Antinociceptive and Antidepressant-Like Profiles of BL-2401, a Novel Enkephalinase Inhibitor, in Mice and Rats

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ABSTRACT—To clarify the properties of BL-2401 ((±)-3-[2-benzyl-3-(propionylthio) propionyl]amino-5-methylbenzoic acid), a novel enkephalinase inhibitor, we examined its antinociceptive and antidepressant-like activities after oral administration, along with their association with endogenous opioid systems. BL-2401 produced an antinociceptive effect after oral administration in the mouse phenylbenzoquinone writhing test (ED₅₀: 12.4 mg/kg) and the rat acetic acid writhing test (ED₅₀: 55.8 mg/kg), the antinociceptive effect being antagonized by naloxone hydrochloride. BL-2401 also relieved arthritis-induced hyperalgesia in rats. In the mouse hot-plate and tail pressure tests, BL-2401 showed significant but modest antinociception at higher doses (200 and 400 mg/kg). In addition, BL-2401 (100 mg/kg) produced a naloxone-reversible antidepressant-like effect in the mouse forced swimming test. As for the mechanism of the action, the active metabolite of BL-2401, BL-2240 ((±)-3-(2-benzyl-3-mercaptopropionyl) amino-5-methylbenzoic acid), selectively inhibited enkephalinase in vitro (IC₅₀: 5.2 nM). Oral administration of BL-2401 to mice significantly inhibited the enkephalinase activity in the striatum and also potentiated the antinociceptive effect of (d-Ala²,Me₅)-enkephalin given intracisternally. These findings indicate that BL-2401 is an orally active enkephalinase inhibitor and may produce antinociceptive and antidepressant-like effects in association with endogenous opioid systems.

Keywords: BL-2401, Enkephalinase inhibitor, Antinociception, Antidepressant-like action, Opioid

The discovery of enkephalins in 1975 (1) accelerated the research on endogenous opioid systems, leading to successive isolation of various opioid peptides and fruitful investigation of their physiological, pathological and pharmacological roles (2, 3). More recently, several groups have succeeded in molecular cloning of μ-, δ- and κ-opioid receptors (4). These findings have been stimulating the development of novel analgesics acting via endogenous opioid systems, which has progressed along two strategies: first, direct ligands to opioid receptors, such as selective κ-opioid receptor agonists (5), and second, modulators of endogenous opioids, such as enkephalinase inhibitors (6).

Enkephalinase (neutral endopeptidase, EC. 3.4.24.11), as well as aminopeptidase, has been shown to play an important role in degrading enkephalins in vivo (7). The inhibitors of enkephalinase have been expected to have an antinociceptive effect (6, 8), but not to elicit morphine-like side effects because they do not bind directly to opioid receptors. Moreover, enkephalinase inhibitors and exogenous enkephalins have been reported to relieve forced swimming-induced immobility and conditioned suppression of motility (9–12), supporting the physiological roles of endogenous enkephalins in stress response, mood or emotion. Various enkephalinase inhibitors have been reported in the literature (6), and they show characteristic profiles; e.g., thiorphan, the prototype of enkephalinase inhibitor, produces an antinociceptive effect after i.v. and i.c.v. administration (13). Acetorphan, a prodrug of thiorphan, shows antinociceptive and antidepressant-like effects after i.v. administration (11). In addition, RB101 (N-[(R,S)-2-benzyl-3(3S)-2-amino-4-methylthio)butyl dithio]-1-oxopropyl]-l-phenylalanine benzyl ester) produces potent antinociceptive and antidepressant-like effects through its activity to inhibit both enkephalinase and aminopeptidase (12, 14). However, few compounds have been demonstrated to show an antinociceptive effect after oral administration;
that is, SCH34826 ((S)-N-[N-[1-[(2,2-dimethyl-1,3-dioxolan-4-yl) methoxy]carbonyl]-2-phenylethyl]-1-phenylalanine]-3-alanine) (15) and ONO9902 (16).

