JPET Fast Forward. Published on March 1, 2004 as DOI: 10.1124/jpet.104.065391

FE200041: A Peripheral Efficacious Kappa Opioid Agonist with unprecedented selectivity

Todd W. Vanderah, Claudio D. Schteingart[1], Jerzy Trojnar[1], Jean-Louis Junien[2], Josephine Lai and

Pierre J-M. Riviere[1]

University of Arizona, Department of Pharmacology, Tucson, Arizona 85724

JPET Fast Forward. Published on March 1, 2004 as DOI: 10.1124/jpet.104.065391
This article has not been copyedited and formatted. The final version may differ from this version.

RUNNING TITLE: A Highly Selective Peripheral Kappa Opioid Agonist

CORRESPONDING AUTHOR:

Todd W. Vanderah, Ph.D.

Department of Pharmacology

University of Arizona Health Sciences Center

Tucson, AZ 85724 (USA)

PHONE (520) 626-7801

FAX: (520) 626-4779

E-MAIL: vanderah@u.arizona.edu

TEXT PAGES: 16

TABLES: 1

FIGURES: 6

REFERENCES: 49

WORD COUNT:

ABSTRACT: 250

INTRODUCTION: 723

DISCUSSION: 1044

ABBREVIATIONS: hKOR, human kappa opioid receptor; hMOR, human mu opioid receptor; hDOR, human delta opioid receptor

JPET Fast Forward. Published on March 1, 2004 as DOI: 10.1124/jpet.104.065391
This article has not been copyedited and formatted. The final version may differ from this version.

TABLE OF CONTENTS: Neuropharmacology or Behavioral Pharmacology

JPET Fast Forward. Published on March 1, 2004 as DOI: 10.1124/jpet.104.065391
This article has not been copyedited and formatted. The final version may differ from this version.

ABSTRACT

The side-effects typically associated with the clinical profiles of opioid μ receptor agonists have driven continuing efforts to identify novel efficacious analgesics including agonists acting at opioid kappa (κ) receptors. Unfortunately, the therapeutic potential of κ- agonists appear limited by significant central nervous system side effects. Kappa (κ) opioid agonists, however, exhibit potent peripherally-mediated antihyperalgesic and antinociceptive effects, suggesting that a peripherally acting κ agonist may be efficacious in pain control with a more desirable safety profile than associated with currently available opioids. Here, we report an all D-amino acid tetrapeptide characterized as a novel, highly selective κ opioid receptor agonist. FE200041 showed selectivity for the hKOR opioid receptor of greater than 30,000- and 68,000-fold vs hMOR and hDOR receptors, respectively, and efficacious agonists activity using in vitro tissue assays. FE200041 produced local, peripheral antinociception in the hindpaw ipsilateral, but not contralateral, to injection. Antinociceptive effects of FE200041in the mouse acetic acid writhing assay lasted over 60 minutes and were antagonized by naloxone and by selective κ, but not μ, opioid receptor antagonists. FE200041 significantly inhibited acetic acid writhing and inhibited formalin-induced flinching in rats. FE200041 did not elicit sedation or motor impairment after systemic administration at a dose 10-fold higher than that needed to achieve antinociception. FE200041 is thus a potent peripherally restricted opioid κ agonist with no demonstrable side-effects typical of κ agonists with CNS activity and with unprecedented selectivity for the opioid κ receptor. The pharmacology of this compound suggests the possibility of therapeutic application.

JPET Fast Forward. Published on March 1, 2004 as DOI: 10.1124/jpet.104.065391
This article has not been copyedited and formatted. The final version may differ from this version.

INTRODUCTION

Three types of opioid receptors termed μ, δ and κ have been identified (Chen et al., 1993; Thompson et al., 1993; Evans et al., 1992; Kieffer et al., 1992; Meng et al., 1993; Fukuda et al., 1993; Zhu et al., 1995; Yasuda et al., 1993; Martin et al., 1976) and found to be expressed in both the CNS and in the periphery (Simonin et al., 1995; Thompson et al., 1993; Bagnol et al., 1997; Stein, 1993). Opioid analgesics that are currently used clinically act primarily at the μ receptor. The unwanted side-effects associated with μ opioids include respiratory depression, euphoria/dysphoria, dependence, constipation and urinary retention (Pasternak, 1993). Previous studies have shown that unlike agonists at δ and μ receptors, agonists at κ opioid receptors do not elicit constipation, urinary retention or euphoria. Hence, κ opioid agonists have been suggested to have potential for treatment of incisional/inflammatory pain, burn injury pain (Field et al., 1999; Wilson et al., 2000), neuropathic pain (Catheline et al., 1998; Przewlocki and Przewlocka, 2001; Walker et al., 1999) visceral pain including dysmenorrhea or gastrointestinal pain (Delgardo-Aros et al., 2002; Kamp et al., 2003), and rheumatoid arthritis (Endoh et al., 2000; Spetea et al., 2002). Clinically-active κ opioid agonists such as pentazocine and butorphanol appear to produce better analgesia in women than men in dental surgery pain (wisdom tooth extraction) (Gear, et al., 1996a,b) suggesting potential advantages of such compounds in some populations of patients. Additionally, studies in humans with enadoline (CI-977)(Hunter et al., 1990) a highly selective and potent opioid κ agonist, resulted in analgesia but was dose-limited due to neuropsychiatric side effects (Pande et al., 1996). In spite of such potential applications, clinical studies have shown that opioid κ receptor agonists elicit severe centrally-mediated side-effects generally described as "dysphoric actions" (Pfeiffer et al., 1986). These side-effects have apparently halted further clinical development for this class of compounds.

Many studies have shown that opiates have peripheral analgesic effects, especially under

JPET Fast Forward. Published on March 1, 2004 as DOI: 10.1124/jpet.104.065391
This article has not been copyedited and formatted. The final version may differ from this version.

inflammatory or hyperalgesic conditions (Barber and Gottschlich, 1992; Junien and Wettstein, 1992 and Stein, 1993). Agonists at κ opioid receptors have been shown to produce analgesia and decrease inflammation in models of rheumatoid arthritis after local administration (Wilson et al., 1996). Restricted CNS penetration is a common strategy to reduce central side-effects of drugs with beneficial peripheral actions. For example, the antihistamines terfenadine, astemizole and mequitazine do not cross the blood-brain barrier at therapeutic doses (Nicholson, 1987). Similar techniques have been attempted in the development of peripherally restricted κ opioid agonists, including ICI 204448 (Shaw et al., 1989), GR 94839 (Rogers et al., 1992) and EMD61753 (asimadoline) (Barber et al., 1994). When asimadoline was tested in humans after postoperative knee surgery the patients tended to report an increase in pain (Machelska et al., 1999). Unfortunately, these compounds were discontinued in clinical trials due to either poor bioavailability, lack of efficacy or CNS side effects at analgesic doses (Barber and Gottschlich, 1997). Peptidic κ opioid agonists including E-2078 (Tachibana et al., 1988) and SK-9709 (Ambo et al., 1995), have been developed as analgesics and utilized years after their synthesis with the idea that a peptide would be less likely to cross the blood brain barrier, however studies demonstrated that both of these dynorphin peptidic fragments crossed the blood brain (Yu et al., 1997; Hiramatsu et al., 2001).

Recently Dooley et al. (1998) reported the discovery of a high affinity (Ki < 1 nM) and selective (μ/κ and δ/κ ratios of >3000) κ opioid agonist using mixture based positional scanning of a combinatorial tetrapeptide library. The tetrapeptide is composed entirely of D-amino acids and was identified as an agonist by its inhibition of forskolin-stimulated cAMP formation using R1G1 thymoma cell line membranes. We have further characterized this tetrapeptide, now termed FE200041, by radioligand binding and in vitro functional assays on transfected cells that express the human κ, μ and δ opioid receptors. Additionally, FE200041 was studied in models of somatic as well as inflammatory pain in both

JPET Fast Forward. Published on March 1, 2004 as DOI: 10.1124/jpet.104.065391
This article has not been copyedited and formatted. The final version may differ from this version.

mice and rats and CNS effects were studies after peripheral administration. The data demonstrate that FE200041 is a highly selective κ opioid antinociceptive agent without central nervous system side effects at doses higher than those needed to elicit antinociception. The peripheral antinociceptive actions of FE200041thus suggest that it is possible to develop peripherally restricted opioid peptides for use in controlling pain.

JPET Fast Forward. Published on March 1, 2004 as DOI: 10.1124/jpet.104.065391
This article has not been copyedited and formatted. The final version may differ from this version.

MATERIALS AND METHODS

Radioligand binding

All radioligands were from NEN (Boston, MA). Radioligand binding analysis was carried out as described previously (Lai et al., 1994) using crude membrane preparations from HN9.10 cells that express the human κ, μ, or δ opioid receptor. The membranes were resuspended in ice-cold Tris-buffer (50 mM, pH7.4) containing 0.5% bovine serum albumin (BSA), 30 μM bestatin, 10 μM captopril, 50 μg/mL bacitracin, 100 μM phenylmethylsulfonylfluoride (PMSF). To characterize the binding affinity of [^{3}H]FE200041 (23.6 Ci/mmol) for the human κ opioid receptor, the rate of association of the radioligand was determined by incubating membranes (~50 μg) from transfected cells that express the human κ opioid receptor with 0.8 nM [^{3}H]FE200041for 0.5, 1, 5, 10, 15, 30, 60, 90 and 120 min at 25°C, done in triplicates. The rate of dissociation was determined by incubating membranes with 0.8 nM [^{3}H]FE200041for 2 hr at 25°C, followed by incubation with 10 μM naloxone for 0.5, 1, 5, 10, 15, 30, 50 and 90 min, done in triplicates. The dissociation constant (k_{-1}) was calculated by plotting $\ln[B_t / B_0]$ against t, where B_0 and B_t are the amounts of radioligand bound at time zero and specific time, t, after the addition of naloxone to obtain the slope of $-k_{-1}$. The association rate constant (k_{+1}) was based on the equation: $k_{on} = k_{-1} + k_{+1}[L]$, where [L] is the concentration of the radioligand, and k_{on} was determined by plotting $\ln[(B\infty - B_t) / B\infty]$ against t, where $B\infty$ is the amount of radioligand bound at equilibrium and B_t is that at time t, to obtain the slope of $-k_{on}$. The dissociation constant (K_D) for [^{3}H]FE200041 was then calculated by the equation: $K_D = k_{-1}[L] / (k_{on} - k_{-1})$ and expressed as the mean ± S.E.M. of 3 independent experiments.

