

## The Opioid Activity and Receptor Selectivity of Fluorinated Leu<sup>5</sup> Enkephalin Analogues *in Vitro* and *in Vivo*

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The opioid activity and the selectivity for opioid receptor (subtypes) of newly synthesized fluorinated enkephalin (Enk) analogues, [2*R*, 4*R*], [2*R*, 4*S*], [2*S*, 4*S*] and [2*S*, 4*R*] trifluoro-Leu<sup>5</sup>-Enk (KKF-31, 32, 33 and 34) were investigated. The inhibitory effect of KKF-compounds on the electrically induced contractions of guinea-pig ileum (GPI) and mouse vas deferens (MVD) were dose-dependent but relatively lower than that of L-Leu<sup>5</sup>-Enk, except that KKF-34 was rather slightly more potent than L-Leu<sup>5</sup>-Enk in MVD preparations. In GPI preparations, the pA<sub>2</sub> values of naloxone for these compounds were higher than those of naltrindole while the values of naltrindole for KKF-compound were higher than those of naloxone in MVD preparations. Intracerebroventricular KKF-31 and KKF-32 at doses of 20 and 10 nmol/mouse, respectively, produced analgesia comparable to 0.1 nmol Tyr-D-Arg-Phe-Lys-NH<sub>2</sub> and 100 nmol Tyr-D-Thr-Gly-Phe-Leu-Thr; however, neither KKF-33 nor 34 produced analgesia up to the doses of 100 nmol/mouse. Both naloxone, 1 mg/kg, i.p., and naltrindole, 10 mg/kg, i.p., antagonized KKF-31- and KKF-32-induced analgesia. The results suggest that the introduction of trifluoromethyl group in Leu<sup>5</sup> results in the alternation of the opioid activity and receptor selectivity. Although KKF-33 and KKF-34 possessed a more potent *in vitro* inhibitory effect than KKF-31 and KKF-32, mediated through  $\mu$ - and  $\delta$ -opioid receptors in both preparations, they did not show any appreciable analgesic effect. KKF-31 and KKF-32 produce naloxone- and naltrindole-reversible analgesia irrespective of *in vitro*  $\mu$ - and  $\delta$ -opioid activity.

**Keywords** — fluorinated enkephalin; guinea-pig ileum; mouse vas deferens; analgesic effect; opioid receptor selectivity; naloxone; naltrindole

### Introduction

Generally, fluorination of a drug molecule modifies the resistance against enzyme degradation and results in an increase in the activity of the drug. Coy *et al.*<sup>1)</sup> and Walker *et al.*<sup>2)</sup> reported enhancement of the pharmacological effect by the fluorination of D-Ala<sup>2</sup>-Met-enkephalinamide and dynorphin 1—13, respectively. On the other hand, we have reported that fluorination of the *p*-position of Phe<sup>4</sup> results in a marked potentiation of the inhibitory effect on electrically induced contractions of guinea-pig ileum (GPI) and mouse vas deferens (MVD), and substitution of fluorine for the hydroxyl group of Tyr<sup>1</sup> or introduction of a trifluoromethyl group at the *p*-position of Phe<sup>4</sup> markedly reduces the inhibitory effect in both preparations.<sup>3)</sup>

Using newly synthesized fluorinated enkephalin (Enk) analogues, [2*R*, 4*R*], [2*R*, 4*S*], [2*S*, 4*S*] and [2*S*, 4*R*] trifluoro-Leu<sup>5</sup>-Enk, the analgesic potency and the selectivity for opioid receptor (subtypes) were investigated *in vivo* and *in vitro*.