We have aimed to develop an orally active enkephalinase inhibitor as an analgesic drug that does not cause morphine-like side effects such as drug dependency (17). A novel compound, BL-2240 ((±)-3-(2-benzyl-3-mercaptopropionyl) amino-5-methylbenzoic acid), was found to inhibit enkephalinase potently, and its prodrug, BL-2401 ([(±)-3-[2-benzyl-3-(propionylthio) propionyl]amino-5-methylbenzoic acid), was synthesized as an orally active antinociceptive compound. BL-2401 was metabolized to BL-2240; a pharmacokinetic study demonstrated that BL-2240 was detected in the plasma and brain of mice and rats after oral administration of BL-2401 (K. Hayashi and M. Miyamoto, unpublished data). Thus, the present study was carried out to characterize the antinociceptive profile of BL-2401 in comparison with codeine and aspirin. We also investigated its antidepressant-like effect that might be of benefit for relief of pain; patients with pain often have depressive disorders (18). In addition, the potency of BL-2401 in inhibiting enkephalinase was evaluated using in vitro, ex vivo and in vivo assays to ascertain the mechanism of its effects.

MATERIALS AND METHODS

Animals

ddY Mice and Wistar rats were purchased from Japan SLC (Hamamatsu) and SD rats from Clea Japan (Osaka). Animals were housed in groups (except for arthritis test) under conditions of controlled temperature (23±2°C), humidity (55±10%) and light (06:00-18:00 hr). Food and water were available ad libitum. Each animal was used only once in all experiments.

Nociceptive tests

Phenylbenzoquinone writhing test in mice (19, 20): Female ddY mice (18-22 g) were used. Writhing was induced by i.p. injection of 10 ml/kg of 0.03% phenyl-p-benzoquinone in a 5% ethanol aqueous solution. The number of writhes was counted for a 15-min period commencing 5 min after the phenylbenzoquinone injection. When the writhes were 50% or less the mean number in the vehicle control group, the dose was considered effective. The anti-writhing ED50 and 95% confidence limits were calculated from the effective rates (% of number of mice in which the dose was effective/number of mice tested) according to the method of Litchfield and Wilcoxon (21). BL-2401 and other test drugs were orally administered 30 min before the injection of phenylbenzoquinone. Intracisternal (i.c.) administration of naloxone hydrochloride was performed in a volume of 10 pl/mouse according to the method of Ueda et al. (22).

Acetic acid writhing test in rats (20, 23): Male Wistar rats (90–130 g) were used. Writhing was induced by i.p. injection of 1 ml of 1% aqueous acetic acid. Rats showing writhing responses within 15 min after the acetic acid injection were used for the experiment. BL-2401 and other test drugs were orally administered 15 min after the acetic acid injection, and the number of writhes was counted for a 20-min period commencing 45 min after the oral administration (1 hr after acetic acid injection). The anti-writhing ED50 was calculated from the effective rates as cited above.

Tail pressure test in mice (20): Male ddY mice (22–30 g) were used. The root of the tail was gradually pressed with the apparatus of Nakamura and Shimizu (24). The pressure to elicit biting behavior was measured as a pain index (1 mm = 12.5 g) at 30-min intervals for 3 hr after oral administration of BL-2401 or other test drugs. The pressure was not over 50 mm to avoid injury. In the Results section, the scale of the pressure threshold was represented by g.

Hot-plate test in mice (15, 25): Male ddY mice (20–26 g) were treated with BL-2401 or other test drugs 30 min before testing. The mouse was put onto a heated surface (50°C), and the latencies of the paw licking and the jump were measured. A plastic cylinder (20-cm-high and 15 cm in diameter) was used to keep the mouse on the heated plate. Cut-off time was set at 400 sec.

Paw pressure test in adjuvant-induced arthritic rats: A 10 mg/ml suspension of adjuvant (Mycobacterium butyricum; Difco, Detroit, MI, USA) was prepared using light mineral oil (Bayol F). A 0.06 ml volume of this suspension was injected intradermally into the root of the tail of female SD rats (8-weeks-old). Two weeks later (day 14), rats showing arthritis in their hind paws were selected, and they were orally administered BL-2401 or codeine phosphate once a day during the following 7 days (days 14–20). The nociceptive threshold of the hind paw for mechanical stimulation was measured with the apparatus cited in the section on the tail pressure test, and the pressure to elicit either a struggle response or paw withdrawal was determined. The cut-off pressure was 50 mm. The pressure test was done before and 1 hr after drug administration on day 14 and at 1 hr after drug administration on days 16, 18 and 20. On days 21 and 28 (1 and 8 days after the last administration), only the pressure test was done. In the Results section, the scale of the pressure threshold was represented by g.

