°C). The preparation was exhaustively washed with ice-cold buffer (50 mM HEPES, pH 7.5, containing protease inhibitors); after the final wash, the synaptosomes were stored in buffer with protease inhibitor and 20% (v/v) glycerol and stored at –80°C. \(\beta\) and \(\mu\)-Opioid receptors were radiolabeled with \([\text{H}]\text{DPDPE}\) (PerkinElmer Life Sciences, Boston, MA; 34.0 Ci/mmol) and \([\text{H}]\text{DAMGO}\) (50.0 Ci/mmol; Amersham Biosciences UK, Ltd., Little Chalfont, Buckinghamshire, UK), respectively, as described in detail previously (Salvadori et al., 1997; Okada et al., 2003). Excess unlabeled peptide (2 \(\mu\)M) established the level of nonspecific binding. Radiolabeled membranes were rapidly filtered onto Whatman GF/C glass fiber filters soaked in 0.1% polyethylenimine to enhance the signal to noise ratio, washed three times with 2 ml of ice-cold buffer (50 mM Tris-HCl, pH 7.5 containing 0.1% bovine serum albumin), dried at 75°C for an hour and radioactivity determined using CytoScint (ICN Pharmaceuticals, Costa Mesa, CA). All analogs were analyzed in duplicate using five to eight dosages and three to five independent repetitions; different synaptosomal preparations were frequently used to ensure statistical significance (\(n\) values are listed in Table 1 in parentheses and results are mean \(\pm\) S.E.M.) The affinity constants \((K)\) were calculated according to Cheng and Prusoff.

### Functional Bioactivity in Isolated Tissue Preparations

Preparations of the myenteric plexus-longitudinal muscle (2–3-cm segments) were obtained from the small intestine of guinea pigs (GPI) for the study of \(\mu\)-opioid activity, whereas a single mouse vas deferens (MVD) was used as a source of biological \(\delta\)-opioid receptors. Both tissues, suspended in a balanced salt solution, were used for field stimulation with bipolar rectangular pulses of supramaximal voltage. Agonists were tested for their inhibition of the electrically evoked twitch and the results are expressed as the IC\(_{50}\) values obtained from dose-response curves and represent the mean \(\pm\) S.E. of five to six separate assays. \([\text{D-Ala}^\text{2}]\text{Deltorphin I and dermorphin analogs were used as internal \(\delta\) and \(\mu\)-opioid peptide standards for MVD and GPI, respectively (Okada et al., 2003).}

### Drugs

Morphine and naloxone HCl were obtained from Sigma-Aldrich (St. Louis, MO); naltinrole hydrochloride (NTI), naloxonazine dihydrochloride (NAZ), and \(\beta\)-funaltrexamine hydrochloride (\(\beta\)-FNA) were purchased from Tocris Cookson Inc. (Ellsville, MO). The opiate antagonists were injected s.c.: naloxone (2 mg/kg) and NTI (3 mg/kg) were administered 30 min before the test compounds, whereas with NAZ (35 mg/kg) and \(\beta\)-FNA (40 mg/kg), the mice were treated 24 h before testing (Paul et al., 1989). Under this experimental paradigm, NAZ antagonizes \(\mu\)-agonists (Ling et al., 1986) and \(\beta\)-FNA affects both \(\mu\)- and \(\mu\)-opioid receptor-mediated effects. The doses of naloxone and NTI were selected by their ability completely block the effect of morphine (0.5 \(\mu\)g/mouse i.c.v.) and deltorphin II (8 \(\mu\)g/mouse i.c.v.), respectively.