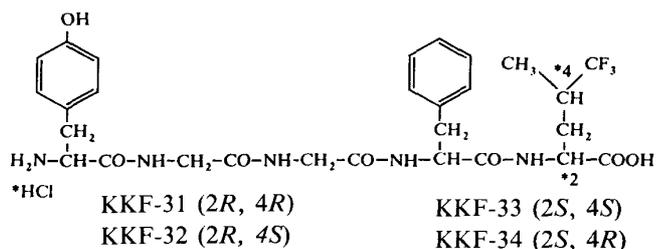


Fig. 1. Chemical Structure of Fluorinated Leu<sup>5</sup>-Enk (KKF-31, 32, 33 and 34)

## Materials and Methods

**Compounds** — Fluorinated D-Leu<sup>5</sup>-Enk analogues, KKF-31 and 32, and L-Leu<sup>5</sup>-Enk analogues, KKF-33 and 34 (Fig. 1) were synthesized by the solution method. L-Leu<sup>5</sup>-Enk, D-Leu<sup>5</sup>-Enk, DALDA (Tyr-D-Arg-Phe-Lys-NH<sub>2</sub>, a typical  $\mu$ -agonist)<sup>4)</sup> and DTLET (Tyr-D-Thr-Gly-Phe-Leu-Thr, a typical  $\delta$ -agonist)<sup>5)</sup> were also synthesized and purified by Kawasaki *et al.* Naloxone-HCl (Sigma) and naltrindole (A gift from H. Nagase, Toray)<sup>6)</sup> were used as specific  $\mu$ - and  $\delta$ -antagonist, respectively.

**In Vitro Experiments** — The longitudinal muscle with myenteric plexus of GPI was prepared as described by Rang,<sup>7)</sup> and MVD was isolated as reported by Hughes *et al.*<sup>8)</sup> Inhibitory effects of the test compounds on the electrically induced contractions of both preparations were estimated from concentration-response curve and expressed as IC<sub>50</sub>. The pA<sub>2</sub> values for each antagonist was calculated according to the method of Schild.<sup>9)</sup> Details of the procedures were reported in our previous paper.<sup>10)</sup>

**In Vivo Experiments** — Male ddY strain mice weighing 22–25 g were used. They were kept in a room maintained at 22 ± 1 °C with free access to food and water. The test compound was dissolved in saline so that the dose was contained in 10  $\mu$ l and injected intracerebroventricularly (i.c.v.) according to the method of Haley and McCormick.<sup>11)</sup> Naloxone, 1 mg/kg, or

naltrindole, 10 mg/kg, was administered intraperitoneally (i.p.) 10 min prior to the injection of the test compound. The analgesic effect of the compounds was measured every 15 min for the duration of 60 min, by the modified Haffner's method.<sup>12)</sup> The effect was expressed as the area under the curve (AUC) by plotting the increases in response time (s) on the ordinate and the time intervals (min) on the abscissa. The analgesic effect (AUC) of the compounds in saline pretreated group were compared with those in naloxone or naltrindole pretreated animals using Student's or Welch's *t*-test, and a *p* value of 0.05 or less was considered significant.

## Results

### In Vitro Experiments

All compounds dose-dependently produced an inhibitory effect on the electrically induced contractions of both GPI and MVD preparations. The IC<sub>50</sub> values and the relative potencies are summarized in Table I. The inhibitory effects of KKF-compounds including D-Leu<sup>5</sup>-Enk were relatively lower than that of L-Leu<sup>5</sup>-Enk in both GPI and MVD preparations, except the inhibitory effect of KKF-34 was 38% more potent than that of L-Leu<sup>5</sup>-Enk in MVD preparations. Expectantly, the inhibitory effect of DALDA was higher in GPI and DTLET in MVD, suggesting the selectivity for different opioid receptors.

In GPI preparations, the effect of all com-

TABLE I. Inhibitory Effect of the Fluorinated Compounds on the Electrically Induced Contractions of Isolated Smooth Muscle Preparations