Antidepressant tests

Forced-swimming test in mice (26): Male ddY mice (25–32 g) were dropped into a vertical cylinder containing 15 cm of water at 21–23°C and left there for 6 min.
The total duration of immobility during the last 4 min was measured. Mice were treated orally with BL-2401 1 hr before the test.

**In vitro enkephalinase inhibition assay**

**Enzyme preparation:** Enkephalin-degrading enzymes were prepared from rat striata according to the method of Gorenstein and Snyder (27) with minor modification. The striata from male Wistar rats (200–300 g) were homogenized in 30 vol. 50 mM Tris-HCl buffer (pH 7.7) with a glass-Teflon homogenizer. The homogenate was centrifuged at 1,000 × g for 15 min, and then the supernatant was centrifuged at 50,000 × g for 15 min. The pellet was washed three times according to the above-described procedure. The resulting pellet was resuspended in 15 vol. 50 mM Tris-HCl buffer (pH 7.7) containing 1% Triton X-100 and then incubated at 37°C for 45 min, followed by centrifugation at 100,000 × g for 1 hr. The supernatant containing solubilized enzymes was applied to a DEAE-cellulose column previously equilibrated with 50 mM Tris-HCl buffer (pH 7.7) containing 1% Triton X-100. Elution was done with two bed volumes of 50 mM Tris-HCl buffer (pH 7.7) containing 1% Triton X-100 followed by a linear salt gradient from 0 to 0.4 M NaCl. These procedures were carried out at 4°C except for the incubation. Under these conditions, enkephalinase activity was detected in the void volume, and aminopeptidase and dipeptidylaminopeptidase were eluted within the NaCl gradient. These fractions were used as enzyme preparations.

**Enzyme assay:** 

$[^3]H$Leucine-enkephalin was used as a substrate. After pre-incubation of enzyme (50 μl) and test compound solution (25 μl) at 37°C for 5 min, $[^3]H$leucine enkephalin (25 μl, final concentration 20 nM) was added. The reaction was performed at 37°C for 1 hr (10 min for dipeptidylaminopeptidase) and stopped with 25 μl of 0.2 N HCl, A 50 μl aliquot of reaction mixture was applied to a Pasteur pipette containing polystyrene beads (Porapak Q) (28). The substrate, but not its metabolites, was retained on the beads. The metabolites were eluted with 3 × 1 ml of water from the column. The reactions progressed linearly at the initial velocity. Test compounds were dissolved in ethanol and diluted with 50 mM Tris-HCl buffer (pH 7.7). IC$_{50}$ values were calculated from inhibitory rates at three to four concentrations according to logit log analysis. The inhibitory rate was estimated from two or three experiments.

**Ex vivo enkephalinase inhibition assay (11)**

Female ddY mice (17–25 g) were treated orally with BL-2401. The mice were sacrificed with cervical dislocation; the right ventricle of the heart was then incised, and saline (20 ml) was injected into the left ventricle to exclude blood from the brain. The striata were dissected and homogenized with 1 ml of 50 mM Tris-HCl buffer (pH 7.4). The homogenate (50 μl) was incubated for 30 min at 25°C with $[^3]H$(D-Ala$^2$,Leu$^5$)-enkephalin (25 μl, final concentration 20 nM), bestatin (10 μM) and captopril (1 μM) and with or without thiorphan (1 μM) (final volume: 100 μl). The reaction was stopped with 25 μl of 0.2 N HCl, and then a 50-μl aliquot of the reaction mixture was applied to a Porapak Q column. The metabolite ($[^3]H$Tyr-d-Ala-Gly) was eluted with 3 × 1 ml of water. Enkephalinase activity was calculated as the difference of metabolite production between the reaction in the presence of thiorphan and that in its absence. Protein was assayed by Lowry's method (29).


DAME administered into the brain is degraded by enkephalinase, but not by aminopeptidase; therefore, enkephalinase inhibitors are able to potentiate the antinociceptive activity of DAME. This test has been used for an in vivo enkephalinase inhibition assay. Male ddY mice (20–26 g) were administered intracisternally with DAME (1 μg/mouse) at various intervals after oral administration of BL-2401. Antinociceptive effect was assessed by the tail pressure test. In the time-course study, DAME was given only once to each mouse pre-treated with BL-2401.