### Opioid Mimetic Substances

Briefly, the optical purity (>98%) of Dmt prepared as described previously (Dygos et al., 1992) was determined by HPLC using a chiral column [CROWN PAC CR(+)] and reaction with d-amino acid and l-amino acid oxidases followed by amino acid analysis. Boc-Dmt-OH was prepared as described previously (Okada et al., 1999). The 3,6-bis-(Tyr-aminooalkyl)-5-methyl-2(1H)-pyrazinones were prepared by published procedures (Okada et al., 1999). Boc-(R)-OH (R: Orn or Lys) coupled with H-(R)-(Z)-CH\(_2\)-Cl (R': Orn or Lys) by a mixed anhydride procedure to yield Boc-(R)-Z-(Z)-CH\(_2\)-Cl. The Boc groups were removed by hydrogen chloride in dioxane, and the amine HCl in MeOH or THF was refluxed for 1 h to give the protected pyrazinone derivatives, which were converted to the corresponding amines (Okada et al., 1999) by catalytic hydrogenation or by HBr/AcOH. The resulting amines were then coupled with Boc-Dmt-OH by using benzotriazolylloxazotxil[pyrrolidin]-phosphonium hexafluorophosphate reagent to protect the compounds 1 to 4. They were treated with TFA to give crude 1 to 4. The final products were purified by semipreparative reverse-phase HPLC, and each compound exhibited a single peak on analytical HPLC (Okada et al., 2003). Analyses by matrix-assisted laser desorption ionization/time of flight mass spectrometry, \(^1\)H and \(^{13}\)C NMR, and HPLC revealed that they were the desired compounds with greater than 98% purity. These compounds were dissolved in physiological saline adjusted to pH 7 for i.c.v. or s.c. injections and dissolved in water for oral administration.

### Table 1

<table>
<thead>
<tr>
<th>Compound</th>
<th>(K_{\mu})</th>
<th>n</th>
<th>(K_{\delta})</th>
<th>n</th>
<th>(\delta_{\mu})</th>
<th>GPI (IC(_{50}))</th>
<th>MVD (IC(_{50}))</th>
</tr>
</thead>
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<tr>
<td>1</td>
<td>0.021 (\pm) 0.003</td>
<td>5</td>
<td>31.9 (\pm) 2.9</td>
<td>5</td>
<td>1519</td>
<td>1.7</td>
<td>25.8</td>
</tr>
<tr>
<td>2</td>
<td>0.114 (\pm) 0.008</td>
<td>3</td>
<td>23.2 (\pm) 2.5</td>
<td>3</td>
<td>204</td>
<td>1.9</td>
<td>1.0</td>
</tr>
<tr>
<td>3</td>
<td>0.043 (\pm) 0.003</td>
<td>3</td>
<td>13.2 (\pm) 1.7</td>
<td>3</td>
<td>307</td>
<td>1.3</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>4</td>
<td>0.051 (\pm) 0.009</td>
<td>5</td>
<td>18.8 (\pm) 2.9</td>
<td>3</td>
<td>369</td>
<td>4.93</td>
<td>&gt;10,000</td>
</tr>
</tbody>
</table>
**Intracerebroventricular Injection.** Intracerebroventricular injection was performed with Hamilton microsyringe fitted with disposable 30-gauge needle; it was inserted 2.5–3 mm in depth as described by Laursen and Belknap (1986). Briefly, the bregma was found by lightly rubbing the point of the needle over the skull until the suture was felt through the skin (about 1–3 mm rostral to a line drawn through the anterior base of the ears). The needle was inserted about 2 mm lateral to the midline, and the total volume injected was 4 μL. Control mice received the same volume of physiological saline. Shortly after testing, the animals were sacrificed according ACUC protocols: a slit was made along the midline of the scalp and mice having needle tract 2 mm lateral from the bregma were counted as having been injected correctly.

**Tail-Flick Test for Spinal Antinociception.** Radiant heat on the dorsal surface of the tail measured the reaction time in a tail-flick instrument (Columbus Instruments, Columbus, OH). The latency for removal of the tail from the onset of the radiant heat is defined as the tail-flick latency (TFL). The baseline TFL was adjusted between 2 and 3 s (preresponse time), and a cutoff time was set at 8 s to avoid external heat-related damage. The analgesic response was measured 10, 15, or 30 min after i.c.v. s.c., or p.o. injections, respectively. Duration time for i.c.v. and s.c. injections, and p.o. administration was 10 and 15 min, respectively, and the test was terminated when TFL was close to the preresponse time. Mice were injected with either saline or different doses of the compounds. Morphine served as a positive control. Minimum effective dose (MED) is the minimum dose of compound showing statistically significant antinociceptive effect expressed as area under the curve (AUC) value in comparison with saline-treated group.

**Statistical Analysis.** Statistical analysis between two independent groups was performed with Student’s t-test. Analysis of variance was used for multiple comparisons. When a significant difference among the treatments was obtained in the analysis of variance, a post hoc Bonferroni's test was used. The AUC was obtained by plotting the response time (seconds) on the ordinate and time (minutes) on the abscissa after administration of the compounds.