JPET Fast Forward. Published on March 1, 2004 as DOI: 10.1124/jpet.104.065391
This article has not been copyedited and formatted. The final version may differ from this version.

For equilibrium saturation analysis, membranes were incubated with 12 concentrations of [^{3}H]FE200041 (12.5 pM - 4000 pM) for 90 min at 25°C in the presence or absence of 10 μM naloxone. The total reaction volume was 1 mL/assay tube and all reactions were carried out in duplicates. At the end of the incubation, reactions were terminated by rapid filtration through Whatman GF/B filters (presoaked in polyethyleneimine) and washed with 2 X 4 mL of ice cold 50 mM Tris. For competition analysis,10 concentrations of FE200041 and either 1.6 nM [^{3}H]U69,593 for κ opioid binding, 1 nM [^{3}H]DAMGO for μ opioid binding or 1 nM [^{3}H]pCl-DPDPE for δ opioid binding, were carried out using membranes from transfected cells that express the κ, μ, or δ receptor, respectively. Non-specific binding was defined as the amount of radioligand bound in the presence of 10 μM naloxone. Reactions were carried out in duplicates for 3 hr at 25°C and terminated by rapid filtration as above. Radioactivity was determined by liquid scintillation counting. Data were fitted by non-linear least squares analysis using GraphPad Prism (v3.0). All analyses were based on 3 independent experiments.

GTPγS Binding

GTPγS binding was done using membranes from cells that express the human kappa opioid receptor. The procedure for this analysis was based on that of Lorenzen et al (1993). Reactions were initiated by the addition of an aliquot of membrane preparation (15 μg) to a final volume of 300 μL incubation mix (50 mM HEPES, pH.7.4, 1 mM EDTA, 5 mM $MgCl_2$, 30 μM GDP, 1 mM dithiothreitol, 100 mM NaCl, 100 μM PMSF, 0.1 % BSA, 0.1 nM [^{35}S]GTPγS (1250 Ci/mmol) and indicated concentration range of agonist and incubated for 60 min at 30°C. Basal level of [^{35}S]GTPγS binding was defined as the amount bound in the absence of agonist. Non-specific binding was determined in the presence of 10 μM unlabeled GTPγS. Reactions were performed in triplicate and terminated by rapid

JPET Fast Forward. Published on March 1, 2004 as DOI: 10.1124/jpet.104.065391
This article has not been copyedited and formatted. The final version may differ from this version.

filtration through Whatman GF/B filters presoaked in water followed by 4 washes with ice-cold wash buffer (50 mM Tris, 5 mM $MgCl_2$, 100 mM NaCl, pH 7.4). The radioactivity was determined by liquid scintillation counting. Data were fitted by non- linear least squares analysis using GraphPad Prism.

Animals

Male ICR mice (20-25g) and male Sprague Dawley rats (200 to 250g) were used in all experiments. Animals were purchased from Harlan (Indianapolis, IN), housed on a regular 12 hr light/dark cycle (lights on at 06:00 h), in a climate-controlled room with food and water *ad libitum.* All procedures were in accordance with the policies and recommendations of the International Association for the Study of Pain and the National Institutes of Health guidelines and use of laboratory animals as well as approved by the Animal Care and Use Committee of the University of Arizona. Groups of 6 to15 were used in all experiments.

Mouse isolated vas deferens

Male ICR mice (20-25g) were used in all experiments. Mice were lightly anesthetized using ether and sacrificed. Tissue preparation was performed as previously described (Hughes et al., 1975). Briefly, the vas deferens was isolated and mounted in organ baths containing 20 ml of oxygenated Krebs buffer solution omitting the $MgSO_4$ at 37°C. Using a Grass S48 stimulator the tissues were stimulated transmurally between platinum wire electrodes at 0.1 Hz, with 2.0 msec pulses at supramaximal voltage. Contractions were recorded on a Grass 7D polygraph. Test compounds were added to the baths in 14-60 μL volumes. Each dose remained in bath for 4 min or until maximal inhibition was reached. Subsequent doses were added cumulatively to the bath at 4 min intervals to produce a concentration-response curve. Tissues were then washed extensively with fresh buffer until the original contraction height was re-established.

JPET Fast Forward. Published on March 1, 2004 as DOI: 10.1124/jpet.104.065391
This article has not been copyedited and formatted. The final version may differ from this version.

Injections

For intravenous (i.v.) administration, animals were placed in restrainers and injections were made using a disposable 1 cc syringe equipped with a 30 gauge disposable needle. The needle was inserted into the tail vein at a 25 degree angle and a small amount of blood drawn back into the syringe before injection of either compounds or vehicle in order to assure injection into the vein. Injection of compounds or vehicle was performed over a 5 sec period for consistency purposes. After injection the needle was removed from the tail vein and gentle pressure was applied at the site of injection in order to prevent loss of fluid from the site of injection. Subcutaneous (s.c.) injections were performed by manually holding the animal and inserting a 30 gauge disposable needle on a disposable 1 cc syringe into the abdominal region of the animal assuring that the needle remained between the muscle and the skin of the animal. Injections of compounds were performed over a 5 sec period and were noted as positive by the development of an out-pocketing of the skin at the site of injection. Intraperitoneal (i.p.) injections were made in similar fashion to the s.c. injections except that the needle was placed completely under the muscle of the abdominal region and an out-pocketing of the skin was not seen. Injections into the hindpaw (i.paw.) of the animal were made using a Hamilton 100 ml syringe and a 30 gauge disposable needle. Injections into the paw were made on the plantar surface just below the skin in a volume of 50 µl/animal.

Acetic Acid Writhing Assay

As a measure of peripheral antinociception, FE200041 as well as the reference κ opioid agonists U50,488H and asimadoline were tested using the acetic acid writhing test. Animals were fasted 12 to 16 hours prior to testing. The nociceptive behavior (writhes) was induced by diluted acetic acid (0.6%, 10 ml/kg, for mice and 2.5%, 0.5 ml/rat) administered intraperitoneally (i.p.) at time 0 min. In all experiments with mice, compounds were given intravenously (i.v.) in the tail 5 minutes prior to acetic acid

JPET Fast Forward. Published on March 1, 2004 as DOI: 10.1124/jpet.104.065391
This article has not been copyedited and formatted. The final version may differ from this version.

administration to determine the activity and potency, as well as at different intervals prior to acetic acid in order to determine their duration of action using a sub-maximally effective dose (A_{80}). In all experiments with rats, compounds were given subcutaneously (s.c.) 15 min prior to acetic acid administration to determine the activity and potency. The number of writhes were counted over a 15 min period starting from the time of acetic acid injection. Activity is expressed as a percentage and is calculated as follows: Antinociception (%) = 100 x [(writhes in control group - writhes in treated group)/writhes in control group]. Potency (A_{50}) is calculated from the full dose response curve. Antagonist studies were performed in mice as above with antagonist administered prior to FE200041. The opioid antagonist naloxone (1 mg/kg) was given by the s.c. route 15 min prior to FE200041. The μ selective, non-equilibrium opioid receptor antagonist β-FNA (10 mg/kg) was administered by the s.c. route 24 hours prior to FE200041 administration (Jiang et al., 1989). The κ opioid receptor antagonist norBNI (10 mg/kg) (Marchand et al., 2003) was administered by the s.c. route 15 min prior to FE 200041 administration (Portoghese et al., 1987;. In addition, to determine a peripheral and non-CNS effect, the κ opioid receptor antagonist norBNI (10 μg/5μl) (Horan et al., 1992) was administered by the i.c.v. route 10 min prior to an A_{90} FE200041 administration. To achieve a time course, separate groups of mice were pretreated with an A_{80} dose of FE 200041 at either 5, 10, 15, 30 ,60 or 120 minutes prior to receiving an i.p. injection of acetic acid. At all time points, the number of writhes were counted for 15 min immediately after the administration of acetic acid.

