Available online at www.sciencedirect.com
ScienceDirect
European Journal of Pharmacology 583 (2008) 62–72
www.elsevier.com/locate/ejphar

Novel D-amino acid tetrapeptides produce potent antinociception by selectively acting at peripheral κ-opioid receptors ☆,☆☆

Todd W. Vanderah [a,*], Tally Largent-Milnes [a], Josephine Lai [a], Frank Porreca [a], Richard A. Houghten [b], Frédérique Menzaghi [c], Kazimierz Wisniewski [d], Jacek Stalewski [d], Javier Sueiras-Diaz [d], Robert Galyean [d], Claudio Schteingart [d], Jean-Louis Junien [e], Jerzy Trojnar [d], Pierre J.-M. Rivière [d]

[a] *Department of Pharmacology, University of Arizona Health Sciences Center, Tucson, AZ, USA*
[b] *Torrey Pines Institute for Molecular Studies, San Diego, CA, USA*
[c] *Cara Therapeutics Inc., Tarrytown, NY, USA*
[d] *Ferring Research Institute Inc., San Diego, CA, USA*
[e] *Centre de Recherche, Fournier Pharma, Daix, France*

Received 30 August 2007; received in revised form 17 December 2007; accepted 14 January 2008
Available online 24 January 2008

Abstract

Kappa-(κ) opioid receptors are widely distributed in the periphery and activation results in antinociception; however supraspinal acting κ-agonists result in unwanted side effects. Two novel, all D-amino acid, tetrapeptide κ-opioid receptor agonists, FE 200665 and FE 200666, were identified and compared to brain penetrating (enadoline) and peripherally selective (asimadoline) κ-agonists as potential analgesics lacking unwanted central nervous system (CNS) side effects. *In vitro* characterization was performed using radioligand binding and GTPγS binding. Antinociception was evaluated in both mice and rats. Rotarod tests were performed to determine motor impairment effects of the κ-agonists. FE 200665 and FE 200666 showed high affinity for human κ-opioid receptor 1 (K_i of 0.24 nM and 0.08 nM, respectively) and selectivity for human κ-opioid receptor 1 (human κ-opioid receptor 1/human μ-opioid receptor/humanδ-opioid receptor selectivity ratios of 1/16,900/84,600 and 1/88,600/>1,250,000, respectively). Both compounds demonstrated agonist activity in the human κ-opioid receptor 1 [^{35}S]GTPγS binding assay (EC_{50} of 0.08 nM and 0.03 nM) and resulted in dose-related antinociception in the mouse writhing test (A_{50}: 0.007 and 0.013 mg/kg, i.v., respectively). Markedly higher doses of FE 200665 and FE 200666 were required to induce centrally-mediated effects in the rotarod assay (548- and 182-fold higher doses, respectively), and antinociception determined in the mouse tail-flick assay (>1429- and 430-fold fold higher doses, respectively) after peripheral administration supporting a peripheral site of action. The potency ratios between central and peripheral activity suggest a therapeutic window significantly higher than previous κ-agonists. Furthermore, FE 200665 has entered into clinical trials with great promise as a novel analgesic lacking unwanted side effects seen with current therapeutics.

Keywords: Antinociception; Kappa opioid agonist; Peptides; Peripheral; Radioligand binding; Rodent models

1. Introduction

Kappa- (κ) opioid receptors are widely distributed in the peripheral, spinal, and supraspinal pain pathways as well as found in immune cells (Carr et al., 1989; Mansour et al., 1995; Taub et al., 1991). Activation of κ-opioid receptors located either in the central nervous system (CNS) or in the periphery results in antinociception (Horan and Porreca, 1993; Millan

☆ This work is attributed to Ferring Research Institute Inc. and the University Of Arizona Department Of Pharmacology.

☆☆ Funding and Financial Support: This work was funded by the Ferring Research Institute Inc., General Atomics Ct, San Diego, CA 92121, USA.

* Corresponding author. University of Arizona, Departments of Pharmacology and Anesthesiology, 1501 N. Campbell Ave., Tucson, AZ 85724, USA. Tel.: +1 520 626 7801; fax: +1 520 626 4779.
E-mail address: vanderah@u.arizona.edu (T.W. Vanderah).