**Results**

**Receptor Binding Affinity and Selectivity.** Table 1 lists data from the competitive binding and in vitro bioassays. All the pyrazinone-containing compounds (1–4), 1 [3-(4′-Dmt-aminobutyyl)-6-(3′-Dmt-aminopropyl)-5-methyl-2(1H)pyrazinone], 2 [3,6-bis(4′-Dmt-aminobutyyl)-5-methyl-2(1H)pyrazinone], 3 [3,6-bis(3′-Dmt-aminopropyl)-5-methyl-2(1H)pyrazinone], and 4 [3-(3′-Dmt-aminopropyl)-6-(4′-Dmt-aminobutyl)-5-methyl-2(1H)pyrazinone], interacted to a high degree with both μ- and δ-opioid receptors in rat brain membrane preparations with selectivity for μ-over δ-opioid receptors. Compound 1 (Fig. 1) displayed μ-opioid receptor affinity that was 2 to 5 times greater than that of compounds 2 to 4.

**Functional Bioactivity in Vitro.** All compounds exhibited potent opioid agonist activity to the μ-opioid receptor in guinea pig ileum (Table 1); however, 1 and 2 also showed modest δ-opioid agonist activity in the mouse vas deferens bioassay system. On the other hand, 3 exhibited the best bioactivity toward the μ-opioid receptor (GPI) and had no activity toward the δ-opioid receptor up to 10 μM in the MVD bioassay.

**Antinociception of Opioid Mimetic Compounds in Mice by Intracerebroventricular, Subcutaneous, and Oral Administration.** A dose-dependent antinociceptive effect in both TF and HP tests was observed after i.c.v. administration of 1. The effect was potent with a MED of 0.7 and 6.9 ng/mouse in the TF and HP tests, respectively, and 65–71 times greater than with morphine (Figs. 2, A and B, and 3, A and B). Compounds 2 and 3 also exhibited potent antinociception in the TF test; they were stronger than morphine by 39–41 and 50–63 fold, respectively. However, 2 and 3 were only 22–89% as effective as 1, depending on the method of measurement (Fig. 4). Compound 4 was not tested in vivo because of its weak bioactivity in the GPI and MVD bioassays (Table 1).

In the TF test, the spinal antinociceptive effect induced by 1 was completely blocked by the nonselective opioid antagonist naloxone to the same degree as in morphine-treated mice (data not shown) and partially (47%) blocked by NTI, a se-

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**Fig. 1.** Chemical structure of 3-(4′-Dmt-aminobutyyl)-6-(3′-Dmt-aminopropyl)-5-methyl-2(1H)pyrazinone (1).

**Fig. 2.** Effect of compound 1 on the tail-flick latency after intracerebroventricular administration in mice. A, time course. B, AUC. Each value is the mean with S.E.M. of five to six mice. The asterisk (*) denotes values that are significantly different from saline-treated mice by Bonferroni’s test (***, p < 0.001; **, p < 0.01; *, p < 0.05).
selective δ-opioid antagonist (Fig. 5A). The irreversible \(\mu_1/\mu_2\)-receptor antagonist \(\beta\)-FNA reduced the antinociception of \(1\) by 74%; however, the \(\mu_1\)-antagonist NAZ had no significant effect (Fig. 5B). The supraspinal analgesic effect of \(1\), determined in the HP test, was completely blocked by naloxone; the same results were obtained with morphine-treated animals (data not shown). However, NTI was ineffective in the HP test (Fig. 6A). On the other hand, \(\beta\)-FNA and NAZ strongly reduced the antinociception of \(1\) to a similar degree, namely, 87 and 82%, respectively (Fig. 6B).

Injection of \(1\) s.c. revealed a dose-dependent antinociception in both the TF and HP tests (Fig. 7); the effect remained significant for at least 2 to 3 h, depending on the dose and measurement paradigm (data not shown). The antinociception measured by compound \(1\) was equivalent to that produced by morphine in the TF test; however, it was only 89% as effective as morphine in the HP test (Fig. 7).

The oral administration of \(1\) exhibited antinociception with a MED at 10 and 30 mg/kg in the TF and HP tests, respectively (Fig. 8). Although \(1\) was 65% as effective as morphine in the TF test, it was not comparable with morphine even at 30 mg/kg; in the HP test, \(1\) demonstrated only 16% of the activity of morphine.