**Materials**

BL-2401, BL-2240, thiorphan and captopril were synthesized in our laboratory. Naloxone hydrochloride and phenyl-p-benzoquinone (Sigma Chemical Co., St. Louis, MO, USA), codeine phosphate (Dainippon Pharmaceutical Co., Osaka), bestatin and DAME (Peptide Institute, Minoh), DEAE-cellulose (Chisso, Chiba), Porapak Q (100–120 mesh; Waters Associates, Milford, MA, USA), $[^3]H$leucine-enkephalin (New England Nuclear, Boston, MA, USA), $[^3]H$(D-Ala$^2$,Leu$^5$)-enkephalin (CEA, Gif-sur-Yvette, France), aspirin (Nacalai Tesque, Kyoto) and other reagents (Nacalai Tesque, and Wako, Osaka) were used. BL-2401, codeine phosphate and aspirin were suspended in 0.5% tragacanth gum for oral administration, and DAME and naloxone hydrochloride were dissolved in saline. These compounds were administered at volumes of 10 ml/kg (mouse; p.o., s.c., i.v.), 5 ml/kg (rat acetic acid writhing test; p.o., s.c.) or 3 ml/kg (rat arthritis test; p.o.).

**Statistical analyses**

Results are presented as the mean with S.E. Statistical difference was estimated by the Dunnett’s test or Bonferroni multiple comparison test (for naloxone antagonism).
Appropriate groups were compared by Student’s t-test.

RESULTS

**Antinociceptive effect**

In the mouse phenylbenzoquinone writhing test, BL-2401 inhibited the writhes at doses of 10–100 mg/kg, p.o. (Table 1), with an ED$_{50}$ value (95% confidence limits: C.L.) of 12.4 (8.4–18.4) mg/kg. ED$_{50}$s of codeine phosphate and aspirin were 15.6 (8.7–28.1) (n = 31) and 126.5 (77.1–207.5) (n = 18) mg/kg, respectively. To estimate the duration of the action, BL-2401 was orally administered 3 and 6 hr before the injection of phenylbenzoquinone. The anti-writhing activity of BL-2401 (50 and 100 mg/kg) was still significant 6 hr after administration; the inhibitory rates were 38.3% (P<0.01) and 54.6% (P<0.01), respectively. The ED$_{50}$ at 3 and 6 hr after administration were 21.4 (11.4–40.0) and 75.4 (52.6–108) mg/kg, respectively. To clarify the involvement of opioid receptors in the action of BL-2401, naloxone antagonism was tested. As shown in Fig. 1, the anti-writhing activity produced by BL-2401 (100 mg/kg, p.o.) was significantly reduced by naloxone hydrochloride at doses of 0.1–0.5 mg/kg, i.v. To further test the involvement of central opioid receptors, naloxone hydrochloride was injected i.c. As shown in Fig. 1, the anti-writhing activity of BL-2401 (100 mg/kg) was also reduced by i.c. injected naloxone hydrochloride at a dose of 0.5 µg/mouse (ca. 0.025 mg/kg), a dose of naloxone hydrochloride which did not show antagonism after i.v. administration. In the rat acetic acid writhing test, BL-2401 produced a dose-related inhibition of writhes with an ED$_{50}$ (95% C.L.) of 55.8 (29.0–107) mg/kg (Table 1). ED$_{50}$s of codeine phosphate and aspirin were 14.2 (8.6–23.5) (n = 32) and 16.9 (8.8–32.5) (n = 18) mg/kg, respectively. Naloxone hydrochloride (0.5 mg/kg, s.c.) significantly reduced the anti-writhing activity of BL-2401 (160 mg/kg, p.o.).