Formalin Flinch Test

As a measure of antinociception in both acute and inflammatory (tonic) pain, FE200041 was tested using the 2% formalin flinch assay in rats. Animals were habituated to the environment for

JPET Fast Forward. Published on March 1, 2004 as DOI: 10.1124/jpet.104.065391
This article has not been copyedited and formatted. The final version may differ from this version.

approximately 30 min prior to testing. The formalin test was carried out in a 30 x 30 x 30 cm chamber with the front and bottom of the chambers made of clear plexiglass. Mirrors were placed at a 45 degree angle under the chambers to give an unobstructed view of the paws. Animals were restrained manually and 2% formalin (made fresh the day of the experiment) was injected subcutaneously (50 μl) into the plantar surface of the left hind paw with a 30 gauge needle. The number of paw flinches was then recorded in 5 min intervals from the time of injection for a 60 min period. Elevations of the paw, licking and biting of the injected paw were counted as "flinches". The acute or "first phase" of the nociceptive response peaked at 5 min after formalin injection and the inflammatory or "second phase" peaked at 30 min after formalin injection. The number of flinches in the first 10 min were representative of the acute phase and the number of flinches from 10 min until 60 min were representative of the second phase. FE200041 or vehicle were administered by the i.v. route 5 minutes prior to formalin injection. Antinociception for either phase I or phase II was calculated as follows: % Antinociception = 100 [(# of flinches in control animal - # of flinches in drug treated animal)/(# of flinches in control animal)]. Potency (A_{50}) is calculated from the full dose response curve for both phase I and phase II.

Radiant heat paw withdrawal assay

In order to evaluate the peripheral activity of FE200041 we tested the local administration of FE200041and hind-paw withdrawal latencies using a thermal stimulus in mice. The method of Hargreaves et al. (1988) was used to assess paw withdrawal latency to a thermal nociceptive stimulus. Animals were placed in 3cm X 3cm plexiglass boxes on top of a glass plate that was maintained at room temperature. Animals were allowed to habituate for a period of 45 min. For baseline paw withdrawal latencies, a heat source that increased in intensity from a non-noxious temperature to a noxious temperature (occurs within 1 to 16 seconds in naive animals) from under the glass plate was reflected onto

JPET Fast Forward. Published on March 1, 2004 as DOI: 10.1124/jpet.104.065391
This article has not been copyedited and formatted. The final version may differ from this version.

the plantar surface of the right hind paw with the focus of the light beam being no larger than a 3 to 5 mm diameter and the time to withdrawal the paw from the heat source was recorded. A maximum cut-off of seconds was used to prevent tissue damage. Animals were administered an A_{80} dose (10 μg/5 μl, i.paw) of FE200041 or vehicle into the dorsal side of the right hind paw and paw withdrawal latencies were tested at 15 min intervals over a 60 minute period. In order to determine a local, peripheral effect of FE200041, the agonist or vehicle was injected into the left hind-paw and paw withdrawal latencies were tested at 15 min intervals over a 60 minute period. Antinociception was calculated as follows: % Antinociception = 100 [(test paw withdrawal latency - baseline paw withdrawal latency)/(cut off for paw withdrawal latency of 40 seconds - baseline paw withdrawal latency)].

Rotarod test

In order to evaluate CNS effects including sedation or non-specific motor effects, animals were tested for their ability to balance on a slowly rotating rod (rate of rotation was 10 revolutions per min; diameter was 1.5 cm for mice and 6.5 cm for rats) after peripheral administration of test compounds or vehicle. Animals were conditioned prior to the experiment and selected animals (those remaining on the rotarod for 180 sec) were injected by either the i.v. (mice) or s.c. (rats) route with test compounds. Animals were tested either 5, 10 and 15 min after i.v. or 15, 20, 25 min after s.c. administration. The latency to fall off the rod was recorded with 180 seconds as the cut-off. Animals falling off the rod before the 180 sec cut-off were considered to have motor impairment. Activity is expressed as a percentage and is calculated as follows; % activity = 100 x [(latency to remain on the rotarod in control group - latency in treated group)/(latency in control group)].

Statistics

Data were analyzed by ANOVA, and where significance was indicated, followed by Student's *t*

JPET Fast Forward. Published on March 1, 2004 as DOI: 10.1124/jpet.104.065391
This article has not been copyedited and formatted. The final version may differ from this version.

test for grouped data. The significance criterion was $p<0.05$ throughout. Results are reported as the mean activity score and S.E.M.

Chemicals

FE 200041 and asimadoline were dissolved in distilled water and provided by Ferring Research Institute Inc. (San Diego, CA). Naloxone was dissolved in distilled water and purchased from Sigma (St. Louis, MO). β-funaltrexamine (β-FNA), (-)U50,488 and nor-binaltorphimine (nor-BNI) were dissolved in distilled water and purchased from Tocris (Ellisville, MO). ICI 174,864 and CTAP (D-Phe-Cys-Tyr-D-Trp- Arg-Thr-Pen-Thr-NH_2) were a gift from the National Institute on Drug Abuse via Multiple Peptide Systems (San Diego, CA.). Radiolabelled [D-Ala^2, $NMePhe^4$, Gly^5-ol]enkephalin (DAMGO) (80 Ci/mmol), U69,593 (69 Ci/mmol), pCl-DPDPE (65 Ci/mmol) and FE200041 (23.6 Ci/mmol) were purchased from NEN (Boston, MA, U.S.A.).

JPET Fast Forward. Published on March 1, 2004 as DOI: 10.1124/jpet.104.065391
This article has not been copyedited and formatted. The final version may differ from this version.

RESULTS

[^{3}H]FE200041 exhibits high affinity and selectivity for human KOR (hKOR)

Association and dissociation analysis of [^{3}H]FE200041using transfected cells that expressed the hKOR showed that the peptide had a dissociation constant of 0.43 ± 0.13 nM based on three independent determinations (Fig. 1a,b). This value is in good agreement with the K_D value of 0.8 nM based on equilibrium saturation analysis (Fig. 1c). The receptor density based on the B_{max} value obtained from the saturation analysis was 3.5 pmol/mg of membrane protein. The equilibrium dissociation constants (K_i) for FE200041 for hKOR, hMOR and hDOR based on competition analysis were 0.15 nM, 4.6 μM and > 10 μM, respectively. Thus, FE200041 was over 30,000 fold selective for hKOR over hMOR and over 68,000 fold selective for hKOR over hDOR (Table 1).

FE200041 is a potent and efficacious agonist at the opioid κ receptor

In vitro analysis based on FE200041-induced specific binding of [^{35}S]GTPγS to hKOR-containing cell membranes showed that the peptide activated GTP binding with an EC_{50} value of 1.1 nM and an E_{max} value of 148% over basal level of GTP binding in the absence of FE200041 (Table 1). These values were similar to the fully efficacious kappa agonist U69,593 (EC50 of 1.6 nM and Emax of 86%)(unpublished observations, Vanderah T.W. and Riviere P .J-M.) suggesting that FE200041 acts as a fully efficacious agonist in vitro. The EC_{50} value thus approximated the affinity based on the dissociation constant of FE200041 for hKOR. *In vitro* bioassay using mouse vas deferens (MVD) preparations showed that FE200041 inhibited the electrically evoked smooth muscle contraction in a dose-dependent manner. A complete washout after FE200041administration restored full electrical-induced contraction. The IC_{50}

JPET Fast Forward. Published on March 1, 2004 as DOI: 10.1124/jpet.104.065391
This article has not been copyedited and formatted. The final version may differ from this version.

for FE200041 was 5.8 nM with an E_{max} value of 96% in the presence of the delta antagonist ICI 174,864 (1μM) and in the presence of the μ opioid antagonist CTAP (1μM). FE200041 inhibition of electrically-induced contraction of the MVD was blocked by 1μM naloxone.

FE200041 PRODUCES POTENT ANTINOCICEPTION THROUGH THE K RECEPTOR

FE200041 exhibited antinociceptive activity in assays of acute and persistent nociception such as the acetic acid-induced writhing and formalin-induced paw flinch tests. FE200041, when administered intravenously (i.v.) 5 min prior to 0.6% acetic acid administration into the abdominal region of mice, resulted in a dose-related inhibition of writhes over a 15 min period when compared to animals receiving only vehicle prior to acetic acid (Figure 2). The A_{50} for FE200041 in the acetic acid writhing test was 0.06 mg/kg, i.v. (95% C.I., 0.04-0.11). For comparison the κ opioid agonist (-)U50,488 and asimadoline were also tested in the mouse acetic acid writhing test after i.v. administration using the same dosing schedule as FE200041. (-)U50, 488 resulted in an A_{50} of 0.10 mg/kg, i.v. (95% C.I.,0.06-0.18) and asimadoline resulted in an A_{50} of 0.19 mg/kg, i.v. (95% C.I., 0.14-0.25)(Figure 2). FE200041 was also tested after intraperitoneal (i.p.) injection 5 min prior to 0.6% acetic acid administration in mice resulting in an A_{50} of 2.3 mg/kg (95% C.I., 1.15- 3.57)(data not shown). A time course for FE200041 using the acetic acid writhing test was performed using an A_{80} dose (0.3 mg/kg, i.v.) and testing groups of animals until the antinociceptive response was reduced to 20%. FE200041 showed significant antinociceptive activity given as much as 60 min prior to acetic acid administration (Figure 3). The opioid antagonist naloxone significantly blocked the antinociceptive effects of i.v. administered FE200041 in the mouse acetic acid writhing test; the A_{50} value of FE200041in the presence of naloxone was 1.78 mg/kg (95% C.I. 0.78-

JPET Fast Forward. Published on March 1, 2004 as DOI: 10.1124/jpet.104.065391
This article has not been copyedited and formatted. The final version may differ from this version.