0014-2999/$ - see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2008.01.011

et al., 1988; Porreca et al., 1987; Stein, 1991; Stein et al., 1989, 1990). Unlike mu- (μ) opioid receptor agonists (e.g., morphine), κ-agonists that reach the CNS induce aversive behavior; however they do not inhibit gastrointestinal transit and do not cause respiratory depression (Di Chiara and Imperato, 1988; Field et al., 1999; Freye et al., 1983; Porreca and Burks, 1983; Porreca et al., 1984; Shippenberg et al., 1988; Unterwald et al., 1987). For these reasons, peripheral κ-opioid receptors are potential targets for novel analgesics that would not present with the side effects of morphine-like compounds such as constipation, respiratory depression and drug abuse liability.

The first generation of κ-agonists made available and tested in clinical trials were orally active, brain penetrating, small organic molecules. Spiradoline and enadoline are typical representatives of the first generation of κ-agonists (Hunter et al., 1990; Von Voigtlander and Lewis, 1988). This class of compounds was clinically efficacious and lacked morphine-like side effects (Pande et al., 1996). However, these molecules were associated with dose limiting neuropsychiatric effects including sedation and dysphoria, and further development of these compounds as analgesics was discontinued (Pande et al., 1996; Reece et al., 1994). These side effects are κ-opioid receptor specific and are attributed to κ-opioid receptors that are located in the CNS behind the blood–brain barrier, perhaps in the nucleus accumbens where receptor activation results in the inhibition of dopamine release (Di Chiara and Imperato, 1988; Donzanti et al., 1992). Because peripheral κ-opioid receptors are also able to induce analgesia, particularly after tissue injury and inflammation, a second generation of compounds was designed to increase peripheral selectivity and decrease brain penetration, in order to allow for antinociception without the centrally-mediated side effects (Millan et al., 1988; Stein, 1991; Stein et al., 1989, 1990). The second generation of so-called peripheral κ-agonists was intended to be active by the oral route of administration and was based on small organic molecules. In most cases, they were chemically related to the first generation of κ-agonists (i.e. arylacetamides with decreased lipophilicity). Asimadoline is the prototype of this second generation of κ-agonists (Barber et al., 1994; Gottschlich et al., 1995). Though shown to have reduced CNS activity when compared to the first generation of κ-agonists in animal models, asimadoline failed to show clinical antinociceptive efficacy at doses which are devoid of central side effects (Barber et al., 1994; Bickel et al., 1998; Gottschlich et al., 1995; Machelska et al., 1999). Thus, it appears that the same physico-chemical properties that facilitate oral absorption are also likely to facilitate CNS penetration, resulting in a very narrow safety margin between the peripheral analgesic effect and the central side effects.

To address this deficiency and improve the therapeutic window of κ-agonists as analgesics, we developed a program to discover a third generation of compounds based on peptides. As peptides are intrinsically hydrophilic and generally do not cross biological membranes passively, they should be able to act on peripheral κ-opioid receptors with minimal or no penetration through the blood–brain barrier. In this approach we focused primarily on safety, as opposed to the previous small molecule programs that had oral bioavailability as a fundamental prerequisite. Our initial lead peptide was discovered by screening a mixture based positional scanning of a combinatorial tetrapeptide library using κ- and μ-opioid receptor radioligand binding analysis (Dooley et al., 1998). Although the library contained both L and D amino acids, the best κ-agonist tetrapeptide identified was an all D-amino-acid tetrapeptide (H-D-Phe-D-Phe-D-Nle-D-Arg-NH_2), which is structurally unrelated to the endogenous κ-opioid receptor selective peptide, dynorphin (Chavkin et al., 1982; Goldstein et al., 1979; Vanderah et al., 2004). This tetrapeptide sequence was further elaborated in search of compounds with improved *in vivo* characteristics (Wisniewski et al., 2001). These new D-amino acid tetrapeptide analogs were evaluated by both *in vitro* and *in vivo* assays for (a) their affinity and selectivity at the cloned κ-, μ- and δ-opioid receptors, (b) their agonist activity at the κ-opioid receptor, (c) their antinociceptive activity in the mouse and rat writhing assays, and (d) their CNS penetration in the mouse and rat rotarod (motor impairment/sedation) and mouse tail-flick (centrally-mediated antinociception) assays.

We report here the pharmacological characterization of two representatives of this class of compounds, FE 200665 (H-D-Phe-D-Phe-D-Nle-D-Arg-NH-4-Picolyl) and FE 200666 (H-D-Phe-D-Phe-D-Leu-D-Orn-Morpholine amide), (Fig. 1). Recently, these two compounds were shown to be peripherally selective κ-agonists with analgesic and anti-inflammatory properties in a rat model of Freund's complete adjuvant-induced inflammation (Binder et al., 2001).