<table>
<thead>
<tr>
<th>Dose (mg/kg, p.o.)</th>
<th>N ( )</th>
<th>Number of writhes</th>
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<tr>
<td></td>
<td></td>
<td>BL-2401</td>
</tr>
<tr>
<td>Mouse phenylbenzoquinone writhing test</td>
<td></td>
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</tr>
<tr>
<td>10</td>
<td>12 (4)</td>
<td>10.1±0.9**</td>
</tr>
<tr>
<td>25</td>
<td>12 (11)</td>
<td>5.4±0.6**</td>
</tr>
<tr>
<td>50</td>
<td>6 (6)</td>
<td>4.3±0.9**</td>
</tr>
<tr>
<td>100</td>
<td>6 (6)</td>
<td>3.7±1.0**</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Dose (µg/mouse)</th>
<th>N</th>
<th>Number of writhes</th>
</tr>
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<tbody>
<tr>
<td>40</td>
<td>8 (3)</td>
<td>11.4±1.1**</td>
</tr>
<tr>
<td>80</td>
<td>8 (5)</td>
<td>8.8±1.0**</td>
</tr>
<tr>
<td>160</td>
<td>8 (7)</td>
<td>6.6±0.8**</td>
</tr>
<tr>
<td>160 + NLX$^{b}$</td>
<td>8 (0)</td>
<td>21.0±1.5$^{f}$</td>
</tr>
</tbody>
</table>

Table 1. Anti-writhing activity of BL-2401 in the phenylbenzoquinone writhing test in mice and in the acetic acid writhing test in rats

### Notes

- ( ) number of mice or rats showing writhing counts 50% or less of each vehicle control.
- NLX: naloxone hydrochloride (0.5 mg/kg) was administered s.c. 45 min after the injection of acetic acid. **P<0.01, significantly different from each vehicle control group.
- P<0.01, significantly different from BL-2401 (160 mg/kg) alone.

Fig. 1. Antagonism of BL-2401-induced anti-writhing activity by naloxone hydrochloride (NLX) given i.v. or i.c. in the phenylbenzoquinone writhing test in mice. BL-2401 (100 mg/kg) was orally administered 30 min before i.p. injection of phenylbenzoquinone. NLX was given i.v. (left panel) or i.c. (right panel) 5 min before phenylbenzoquinone injection. Eight mice were used for each group (6 for NLX alone). *P<0.05, **P<0.01, significantly different from each vehicle control group.
p.o.) produced a modest antinociceptive effect (Fig. 2), while codeine phosphate produced a marked antinociceptive effect at doses of 20–80 mg/kg. Aspirin (800 mg/kg) had no significant effect. In the mouse hot-plate test, BL-2401 significantly prolonged the jump latency at doses of 200 and 400 mg/kg (Fig. 3). Codeine phosphate showed significant prolongation at doses of 20 and 40 mg/kg (Fig. 3). Aspirin had no effect; the mean and S.E. for 12 mice were 147.8±14.8 and 163.0±15.8 (sec) for vehicle and 800 mg/kg, respectively. The licking latency was not affected by any doses.

In the adjuvant-induced arthritic rats, hyperalgesia was observed throughout the test period; the pressure threshold for arthritic rats (119±10 g, n=20, day 14) was

![Figure 2: Antinociceptive activities of BL-2401 and codeine phosphate in the tail pressure test in mice.](image1)

**Fig. 2.** Antinociceptive activities of BL-2401 and codeine phosphate in the tail pressure test in mice. Left panel: ○, vehicle; ●, BL-2401 400 mg/kg. Right panel: ○, vehicle; △, codeine phosphate, 20 mg/kg; □, 40 mg/kg; ●, 80 mg/kg. Each dose was administered orally. n=6–9. *P<0.05, **P<0.01, significantly different from each vehicle control group. Data were previously analyzed by repeated measures analysis of variance, indicating significant (P<0.01) interaction between treatment and time in both experiments.

![Figure 3: Antinociceptive effects of BL-2401 and codeine phosphate in the hot-plate test in mice.](image2)

**Fig. 3.** Antinociceptive effects of BL-2401 and codeine phosphate in the hot-plate test in mice. Each dose was administered orally 30 min before testing. n=11–12. *P<0.05, **P<0.01, significantly different from each vehicle control group.
significantly lower than that for normal rats (212±11 g, n=20, day 14). BL-2401 partially but significantly relieved the hyperalgesia at doses of 30 and 100 mg/kg (Fig. 4). However, its dose-response relation was not clear. Codeine phosphate (100 mg/kg) markedly increased the pressure threshold, which rose over the normal level. One and 8 days after the last administration, the thresholds in all treated groups returned to the level for the vehicle control group. BL-2401 and codeine phosphate did not affect the hind paw volume.