2.18), indicating a 29.6 fold rightward shift in the presence of naloxone. Likewise, an A_{90} (1 mg/kg, i.v.) antinociceptive dose of FE200041 (95.3 ± 1.8% activity) in the mouse acetic acid writhing test was significantly attenuated by pretreatment with the opioid κ receptor selective antagonist, nor-BNI (10 mg/kg, s.c.)(Portoghese et al., 1987), administered 15 min prior to FE200041(29.8 ± 8.7 % activity)(Figure 4). However, the opioid μ receptor selective antagonist β-FNA (10 mg/kg, s.c.)(Jiang et al., 1989), given as a pretreatment 24 hr prior to testing did not attenuate the antinociceptive activity of FE200041 (80.5 ± 5.3 % activity) (Figure 4). In order to elucidate whether the antinociceptive effects of systemic FE200041 was due to penetration into the central nervous system, the selective opioid κ receptor antagonist, nor-BNI (10 μg) was administered directly in to the central nervous system by the i.c.v. route just prior to the systemic administration of FE200041. The A_{90} (1 mg/kg, i.v.) antinociceptive dose of FE200041 in the mouse acetic acid writhing test was not significantly attenuated by the pretreatment with nor-BNI (10 μg, i.c.v.) (88.4 ± 8.4% activity). Antagonists alone had no effect on the number of acetic acid-induced writhes. The s.c. administration of FE200041 also resulted in a dose related inhibition of writhes in rats over a 15 min period when compared to animals receiving only vehicle. Animals were pretreated (15 min) with FE200041 prior to 2.5% acetic acid administration into the abdominal region. The A_{50} for FE200041 in the rat acetic acid writhing test was 0.09 mg/kg, s.c. (95% C.I., 0.02-0.48).

In the formalin test, FE200041 resulted in a dose-related inhibition of 2% formalin-induced flinching in both phase I and phase II of the assay. Hindpaw injection of 2% formalin in vehicle treated animals resulted in a typical response with two phases. The first phase was seen during the first 10 min and the second phase was seen during the period from 10 to 60 min. A dose of 1 mg/kg, i.v., of FE200041 resulted in approximately 95% inhibition of phase I flinching and approximately 80% inhibition in phase

JPET Fast Forward. Published on March 1, 2004 as DOI: 10.1124/jpet.104.065391
This article has not been copyedited and formatted. The final version may differ from this version.

II flinching (Figure 5a,b). The A_{50} for FE200041 in the rat 2% formalin flinching assay was 0.39 mg/kg, i.v. (95% C.I., 0.29-0.55) for phase I and 0.55 mg/kg, i.v. (95% C.I., 0.42-0.73) for phase II.

In order to further characterize the peripheral actions of FE200041, the radiant heat paw withdrawal assay was performed and FE200041(10 μg/rat) was administered to the paw either ipsilateral or contralateral to that paw being tested. The i.paw administration of FE200041 resulted in a fully antinociceptive effect (79.9 ± 7.7% activity) when administered into the ipsilateral paw (the paw of thermal testing). The paw withdrawal latency of the ipsilateral paw after FE200041 administration was 36.2 ± 2.7 sec, significantly higher than the pre-FE200041 administration baseline latency of 14.0 ± 0.5 sec. When the paw contralateral to the side of FE200041 injection was tested, no change in paw withdrawal latency was observed (i.e., 13.8 ± 0.3 sec vs. 13.5 ± 0.4 sec) (Figure 6). Administration of vehicle into the paw either ipsilateral or contralateral to the side of thermal testing did not result in significant changes from baseline paw withdrawal latencies.

FE200041 DID NOT ELICIT SIGNIFICANT CNS EFFECTS

In order to evaluate possible CNS activity of FE200041, mice or rats given the peptide were monitored for signs of sedation or motor impairment based on the rotarod test. For these experiments, FE200041 was administered to mice intravenously (i.v.) at antinociceptive doses and at doses several times higher than those needed to produce antinociception. Antinociceptive doses of FE200041 did not interfere with rotarod performance for the duration of the experiment (180 sec). Similarly, doses of FE200041 10-fold higher than those necessary to achieve antinociception did not interfere with rotarod performance in mice. The A_{50} for FE200041 in the mouse rotarod test was 5.04 mg/kg, i.v. (95% C.I., 3.68-6.89) measured at 5 min after administration, the time of peak antinociceptive action. Thus, the sedation/motor impairment dose response curve for FE200041 in the mouse rotarod test showed greater than a 84-fold

JPET Fast Forward. Published on March 1, 2004 as DOI: 10.1124/jpet.104.065391
This article has not been copyedited and formatted. The final version may differ from this version.

shift to the right when compared to the antinociceptive dose-response curve in the mouse acetic acid writhing test. However, when the κ agonists (-)U50,488 and asimadoline were tested in the mouse rotarod test over the dose range in which these compounds were found to be active in the writhing test only a 2-fold shift to the right for (-)U50,488 (rotarod A_{50} 0.19 mg/kg, i.v.) and a 10-fold shift to the right for asimadoline (rotarod A_{50} 1.94 mg/kg, i.v.) was observed.

Similar results were found in rat rotarod performance. Rats remained on the rotarod for the duration of the experiment (180 sec) at antinociceptive doses of FE200041. Every animal tested remained on the rotarod until cut-off (180 sec) at all doses of FE200041 tested including 1, 3 and 10 mg/kg, s.c. (N=6 for ea. dose). These doses were 10-fold higher than those necessary to produce antinociception in both the rat acetic acid writhing and 2% formalin flinch assay. Thus, no sedation or motor impairment was seen in any of the rats over the duration of the experiment.

JPET Fast Forward. Published on March 1, 2004 as DOI: 10.1124/jpet.104.065391
This article has not been copyedited and formatted. The final version may differ from this version.

DISCUSSION

Pharmacological characterization of FE200041 presented here shows that the all D-amino acid tetrapeptide, FE200041, exhibits high affinity, selectivity and agonist activity at the human κ opioid receptor. The selectivity of this peptide for the hKOR over the hMOR or hDOR of 31,000-68,000-fold is unprecedented, and represents the most selective κ opioid agonist identified to date. In addition, in vitro and in vivo analyses reveal that FE200041 is a potent and efficacious agonist both at the cloned hKOR as well as at the rodent receptor in vivo. These findings support the possibility that FE200041 would likely be active as an analgesic agent in humans.

Previous studies have suggested a potential for therapeutic application of opioid κ agonists. For example, studies with enadoline (CI-977)(Hunter et al., 1990) a highly selective and potent opioid κ agonist, showed that this compound was as active as morphine (10 mg) as an analgesic at a dose of 25 μg in female patients (Pande et al., 1996). However, clinical use of enadoline was dose-limited due to neuropsychiatric side effects (Pande et al., 1996). Asimadoline resulted in a trend toward hyperalgesia on postoperative pain in patients who underwent knee surgery (Machelska et al., 1999). This may very likely be due to the limited doses of asimadoline that could be administered in order to prevent unwanted CNS-induced dysphoric effects. These findings, together with previous reports of dysphoric actions of CNS-penetrating κ agonists (Pfeiffer et al., 1986) as well as the many reports from preclinical investigations showing peripheral antinociceptive actions of κ agonists (Barber et al., 1993; 1994), led to investigations of compounds with restricted access to the CNS.

The present study shows that FE200041 exhibits potent antinociceptive actions following systemic administration. In the acetic acid induced writhing test, which models persistent pain of moderate

JPET Fast Forward. Published on March 1, 2004 as DOI: 10.1124/jpet.104.065391
This article has not been copyedited and formatted. The final version may differ from this version.

intensity, a 1 mg/kg, i.v. dose of FE200041 produced a full antinociceptive effect in both mice and rats. This effect of FE200041 was mediated by opioid receptors as the antinociception was fully blocked by naloxone. The peripheral effects of FE200041 was demonstrated by administering the selective κ antagonist either systemically or centrally. The κ selective antagonist nor-BNI given peripherally reversed the antinociceptive effect of FE200041 but failed to antagonize the antinociceptive actions of FE200041 when the antagonist was administered by the i.c.v. route. These data, together with the failure of the peripherally administered μ opioid receptor antagonist β -FNA, given at doses and times which have previously been shown to block the effects of selective μ opioid agonists (Portoghese et al., 1987; Jiang et al., 1989; Horan et al., 1992; Marchand et al., 2003) to antagonize the antinocicpetive effects of FE200041, provides further confirmation that the actions of FE200041 in vivo are the result of peripheral κ receptor activation. This conclusion is consistent with the selectivity of FE200041 for κ receptors in the in vitro assays as well as the lack of typical kappa opioid induced CNS side effects such as sedation as seen in the rotarod test.

The formalin flinch test (Dubuisson and Dennis, 1977; Dickenson and Sullivan, 1987; Wheeler-Aceto and Cowan, 1991) was employed to determine the possible effect of FE200041 on acute C/Aδ sensory afferent fiber activity (phase I) and subsequent tonic inflammatory pain (phase II). Both μ and κ opioid agonists such as morphine and enadoline, respectively, are effective in the inhibition of formalin-induced nociception in both the acute and tonic phase of this assay (Barber et al., 1994; Dickenson and Sullivan, 1987; Wheeler- Aceto and Cowan, 1991) suggesting that opioids may directly inhibit the activity of afferent fibers in normal and in inflammatory states. FE200041 at a dose of 1 mg/kg, i.v. resulted in complete inhibition of 2% formalin-induced flinching in phase I and over 80% inhibition in phase II at 1

JPET Fast Forward. Published on March 1, 2004 as DOI: 10.1124/jpet.104.065391
This article has not been copyedited and formatted. The final version may differ from this version.

mg/kg after systemic administration in the rat. Thus, these data further substantiate the activity of FE200041 as an efficacious, systemically active κ selective agonist. The antinociceptive effect of i.v. FE200041at a subeffective (A_{80}) dose persisted for over 60 min, suggesting sufficient duration of action which might be suitable for potential clinical application.