### Discussion

The goal of developing highly potent opioid mimetics with strong antinociception and lacking tolerance, and physical and psychological dependence led many researchers to design new opioid mimetics by initially modifying the address and message domains of naturally occurring mammalian ligands. Later alterations in opioid structure, in particular the substitution of the N-terminal residue Tyr by Dmt (Chandrakumar et al., 1992; Hansen et al., 1992; Pitzele et al., 1994; Guerrini et al., 1996; Salvadori et al., 1997; Sasaki et al., 1999; Schiller et al., 2000; Bryant et al., 2003) and dimerization of Dmt through alkyldiamine (Okada et al., 2003), led to increases in the binding to opioid receptors and bioactivities in vitro and in vivo (Bryant et al., 2003). This study provided evidence that a pyrazinone ring connecting to two symmetric Dmt N termini by alkyl chains might serve a dual role in opioid peptides as both message and address domains. Furthermore, the data revealed interesting differences in the bioactivity in vitro and in vivo to known opioid peptides.

### Tolerance by the Opioid Mimetic Compound 1 and Morphine

To assess the development of tolerance, mice were injected s.c. with 3 mg/kg of \(1\) or morphine daily for 7 days, and TF and HP latencies were measured. On day one of the injection regime, both compound \(1\) and morphine showed antinociception to a similar degree in the TF test with an
affinity, excellent selectivity for µ-opioid receptors, and potent bioactivity in vitro and in vivo.

The in vivo studies revealed that 1 was a potent analgesic acting at µ-opioid receptors through supraspinal and spinal mechanisms, and the antinociception induced by 1 was reversible by the general opioid antagonist naloxone, which confirmed that it acted through opioid receptors (Sawynok et al., 1979). Although 1 is more selective for µ- than δ-opioid receptors (Table 1), the fact that the selective δ-opioid antagonist NTI (Portoghese et al., 1988) partially blocked the i.c.v. antinociceptive effect measured by TF, not by HP tests, suggests that the spinal effect of 1 is mediated by both µ- and δ-opioid receptors; however, only µ-opioid receptors were responsible for the supraspinal mechanism, which is defined by the HP test. Similarly, Heyman et al. (1988) reported that the δ-opioid receptor antagonist ICI-154,129 is a more potent inhibitor of spinal DPDPE- than spinal DAMGO-induced antinociception and that the irreversible µ-antagonist β-FNA blocks the antinociceptive effects of spinally injected DAMGO, but not DPDPE. Those data supported the involvement of µ- and δ-opioid receptors on spinal antinociception. Other reports also provided substantial evidence to validate our observations that both δ- and µ-receptors are involved in the appearance of antinociception at the level of the spinal cord (Porreca et al., 1984; Heyman et al., 1987; Paul et al., 1989).