**Antidepressant-like effect**

In the mouse forced-swimming test, BL-2401 significantly reduced the duration of immobility at a dose of 100 mg/kg (Fig. 5). The decrease induced by BL-2401 (100 mg/kg) was reversed by s.c. administered naloxone hydrochloride (0.5 mg/kg).

**Enkephalinase inhibitory activity**

BL-2240 was used as an active compound of BL-2401 in the in vitro experiment. BL-2240 inhibited enkephalinase in a concentration-dependent manner with an IC₅₀ of 5.2 nM. The kinetic experiment using various concentrations of substrate and BL-2240 demonstrated that the inhibition by BL-2240 was competitive. BL-2240 also inhibited dipeptidylaminopeptidase with an IC₅₀ of 8.3 μM, although the potency was 1/1000 times weaker than that against enkephalinase. BL-2240 did not inhibit aminopeptidase up to 100 μM. BL-2401 showed weak inhibitory effect on only enkephalinase with an IC₅₀ of 74 nM. Thiorphan, a prototype enkephalinase inhibitor, inhibited enkephalinase with an IC₅₀ of 2.6 nM. In the ex vivo experiment, enkephalinase activity in the striata was measured 30 min after oral administration of BL-2401 at doses of 5, 25 and 100 mg/kg. The enkephalinase activ-
ity in the striata was dose-dependently inhibited (Fig. 6); the inhibitory rates were 24.0%, 41.1% and 63.6% for 5, 25 and 100 mg/kg, respectively. Furthermore, the time-course study showed that BL-2401 (100 mg/kg) produced potent inhibition throughout 8 hr after oral administration; the inhibitory rates were 80.5%, 76.6%, 67.2% and 48.4% at 1, 2, 4 and 8 hr after administration, respectively. In the DAME potentiation test, BL-2401, when given 30 min before DAME, dose-relatedly potentiated the antinociceptive effect of DAME (Fig. 7). In addition, time-course studies of the potentiation induced by BL-2401 were performed at doses of 50 and 100 mg/kg (p.o.). As shown in Fig. 8, significant potentiation was still detected 8 hr after the administration of either dose of BL-2401. BL-2401 alone (up to 100 mg/kg, p.o.) did not significantly affect the pressure threshold.

DISCUSSION

In the nociceptive tests using acute experimental pain models, BL-2401 produced an antinociceptive effect after oral administration, and its antinociceptive profile was different from that of codeine phosphate, an opioid agonist, and aspirin, a non-steroidal anti-inflammatory drug (NSAID). The minimum effective dose (MED) of BL-2401 was 10 mg/kg (phenylbenzoquinone writhing test), 40 mg/kg (acetic acid writhing test), 200 mg/kg (hot plate test) and 400 mg/kg (tail pressure test). The difference in effective doses among tests was larger in comparison with that of codeine phosphate; the MEDs of codeine were 5, 10, 20 and 20 mg/kg, respectively. Furthermore, in the hot plate and the tail pressure tests, the effect elic-
ntestinal side-effects. BL-2401 may be without such
action via inhibiting cyclooxygenase and often cause gastro
edema of the arthritic paws. NSAIDs
inflammatory effect, unlike the known action of NSAIDs,
related to the distinct mechanism of actions of BL-2401
threshold for normal rats. This different potency may be
induced by BL-2401 or BL-2401 in the body after repeated
administration, the pressure thresholds in the BL-2401-treated
groups reduced to the level in the vehicle group. The effect
induced by BL-2401 was slight, unlike the effect of
codeine phosphate, which elicited an increase over the
threshold for normal rats. This different potency may be
related to the distinct mechanism of actions of BL-2401
and opioid analgesics. BL-2401 did not show an anti-
inflammatory effect, unlike the known action of NSAIDs,
which decrease the edema of the arthritic paws. NSAIDs
produce their antinociceptive and anti-inflammatory ac-
tion via inhibiting cyclooxygenase and often cause gastro-
intestinal side-effects. BL-2401 may be without such
side-effects because of the different mechanism of action.
These findings suggest that BL-2401 may have an anti-
 nociceptive effect against chronic pain.