Importantly, the antinociceptive activity of FE200041 is likely to be mediated peripherally as the antinociceptive dose elicited no motor impairment in either mice or rats. Likewise, the antinociceptive dose of FE200041did not result in signs of apparent sedation as measured in the rotarod assay. In contrast, the κ opioid agonists (-)U50,488H and asimadoline, at doses that are antinociceptive in the writhing test, resulted in motor impairment as demonstrated by reduced latencies on the rotarod. This conclusion is supported by the observation that the antinociceptive effects of systemically administered FE200041 were not antagonized by the i.c.v. administration of the κ antagonist norBNI suggesting that the antinociceptive effects of FE200041 are via its peripheral actions. Furthermore, FE200041 was tested by local administration into either the hind paw ipsilateral or contralateral to testing with a thermal stimulus. These data demonstrated that FE200041 was active in the ipsilateral but not the contralateral paw further demonstrating a local, peripheral antinociceptive effect. Thus, the probable peripheral site of antinociceptive activity of FE200041 coupled with a lack of CNS actions strongly suggest that this peptide has the potential to be developed as an analgesic without central side effects associated with μ opioid agonists such as morphine, or other κ agonists that have access to the CNS at analgesic doses, The present data also indicate that modified peptides with all D-amino acids may have increased bioavailability based on the long-lasting activity of FE200041. FE200041 showed antinociceptive actions for approximately 60 min after i.v. administration. Thus, FE200041 represents a prototypic opioid peptide that is highly

JPET Fast Forward. Published on March 1, 2004 as DOI: 10.1124/jpet.104.065391
This article has not been copyedited and formatted. The final version may differ from this version.

efficacious as a peripherally-acting, κ selective compound that is likely to have few side effects associated with conventional opioid agonists.

In summary, our data show that FE200041 is (a) highly selectivity for human κ opioid receptors, with in vitro and in vivo activity in the nanamolar and micromolar range, respectively; (b) is a fully efficacious and peripherally active antinociceptive agent; (c) does not elicit typcial CNS side-effects associated with kappa agonists and (d) demonstrates a long-lasting time course of action as compared to other peptidic kappa agonists suggesting suitability for systemic administration. These features suggest the possibility of future efforts to develop such peripherally restricted kappa agonists as therapeutic agents for man.

JPET Fast Forward. Published on March 1, 2004 as DOI: 10.1124/jpet.104.065391
This article has not been copyedited and formatted. The final version may differ from this version.

REFERENCES:

Ambo A, Adachi T and Sasaki Y (1995) Synthesis and opioid activities of [D-Leu^8] dynorphin (1-8) fragments containing a reduced peptide bond, ψ (CH_2NH). *Chem. Pharm. Bull*., **43**: 1547-1550.

Bagnol D, Mansour A, Akil H and Watson S.J (1997) Cellular Localization and distribution of the cloned mu and kappa opioid receptors in rat gastrointestinal tract. *Neurosci* **81**:579-591.

Barber A., Bartoszyk GD, Bender HM, Gottschlich R, Greiner, HE, Harting J, Mauler F, Minck K-O, Murray RD, Simon M and Seyfried CA (1994) A pharmacological profile of the novel, peripherally-selective k-opioid receptor agonist, EMD 61753. *Br J Pharmacol* **113**:1317-1327.

Barber A and Gottschlich R (1992) Opioid agonists and antagonists: an evaluation of their peripheral actions in inflammation. *Med Res Rev* **12**:525-562.

Barber A and Gottschlich R (1997) Novel developments with selective, non-peptidic kappa-opioid receptor agonists. *Exp Opin Invest Drugs* **6**:1351-1368.

Catheline G, Guilbaud G and Kayser V (1998) Peripheral component in the enhanced antinociceptive effect of systemic U-69,593, a κ-opioid receptor agonist in mononeuropathic rats. *Eur J Pharmacol* **357**:171-178.

Chen Y, Mestek A, Liu J, Hurley J and Yu L (1993) Molecular cloning and functional expression of a mu-opioid receptor from rat brain. *Mol Pharmacol* **44**:8-12.

Delgado-Aros S, Chial HJ, Camilleri M, Szarka LA, Weber FT, Jacob J, Ferber I, McKinzie S, Burton DD and Zinsmeister AR (2002) Effects of a κ-opioid agonist, asimadoline, on satiation and GI motor and sensory functions in humans. *Am J Physiol Gastrointest Liver Phsyiol* **284**:G558-G566.

Dickenson AH and Sullivan AF (1987) Subcutaneous formalin-induced activity of dorsal horn neurons in

the rat: different responses to an intrathecal opiate administered pre- or post-formalin. *Pain* **30**:339-348.

Dooley CT, Ny P, Bidlack JM and Houghten RA (1998) Selective ligands for the μ, δ, and κ opioid receptors identified from a single mixture based tetrapeptide positional scanning combinatorial library. *J Biol Chem* **273**:18848-18856.

Dubuisson D and Dennis SG (1977) The formalin test: a quantitative study of the analgesic effects of morphine, meperidine and brain-stem stimulation in rats and cats. *Pain* **4**:161-174.

Endoh T, Tajima A, Suzuki T, Kamei J, Suzuki T, Narita M, Tseng L and Nagase H (2000) Characterization of the antinociceptive effects of TRK-820 in the rat. *Eur J Pharmacol* **387**:133-140.

Evans C, Keith JD, Morrison H, Magendzo K and Edwards R (1992) Cloning of a delta opioid receptor by functional expression. *Science* **258**:1952-1955.

Field MJ, Carnell AJ, Gonzalez MI, McCleary S, Oles RJ, Smith R, Hughes J and Singh L (1999) Enadoline, a selective κ-opioid receptor agonist shows potent antihyperalgesic and antiallodynic actions in a rat model of surgical pain. *Pain* **80**:383-389.

Gear RW, Gordon NC, Heller PH, Paul SM, Miaskowski C and Levine JD (1996) Gender differences in analgesic response to the kappa-opioid pentazocine. *Neurosci Lett* **205**:207-209.

Gear RW, Miaskowski C, Gordon NC, Paul SM, Heller PH and Levine JD (1996) Kappa opioids produce significantly greater analgesia in women than men. *Nature Med* **2**:1248-1250.

Hargreaves K, Dubner R, Brown F, Flores C and Joris J (1988) A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. *Pain* **32**: 77-88.

Hiramatsu M, Inoue K, Ambo A, Sasaki Y and Kameyama T (2001) Long-lasting antinociceptive effects of a novel dynorphin analogue, Tyr-D-Ala-Phe-Leu-Arg ψ (CH_2NH) Arg-NH_2, in mice. *Br J Pharmacol* **132**:1948-1956.

Horan P, Taylor J, Yamamura H and Porreca F (1992) Extremely long-lasting antagonistic actions of nor-binaltorphimine (nor-BNI) in the mouse tail-flick test. *J Pharmacol Exp Ther* **260**:1237-1243.

Hughes J, Kosterlitz HW and Leslie FM (1975) Effect of morphine on adrenergic transmission in the mouse vas deferens. Assessment of agonist and antagonist potencies of narcotic analgesics. *Br J Pharmacol* **53**:371-81.

Hunter JC, Leighton GE, Meecham KG, Boyle SJ, Horwell DC, Rees DC and Hughes J (1990) CI-977, a novel and selective agonist for the kappa-opioid receptor. *Br J Pharmacol* **101**:183-189.

Jiang Q, Heyman JS and Porreca F (1989) Mu antagonist and kappa agonist properties of b-funaltrexamine (b-FNA): long lasting spinal antinociception. *NIDA Research Monograph* **95**:199-205.

Junien JL and Wettstein JG (1992) Role of opioids in peripheral analgesia. *Life Sci* **51**:2009-2018.

Kamp EH, Carter R, Jones W, Tillman SR and Gebhart GF (2003) Quantitative assessment and characterization of visceral nociception and hyperalgesia in mice. *Am J Physiol Gastrointest Liver Phsyiol* **284**:G434-G444.

Kieffer B, Befort K, Gaveriaux-Ruff C and Hirth C (1992) The delta opioid receptor: isolation of a cDNA by expression cloning and pharmacological characterization. *Proc Natl Acad Sci U.S.A.* **89**:12048-12052.

Lai J, Ma SW, Zhu RH, Rothman RB, Lentes KU and Porreca F (1994) Pharmacological characterization

of the cloned kappa opioid receptor as a kappa 1b subtype. *Neuroreport* **5**:2161-4.

Lorenzen A, Fuss M, Vogt H and Schwabe U (1993) Measurement of guanine nucleotide-binding protein activation by A1 adenosine receptor agonists in bovine brain membranes: stimulation of guanosine-5'- O-(3-[^{35}S]thio)triphosphate binding. *Mol. Pharmacol.* **44**:115-23.

Machelska H, Pfluger M, Weber W, Piranvisseh-Volk M, Daubert JD, Dehaven R and Stein C (1999) Peripheral effects of the κ-opioid agonist EMD 61753 on pain and inflammation in rats and humans. *J Pharmacol Exp Ther* **290** 1:354-361.

Marchand F, Ardid D, Chapuy E, Alloui A, Jourdan D and Eschalier A (2003) Evidence for an involvement of supraspinal δ- and spinal μ-opioid receptors in the antihyperalgesia effect of chroically administered clomipramine in mononeuropathic rats. *J Pharmacol Exp Ther* **307**:268-274.

Martin WR, Eades CG, Thompson JA, Huppler RE and Gilbert PE (1976) The effects of morphine- and nalorphine-like drugs in the nondependent and morphine-dependent chronic spinal dog. *J Pharmacol Exp Ther* **197**:517-532.