![Fig. 5. Effect of opioid receptor antagonists naloxone, NTI, NAZ, and β-FNA on antinociception induced by compound 1 (6.9 ng i.c.v.) in the tail-flick test in mice. A, naloxone (2 mg/kg s.c.) and NTI (3 mg/kg s.c.). B, NAZ (35 mg/kg s.c.) and β-FNA (40 mg/kg s.c.). Each point represents the mean and S.E.M. determined using five to six mice in A and 7 to 10 mice in B. The asterisk (*) denotes values that were significantly different than saline-treated mice by Student’s test (***, p < 0.001), and # denotes values that were significantly different from 1-treated mice by Bonferroni’s test (###, p < 0.001; ##, p < 0.01; #, p < 0.05).](image-url)
Opioid receptors are important in both spinal and supraspinal antinociception (Ling and Pasternak, 1983; Porreca et al., 1984; Bodnar et al., 1988; Heyman et al., 1988) and are divided into two distinct pharmacological subtypes. The $\mu_1$-type binds morphine and most enkephalin analogs, and the $\mu_2$-type interacts primarily with morphine (Wolozin and Pasternak, 1981; Nishimura et al., 1984; Goodman and Pasternak, 1985; Moskowitz and Goodman, 1985a,b; Pasternak and Wood, 1986). Heyman et al. (1988) and Paul et al. (1989) suggest that different $\mu$-opioid receptor subtypes mediate antinociception at the spinal and supraspinal levels: whereas the $\mu_1$-opioid receptor subtype is more important for supraspinal, the $\mu_2$-subtype is involved in spinal antinociception (Ling and Pasternak, 1983; Porreca et al., 1984; Bodnar et al., 1988; Heyman et al., 1988; Paul et al., 1989). However, recent studies with $\mu$-receptor knockout mice show that the $\mu$-receptor agonists, such as heroin and morphine-6-$\beta$-glucuronide, induce antinociception by a mechanism different from that of morphine, which had no effect on CXBK mice lacking $\mu_1$-opioid receptors, whereas heroin and morphine-6-$\beta$-glucuronide showed potent antinociception (Rossi et al., 1996). Narita et al. (2002) also showed that antinociception induced by fentanyl, the anilidopiperidine class of opioids, may be mediated predominantly through $\mu_1$-opioid receptors at both supraspinal and spinal sites in mice. Therefore, to examine the relative roles of $\mu_1$- and $\mu_2$-receptors in spinal and supraspinal antinociception of 1, we assessed the effects of NAZ, a selective $\mu_1$-opioid receptor antagonist and $\beta$-FNA, an irreversible $\mu_1/\mu_2$-opioid antagonist. $\beta$-FNA reduced the antinociception of 1 in the TF test; however, NAZ had no significant effect, which suggesting that $\mu_2$-opioid receptors are mainly involved in the spinal mechanism of 1. On the other hand, $\beta$-FNA and NAZ reduced the antinociception of 1 to a similar degree in the HP test, indicating that supraspinal antinociception is mediated through $\mu_1$-opioid receptors. Nor-binaltorphimine, a selective $\kappa$-opioid antagonist, did not block the effect of 1 in TF and HP tests (data not shown). Thus, we conclude that 1 is an opioid mimic with $\mu$- and

![Fig. 8. Effect of orally administered compound 1 on the tail-flick (A) and hot-plate (B) latencies in mice. Each point represents the mean and S.E.M. derived from the use of five to eight mice. The asterisk (*) denotes values that were significantly different than saline-treated mice by Bonferroni’s test (+++, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$)]

![Fig. 9. Antinociceptive effect of subcutaneously injected compound 1 (3 mg/kg) or morphine (3 mg/kg) for 7 days measured by the tail-flick (A and B) and hot-plate (C and D) tests. A and C represent the time course (open symbols: circles, morphine; squares, 1-treated mice. Closed symbols: day 1; open symbols, day 7). B and D represent AUC. Each point represents the mean and S.E.M. obtained by using seven mice. The asterisk (*) denotes values that were significantly different from those on day 1 for 1- or morphine-treated mice by Student’s test (+++, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$).]
δ-opioid receptor agonist activities acting spinally and supraspinaly. Spinal effects occurred through δ- and the combined action of both μ₁- and δ-opioid receptors with a preference of μ₁ over δ receptor subtypes, whereas supraspinally effects arise primarily through the action of δ-opioid receptors.

Administration of 1 either by s.c. or p.o. exhibited a different degree of antinociception depending on measurement test, which suggested that 1 crossed both the gastrointestinal epithelial and blood-brain barriers into the brain to produce a central nervous system-mediated antinociception. Although 1 was 65 to 71 times more potent than morphine after i.v. administration, it did not show the same degree of antinociception after peripheral injection, which may be explained by diverse factors, such as lipophilicity, molecular weight, metabolic and protease stabilities, and transport mechanisms, which might limit transit of 1 through membrane barriers (Banks and Kastin, 1990; Smith et al., 1992; Ermisch et al., 1993; Samii et al., 1994).

Morphine is the one of the most potent opiates that is widely used to relieve pain. However, the development of tolerance, and physical and psychological dependence during long-term therapy, and its respiratory depressant effects, sedation, and withdrawal symptoms are serious side effects (Yakhsh et al., 1977). When we investigated the tolerance of 1 after s.c. injection, it produced an effect similar to morphine, which suggests that both compounds seemed to act by the same mechanism; however, because 1 is more potent, lower concentrations could be used for postoperative pain, or in the treatment of acute or chronic cancer neuropathies. Thus, the pyrazinone ring may be an ideal platform on which to develop stable opioids with sufficient lipophilicity suitable for clinical and therapeutic applications, albeit other biological endpoints must be studied to determine whether these compounds have a similar spectrum of detrimental effects as morphine.

References