Pain, especially a chronic one, and emotions are
thought to form a vicious circle (32). Emotions such as
anxiety, fear and depression often amplify pain; mean-
while, pain may serve as a stressor that causes such emo-
tions. This is supported by the application of anxiolytics
and antidepressant drugs as adjuvants for clinical chronic
pain (33). The antidepressant-like activity of BL-2401 was
examined in the forced swimming test which is useful for
screening of antidepressants. In this test, BL-2401
decreased the duration of immobility. It is reported,
however, that some compounds with locomotor stimulant
activity might cause false-positive effect in this
test (26). BL-2401 slightly increased locomotor activity in
mice (Y. Seto et al., unpublished data), though not inten-
vively like morphine. In addition, BL-2401 inhibited the
incidence of muricide behavior observed in olfactory-
bulbectomized rats (Y. Seto, unpublished data). These
results indicate that BL-2401 possess an antidepressant-
like effect after oral administration. We are interested in
the emotional influence on the pain suppression and the
roles of endogenous opioids in emotion, and further stud-
ies are necessary to clarify these points.

In the present study, the antinociceptive and anti-
edressant-like effects of BL-2401 were antagonized by
naloxone hydrochloride, suggesting the involvement of
endogenous opioid systems in the effects of BL-2401.
Since BL-2401 and BL-2240 did not show direct binding
activity in an opioid receptor assay using isolated guinea
pig ileum longitudinal muscle (A. Kita, unpublished
data), the actions of BL-2401 may be mediated by en-
dogenous opioids, indicating the difference between BL-
2401 and codeine phosphate as to the mode of the anti-
ociceptive action. Chipkin (8) has postulated that
enkephalinase inhibitors produce an antinociceptive
effect, only when endogenous enkephalinergic systems
are active in response to noxious stimuli. The activation
of endogenous opioid systems is estimated by certain
features, i.e., hyperalgesia induced by naloxone, and
increase in the release of endogenous opioids elicited
by noxious stimuli. It has been previously reported that
naloxone hydrochloride (1 mg/kg, i.p.) shows an algesic
effect in the writhing test and the hot plate test (only jump
latency) (34), and acetic acid stimulus increases the release
of enkephalin (35). The positive effects of BL-2401 in
the above-mentioned tests suggest that the antinocicep-
tion elicited by BL-2401 depends on the activation of the en-
dogenous opioid systems.

To further clarify the mechanism responsible for
the actions of BL-2401, its inhibitory activity on
enkephalinase was investigated using in vitro, ex vivo and
in vivo assays. In the in vitro study, BL-2240 inhibited
enkephalinase selectively among the enkephalin-de-
grading enzymes. The ex vivo study indicated that
BL-2401 given orally reached the brain and inhibited
enkephalinase there. In addition, based on the ability
of BL-2401 to potentiate DAME-induced antinociception, it
is considered that BL-2401 may inhibit the degradation of
DAME, and that of endogenous enkephalins as well. The
effective doses and the time course for ex vivo and in vivo
enkephalinase inhibition elicited by BL-2401 were similar
to those for its anti-writhing activity, suggesting that the
antinociceptive effect of BL-2401 is due to its inhibitory
action on enkephalinase.

As already reported, RB101, which inhibits both
enkephalinase and aminopeptidase, shows a potent anti-
 nociceptive effect against thermal or electrical nocicep-
tive stimuli (14) after parenteral administration. In con-
trast, the antinociceptive potency of BL-2401 on the acute
thermal or mechanical stimuli was modest, similar to a
selective enkephalinase inhibitor such as SCH34826 (15). However, BL-2401 showed both antinociceptive and antidepressant-like effects after oral administration.

In conclusion, the property of BL-2401 as a potent and selective enkephalinase inhibitor was demonstrated in the ex vivo and in vivo studies. When administered orally, BL-2401 produced an antinociceptive effect mediated by opioid systems, although the effect was modest against acute mechanical and physical stimuli. The different efficiency of BL-2401 from opioid analgesics and NSAIDs in various antinociceptive tests may be based on the different mechanism of action. Furthermore, BL-2401 produced antidepressant-like effects via opioid systems after oral administration to mice.

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