Meng F, Xie G-X, Thompson R, Mansour A, Goldstein A, Watson SJ and Akil H (1993) Cloning and pharmacological characterization of a rat kappa opioid receptor. *Proc Natl Acad Sci U.S.A.* **90**:9954- 9958.

Nicholson AN (1987) New antihistamines free of sedative side-effects. *Trends Pharmacol Sci* **8**:247-249.

Pande AC, Pyke RE, Greiner M, Cooper SA, Benjamin R and Pierce MW (1996) Analgesic efficacy of the kappa-receptor agonist, enadoline, in dental surgery pain. *Clinical Neuropharmacol* **19**:92-7.

Pasternak GW (1993) Pharmacological mechanism of opioid analgesia. *Clin Neuropharmacol* **16**:1-18.

Pfeiffer A, Brantl V, Herz A and Emrich HM (1986) Psychotomimesis mediated by kappa opiate receptors. *Science* **233**:774-776.

Portoghese PS, Lipkowski AW and Takemori AE (1987) Bimorphinans as highly selective potent kappa receptor antagonists. *J Med Chem* **30**:238-239.

Przewlocki R and Przewlocka B (2001) Opioids in chronic pain. *Eur J Pharmacol* **429**:79-91.

Rogers H, Birch PJ, Harrison SM, Palmer E, Manchee GR, Judd DB, Naylor A, Scopes DIC and Hayes AG (1992) GR 94839, a κ-opioid agonist with limited access to the central nervous system, has antinociceptive activity. *Br J Pharmacol* **106**:783-789.

Shaw JS, Carroll JA, Alcoc P and Main BG, (1989) ICI 204448: a k-opioid agonist with limited access to the CNS. *Br J Pharmacol* **96**:986-992.

Simonin F, Gaveriaux-Ruff C, Befort K, Matthes H, Lannes B, Micheletti G, Mattei M-G, Charron G, Bloch B and Kieffer B (1995) κ-opioid receptor in humans: cDNA and genomic cloning, chromosomal assignment, functional expression, pharmacology, and expression pattern in the central nervous system. *Proc Natl Acad Sci U.S.A.* **92**:7006-7010.

Spetea M, Rydelius G, Nylander I, Ahmed M, Bileviciute-Ljungar I, Lundeberg T, Svensson S and Kreieberg, A (2002) Alteration in endogenous opioid systems due to chronic inflammatory pain conditions. *Eur J Pharmacol* **435**:245-252.

Stein C (1993) Peripheral mechanisms of opioid analgesia. *Anesth Analg* **76**:182-191.

Tachibana S, Yoshino H, Arakawa Y, Nakazawa T, Kaneko T, Yamatsu K and Miyagawa H (1988) Design and synthesis of metabolically stable analogues of dynorphin A and their analgesic characteristics. In *Biowarning System in the Brain* ed. Takagi H, Oomura Y, Ito M and Otsuka M.

pp.101-109, Tokyo: University of Tokyo Press.

Thompson RC, Mansour A, Akil H and Watson SJ (1993) Cloning and pharmacological characterization of a rat m opioid receptor. *Neuron* **11**:903-913.

Walker J, Catheline G, Guilbaud G and Kayser V (1999) Lack of cross-tolerance between the antinociceptive effects of systemic morphine and asimadoline, a peripherally-selective κ-opioid agonist, in CCI- neuropathic rats. *Pain* **83**:509-516.

Wheeler-Aceto H and Cowan A (1991) Standardization of the rat paw formalin test for the evaluation of analgesics. *Psycopharmacology* **104**:35-44.

Wilson JL, Carmody JJ and Walker JS (2000) The importance of the hypothalamo-hypophyseal-adrenal axis to the anti-inflammatory actions of the κ-opioid agonist PNU-U50,488H in rats with adjuvant arthritis. *J Pharmacol Exper Ther* **294**:1131-1136.

Wilson JL, Nayanar V and Walker JS (1996) The site of anti-arthritic action of the kappa agonist, U50,488H: Importance of local administration. *Br J Pharmacol* **118**:1754-1760.

Yasuda K, Raynor K, Kong H, Breder CD, Takeda J, Reisine T and Bell GI (1993) Cloning and functional comparison of k and d opioid receptors from mouse brain. *Proc Natl Acad Sci U.S.A.* **90**: 6736-6740.

Yu J, Butelman ER, Woods JH, Chait BT and Kreek MJ (1997) Dynorphin A (1-8) analog, E-2078, crosses the blood-brain barrier in rhesus monkeys. *J Pharmacol Exper Ther* **282**:633-638.

Zhu J, Chen C, Xue J-C, Kunapuli S, DeRiel K and Liu-Chen L-Y (1995) Cloning of a human κ opioid receptor from the brain. *Pharmacol Lett* **56**: PL201-207.

Footnotes:

These studies were supported by Ferring Research Institute Inc. San Diego, CA

Reprint info: Todd W. Vanderah, Ph.D.

University of Arizona, COM

1501 N. Campbell Ave., LSN Rm560

Tucson, AZ 85724

[1] Ferring Research Institute Inc., San Diego, California;

[2] Laboratories Fournier , Dijon, France

Acknowledgments: We thank Peg Davis, Rachel Johnson, Shou-Wu Ma, and Dinesh Srinivasan for their technical assistance.

JPET Fast Forward. Published on March 1, 2004 as DOI: 10.1124/jpet.104.065391
This article has not been copyedited and formatted. The final version may differ from this version.

FIGURE LEGENDS

Figure 1. Time-course of association (A) and dissociation (B) of [^{3}H]FE200041 in membrane preparations from transfected cells that express the hKOR. Data shown are representative of 3 independent determinations. The K_D value determined from this experiment was 0.71 nM. Each data point is the mean of triplicate assay.

Saturation radioligand binding using [^{3}H]FE200041 on mouse cancer cells HN.9.10 stably transfected with the human kappa opioid receptor (C). The binding capacity (B_{max}) and dissociation constant (K_D) for [^{3}H]FE200041 on hKOR is 3.5 pmol/mg and 0.77 nM , respectively (n=3).

Figure 2. Dose-related inhibition of acetic acid (0.6%, 10 ml/kg, i.p.)-induced writhing in mice by FE200041 (filled circle), (-)U50,488 (filled triangle) or asimadoline (filled square) by the i.v. route of administration. Intravenous administration of FE200041 resulted in an A_{50} of 0.06 mg/kg (95% C.I., 0.04-0.11). (-)U50, 488 resulted in an A_{50} of 0.10 mg/kg (95% C.I.,0.06-0.18) and asimadoline resulted in an A_{50} of 0.19 mg/kg (95% C.I., 0.14-0.25)(n=6 to 8 for each point).

Figure 3. Time related inhibition of acetic acid-induced writhing in mice by an A_{80} dose of FE200041 was performed. The dose of 0.3 mg/kg, i.v. of FE200041 was administered either 5, 10, 15, 30, 60 or 120 minutes prior to acetic acid administration in separate animals and the number of writhes were recorded. FE 200041 resulted in a significant antinociceptive effect (>20%) over a period of 60 minutes (n=6 for

JPET Fast Forward. Published on March 1, 2004 as DOI: 10.1124/jpet.104.065391
This article has not been copyedited and formatted. The final version may differ from this version.

each point).

Figure 4. Inhibition of acetic acid-induced writhing in mice by FE200041 (1 mg/kg, i.v.) was performed in the absence and presence of either the selective μ opioid receptor antagonist β-FNA (10 mg/kg, s.c.) or the selective κ opioid receptor antagonist nor-BNI (10 mg/kg, s.c.)(10 μg/5 μl, i.c.v.). β-FNA was administered 24 hours prior to FE200041 and did not block the antinociceptive effects of FE200041. In contrast, nor-BNI given s.c., 15 min prior to FE200041 significantly attenuated the antinociceptive effects of FE200041 from 96% activity in control animals to 28% activity (n=8 for each bar). However, nor-BNI given i.c.v., 5 min prior to FE200041 had no effect on the antinociceptive effects of FE200041 from 96% activity in control animals to 88 % activity (n=8 for each bar).

Figure 5. (A)Dose related inhibition of formalin-induced flinching in rats by FE200041 by the i.v. route of administration, vehicle (filled circle), 0.3 mg/kg (filled square), 0.6 mg/kg (filled triangle), 1 mg/kg (filled diamond) . (B)FE200041 significantly attenuated both phase I and Phase II of the formalin-induced paw flick assay with and A_{50} of 0.39 mg/kg, i.v. (95% C.I., 0.29-0.55) for phase I (closed circle)and 0.55 mg/kg, i.v. (95% C.I., 0.42-0.73) for phase II (closed square)(n=6).

Figure 6. Local analgesic effects of FE200041using the radiant heat test. FE200041(10 μg/rat) was administered in either the ipsilateral hindpaw or contralateral hindpaw to testing. FE200041 resulted in a significant increase in the ipsilateral paw withdrawal latency from a radiant heat source whereas when administered in the contralateral paw to testing there was no difference in paw withdrawal latencies from

baseline (n=6).