| Compound                | GPI |                       |                   | MVD |                       |                   | IC <sub>50</sub> (GPI)/<br>IC <sub>50</sub> (MVD) |
|-------------------------|-----|-----------------------|-------------------|-----|-----------------------|-------------------|---|
|                         | N   | IC <sub>50</sub> (nM) | Relative activity | N   | IC <sub>50</sub> (nM) | Relative activity |   |
| D-Leu <sup>5</sup> -Enk | 13  | 1000 ± 60             | 21                | 10  | 21.8 ± 1.5            | 47                | 45.9  |
| KKF-31                  | 3   | 2650 ± 430            | 8                 | 5   | 113 ± 6.5             | 9                 | 23.5  |
| KKF-32                  | 4   | 4310 ± 1310           | 5                 | 4   | 74 ± 10.6             | 14                | 58.2  |
| L-Leu <sup>5</sup> -Enk | 7   | 209 ± 30              | 100               | 10  | 10.2 ± 1.3            | 100               | 20.5  |
| KKF-33                  | 4   | 398 ± 74              | 53                | 6   | 25 ± 1.4              | 41                | 15.9  |
| KKF-34                  | 3   | 1010 ± 20             | 21                | 7   | 7.4 ± 0.8             | 138               | 136   |
| DALDA                   | 6   | 155 ± 13              | 135               | 8   | 830 ± 91.3            | 1                 | 0.19  |
| DTLET                   | 10  | 19.7 ± 1.6            | 1060              | 6   | 0.07 ± 0.001          | 14600             | 281   |

TABLE II. Antagonism by Naloxone and Naltrindole to the Inhibitory Effect of Test Compounds on the Electrically-Induced Contractions of Isolated Smooth Muscle Preparations

| Compound                | GPI         |             | MVD         |              |
|-------------------------|-------------|-------------|-------------|--------------|
|                         | Naloxone    | Naltrindole | Naloxone    | Naltrindole  |
| D-Leu <sup>5</sup> -Enk | 8.72 ± 0.06 | 7.76 ± 0.09 | 8.39 ± 0.03 | 9.41 ± 0.04  |
| KKF-31                  | 8.67 ± 0.07 | 7.77 ± 0.03 | 8.22 ± 0.02 | 8.99 ± 0.15  |
| KKF-32                  | 8.49 ± 0.11 | 7.74 ± 0.05 | 8.20 ± 0.10 | 8.93 ± 0.05  |
| L-Leu <sup>5</sup> -Enk | 8.56 ± 0.03 | 7.45 ± 0.03 | 7.45 ± 0.03 | 8.79 ± 0.10  |
| KKF-33                  | 8.54 ± 0.07 | 7.71 ± 0.08 | 7.97 ± 0.03 | 9.15 ± 0.12  |
| KKF-34                  | 8.71 ± 0.05 | 7.90 ± 0.03 | 7.84 ± 0.11 | 9.20 ± 0.11  |
| DALDA                   | 8.95 ± 0.13 | 7.44 ± 0.13 | 8.89 ± 0.05 | 7.96 ± 0.06  |
| DTLET                   | 8.21 ± 0.07 | 8.43 ± 0.01 | 7.50 ± 0.10 | 10.23 ± 0.25 |

The  $pA_2$  values for the antagonists were calculated according to the method of Schild and expressed as the mean  $\pm$  S.E.M.

pounds were antagonized by naloxone with relatively high  $pA_2$  values and, except DTLET, were rather resistant against naltrindole with one order less  $pA_2$  values. On the other hand, in MVD preparations the  $pA_2$  values of naltrindole for KKF-compounds were comparably higher than the values of naloxone. Naltrindole for DTLET showed the highest  $pA_2$  value while

that for DALDA the lowest, and the  $pA_2$  values of naloxone for these typical compounds were opposite to the result of naloxone (Table II).

#### In Vivo Experiments

The antinociceptive effect of DALDA, DTLET, L-Leu<sup>5</sup>-Enk, D-Leu<sup>5</sup>-Enk and KKF-compounds following i.c.v. administration was measured. DALDA, DTLET, D-Leu<sup>5</sup>-Enk,

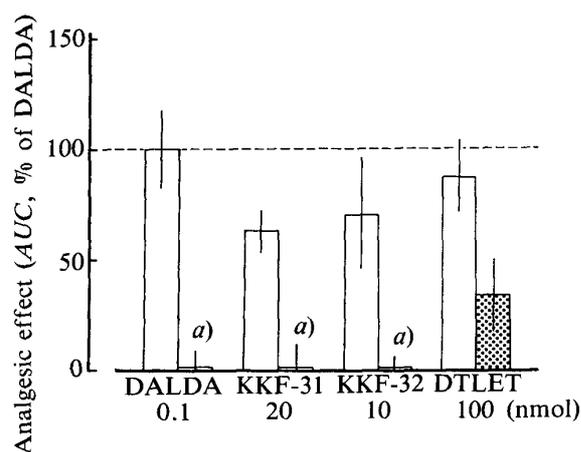


Fig. 2. Effect of Naloxone on i.c.v. DALDA, KKF-31, KKF-32 and DTLET Induced Analgesia