Table 1. *In vitro* characterization of FE 200041				
Binding Ki (nM)			**Selectivity**	
κ	**μ**	**δ**	**μ/κ**	**δ/κ**
0.146	4570	<10,000	31,300	68,500
hKOR GTPγS				
EC50	1.06 nM	**Emax**	147.70%	
MVD				
IC50	5.839 nM	**Emax**	95.70%	

Figure 1

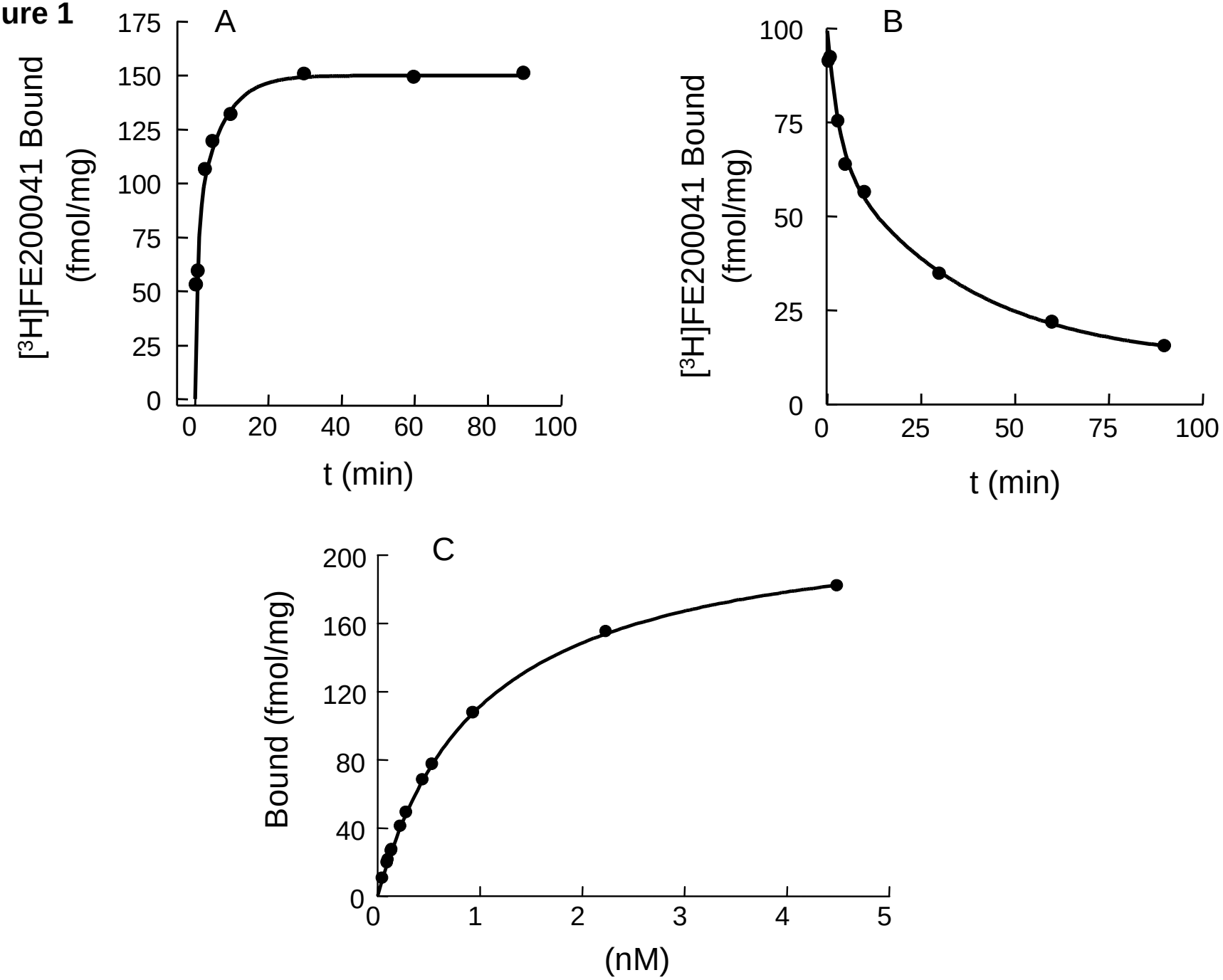

Figure 2

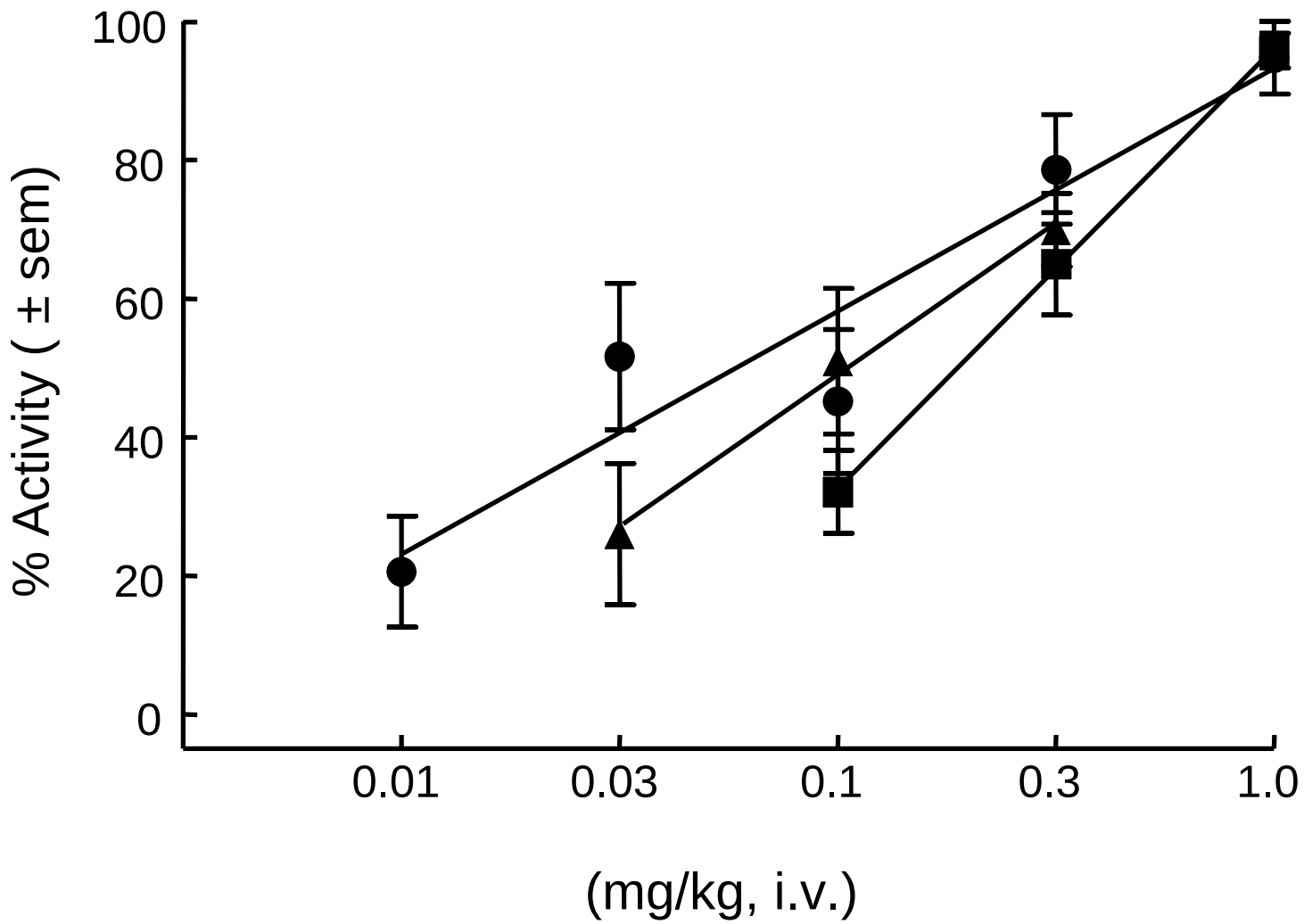

Figure 3

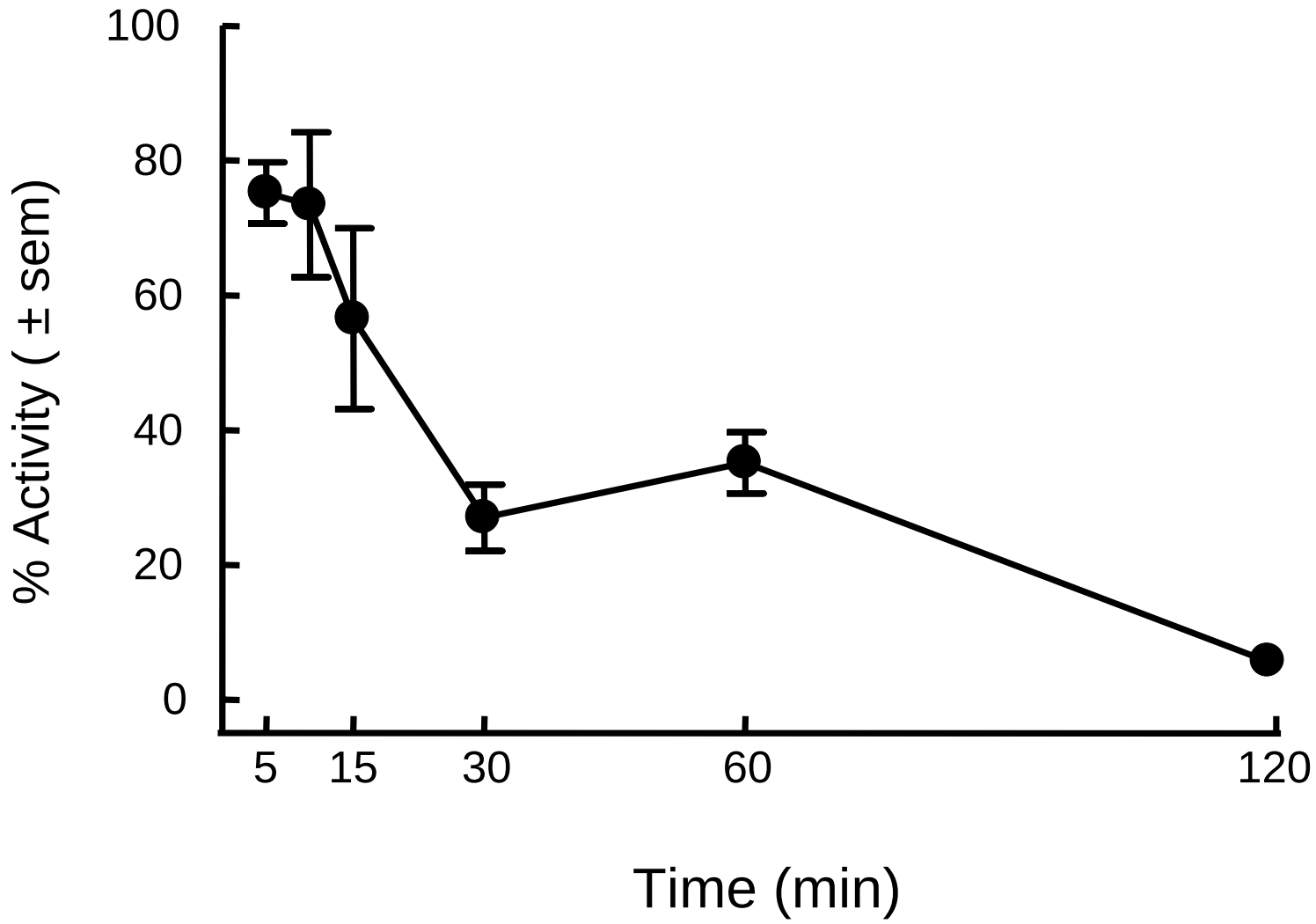

Figure 4

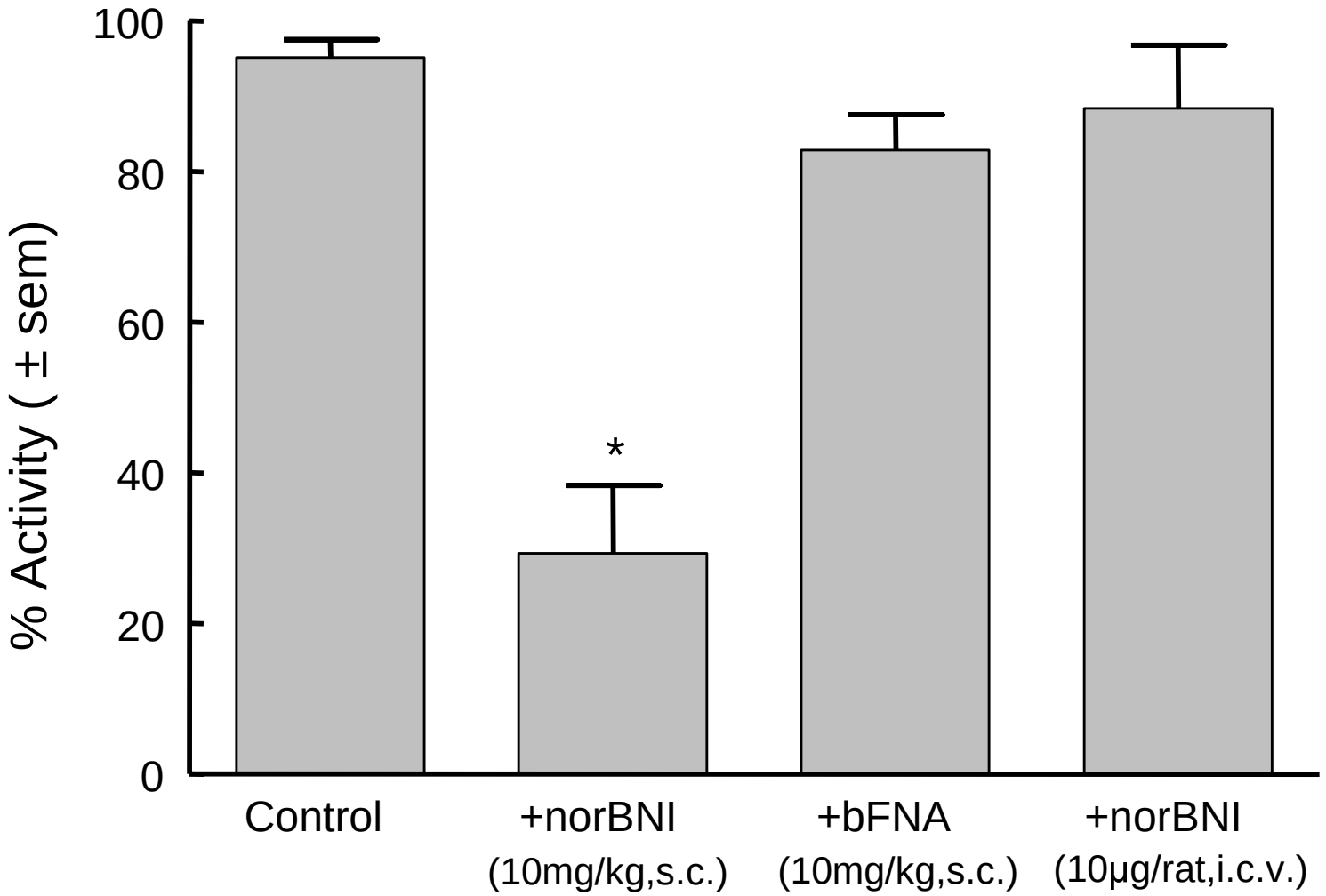

Figure 5

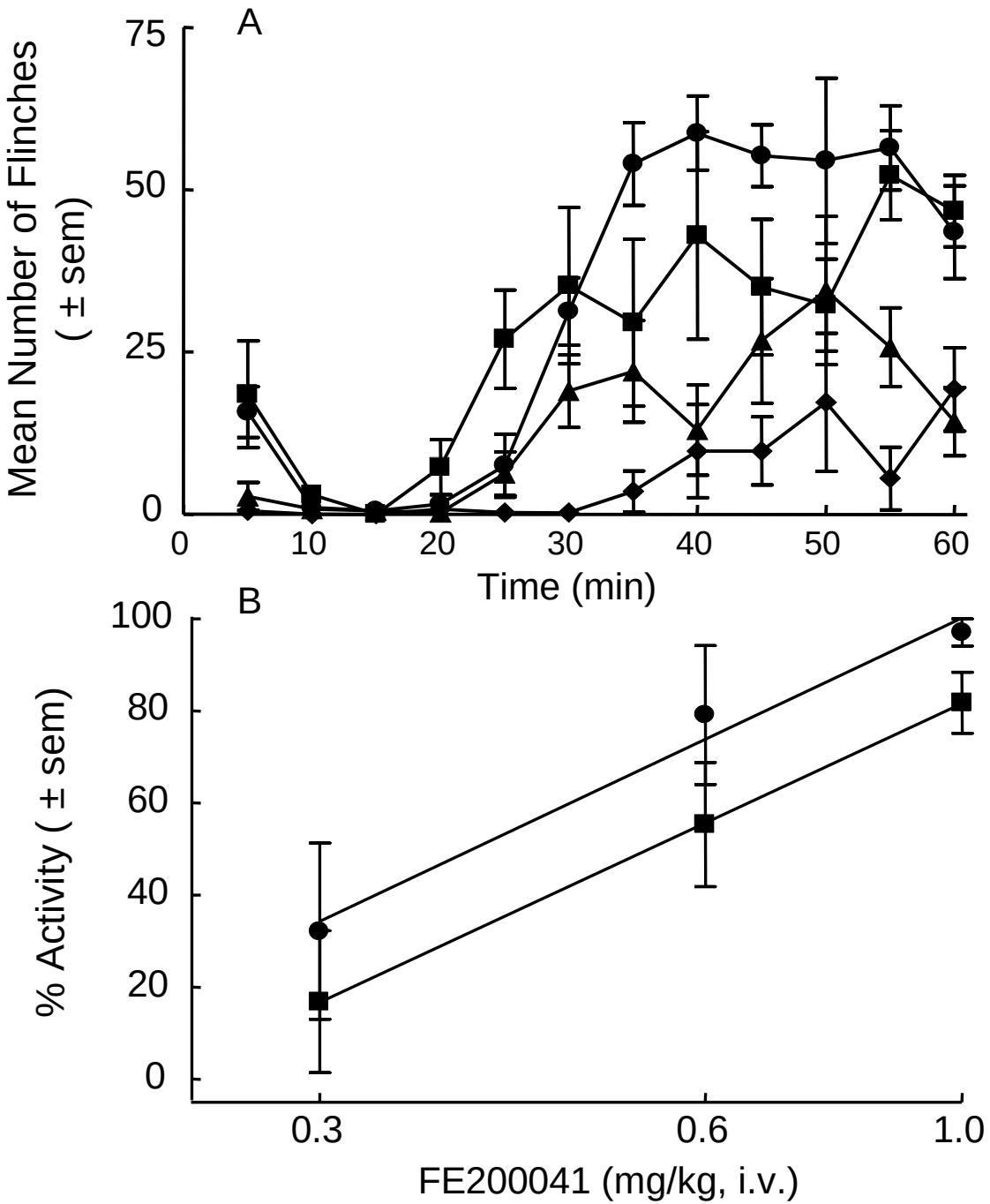

Figure 6