Mice were pretreated with saline ( $\square$ ) or with 1 mg/kg of naloxone ( $\text{hatched}$ ) i.p., 10 min before the test compound given by i.c.v. The analgesic effect was measured by the tail pinch method and was expressed as the % of the effect of DALDA shown by AUC. Figures are the doses of compounds. Each value indicates the mean  $\pm$  S.E.M. of at least 10 mice. *a*)  $p < 0.01$ , compared with saline pretreated group.

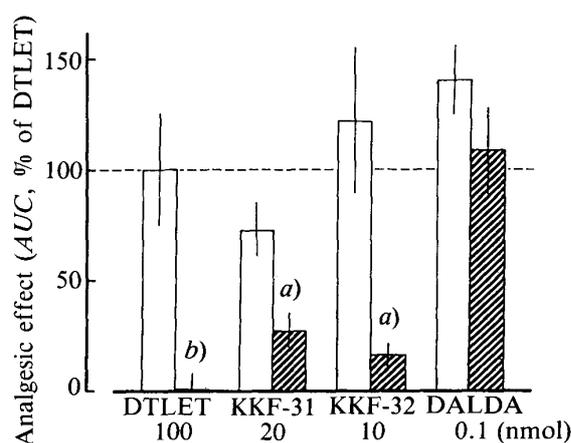


Fig. 3. Effect of Naltrindole on i.c.v. DTLET, KKF-31, KKF-32 and DALDA Induced Analgesia

Mice were pretreated with saline ( $\square$ ) or with 10 mg/kg of naltrindole ( $\text{hatched}$ ), i.p., 10 min before the test compound given by i.c.v. The analgesic effect was measured by the tail pinch method and was expressed as the % of the effect of DTLET shown by AUC. Figures are the dose of compound. Each value indicates the mean  $\pm$  S.E.M. of at least 10 mice. *a*)  $p < 0.05$ , *b*)  $p < 0.01$ , compared with saline pretreated group.

KKF-31 and KKF-32 dose-dependently produced analgesia lasting for 45–60 min with a peak time of analgesia 15–30 min after the injection at appropriate doses up to 100 nmol/mouse. The *AUCs* of KKF-31 and KKF-32 at doses of 20 and 10 nmol/mouse were  $53.1 \pm 11.0$  and  $60.5 \pm 21.2$ , respectively. These effects were comparable to DALDA (0.1 nmol/animal, *AUC*  $84.1 \pm 20.0$ ) and DTLET (100 nmol/mouse, *AUC*  $73.4 \pm 15.2$ ). However, no appreciable analgesic effect of KKF-33 and 34 was found up to the doses of 100 nmol/mouse. Pretreatment with naloxone, 1 mg/kg, i.p., completely antagonized the analgesic effect of KKF-31, KKF-32 and DALDA, and partially but not significantly that of DTLET (Fig. 2). On the other hand, naltrindole, 10 mg/kg, i.p., had no effect on the analgesia induced by DALDA, while it significantly suppressed DTLET-, KKF-31- and 32-induced analgesia (Fig. 3). Meanwhile, D-Leu<sup>5</sup>-Enk at a dose of 100 nmol produced analgesia (*AUC*  $82.2 \pm 21.3$ ) which is about 1/10 as potent as that of KKF-32; L-Leu<sup>5</sup>-Enk did not show any appreciable analgesia at the same dose (*AUC*  $11.3 \pm 22.2$ ).

### Discussion

In line with the earlier reports on DALDA,<sup>5</sup> and DTLET,<sup>6</sup> we found that DALDA possesses a typical  $\mu$ -opioid receptor agonistic property and DTLET a typical  $\delta$ -receptor agonist characteristic, from the fact that  $IC_{50}(\text{GPI})/IC_{50}(\text{MVD})$  ratio of DALDA and DTLET was 0.19 and 281, respectively, as shown in Table I. The  $pA_2$  values also support the selectivity of these typical compounds for the receptors. The KKF-compounds were also studied in *in vitro* experiments to estimate the  $IC_{50}$  values, and  $pA_2$  values of naloxone and naltrindole. The  $IC_{50}$  values of GPI and MVD indicate all KKF-compounds, compared with the respective parent compound L-Leu<sup>5</sup>-Enk and D-Leu<sup>5</sup>-Enk, showed decreased potencies in inhibiting the contractions of the tissue preparations with the exception of a slight increase in potency of KKF-34 in MVD preparations. The results, taken together, indicate that the L-configuration at Leu<sup>5</sup> retains relatively higher activity in both

preparations than a D-configuration at Leu<sup>5</sup> with or without fluorination at position 4 of Leu<sup>5</sup>. On the other hand, the selectivity of KKF-34 for  $\delta$ -receptor was 7 times as high as that of L-Leu<sup>5</sup>-Enk, whilst there was no substantial differences in the selectivity between KKF-33 and L-Leu<sup>5</sup>-Enk, and KKF-31, 32 and their parent compound D-Leu<sup>5</sup>-Enk. Thus, in the case of L-Leu<sup>5</sup>-Enk the conformational changes of the peptide rather than the alternation of molecular size and/or electron density due to the introduction of fluorine at Leu<sup>5</sup> may affect the opioid receptor selectivity *in vitro*.

The analgesic effect induced by DALDA, KKF-31 and 32 was antagonized by naloxone. Naltrindole completely suppressed DTLET- and KKF-32-induced analgesia, and partially but significantly attenuated the KKF-31-induced analgesia. The results suggest that the analgesic effect of KKF-31 and KKF-32 is produced by the mediation of not only  $\mu$ - but also  $\delta$ -opioid receptor mechanisms.

Interestingly, i.c.v. KKF-31 and 32 at doses of 20 and 10 nmol, respectively, as well as i.c.v. DALDA, 0.1 nmol, and DTLET, 100 nmol, produced analgesic effect; however, no appreciable analgesic effect was produced by KKF-33 or KKF-34 up to the doses of 100 nmol/animal. As shown in Table I, KKF-33 and KKF-34 which are incapable of producing analgesia possess higher affinity for  $\mu$ - and  $\delta$ -opioid receptors than KKF-31 and KKF-32 in the inhibitory effect on the contractions in GPI and MVD. Thus, it is unlikely that the inhibitory effect of KKF-31, 32 and their selectivity for opioid receptors in the tissue preparations reflect the analgesic effect of the compounds.

Although the pharmacological mechanism through which KKF-31 and KKF-32 produce the analgesic effect is uncertain, it may be possible to explain the differences between KKF-31, 32 and KKF-33, 34 in producing analgesia as attributable to two distinct parent fluorinated compounds, *i.e.* D-Leu<sup>5</sup>-Enk for KKF-31 and 32, and L-Leu<sup>5</sup>-Enk for KKF-33 and 34. In fact, we demonstrated here that i.c.v. D-Leu<sup>5</sup>-Enk was capable of producing analgesia but L-Leu<sup>5</sup>-Enk was not, presumably resulting from the stability of D-Leu<sup>5</sup>-Enk for enzyme degradation, com-

pared with that of L-Leu<sup>5</sup>-Enk. It is therefore suggested that the fluorination of D-Leu<sup>5</sup>-Enk, differed from the *in vitro* effect, results in the potentiation of its analgesic activity *in vivo*.

Thus, KKF-31 and KKF-32 produce naloxone- or naltrindole-reversible analgesia irrespective to *in vitro*  $\mu$ - and  $\delta$ -opioid activity, and it is evident that caution must be given to the extrapolation of the nature of opioid activity and selectivity of compounds *in vivo* from their profile *in vitro*.

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