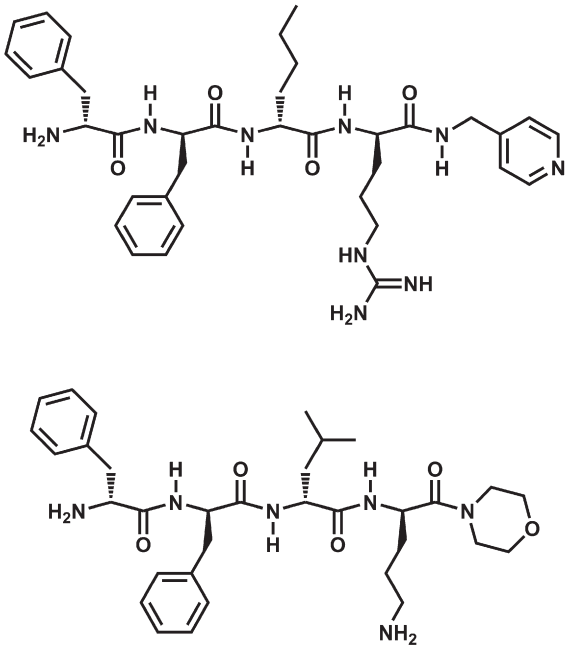

Fig. 1. Structures of FE 200665 (top) and FE 200666 (bottom).

We evaluated FE 200665 and FE 200666 extensively in *in vitro* and *in vivo* assays in comparison with the reference opioid agonists: enadoline, asimadoline and morphine. FE 200665 and FE 200666 have high affinity and unprecedented selectivity for the κ-opioid receptors, as well as demonstrate high potency, efficacy and duration of action in experimental pain models. They also exhibit a more than 100-fold increase in peripheral selectivity as compared to the second generation small molecule κ-agonist asimadoline. FE 200665, a.k.a. CR665, is now under clinical development by Cara Therapeutics. Preliminary disclosure by the company reported that CR665 was safe and well tolerated in volunteers, hence being devoid of CNS side-effects associated with brain penetrating compounds.

2. Materials and methods

All *in vivo* experimental procedures were in accordance with the policies and recommendations of the *Ethical Guidelines for Investigations of Experimental Pain in Conscious Animals* edited by International Association for the Study of Pain and of the *Guiding Principles in the Care and Use of Animals* approved by the Council of The American Physiological Society. All *in vivo* experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Arizona (approval number 02-160).

2.1. Compounds

The novel tetrapeptide κ-agonists were compared to reference compounds. Enadoline was used as a brain penetrating reference κ-agonist, asimadoline as a reference peripheral κ-agonist and morphine as a preferred μ-agonist reference. The newly introduced κ-agonist, TRK-820 was also evaluated (Endoh et al., 1999). Naloxone was used as a non-selective opioid receptor antagonist. FE 200665, FE 200666, asimadoline, and TRK-820 were prepared by the Department of Chemistry at the Ferring Research Institute Inc., San Diego. FE 200665 and FE 200666 were prepared as acetate salts (Fig. 1). Enadoline was generously supplied by Parke-Davis Warner-Lambert, Ann Arbor, MI. (−) U50488 was purchased from Tocris (Ellisville, MO). Morphine and naloxone were obtained from Sigma (St. Louis, MO). All drug doses are expressed as free base peptide for peptidic substances or as free base for non-peptidic substances. Distilled water was used as vehicle and control treatment (10 ml/kg BW, i.v. in mice, 1 ml/kg BW, s.c. for rats).

2.2. In vitro assays

2.2.1. Radioligand binding assays

All radioligands were purchased from NEN (Boston, MA). Radioligand binding analysis was carried out as described previously using crude membrane preparations from HN9.10 cells that have been transfected with the human κ-, δ-, or the μ- opioid receptor cDNA, and each transfected cell line expressed constitutively a stable level of these receptors after clonal selection (Lai et al., 1994). The membranes were resuspended in ice-cold Tris–buffer (50 mM, pH7.4) containing 0.5% bovine serum albumin (BSA), and the following protease inhibitors: 30 μM bestatin, 10 μM captopril, 50 μg/ml bacitracin, 100 μM phenylmethylsulfonyl-fluoride (PMSF). Radioligand competition analysis for FE 200665 and FE 200666 was carried out using membranes prepared from each of the cell lines that expressed either κ-, δ-, or μ-opioid receptors. [^{3}H]U69, 593 (1.6 nM) was used to label the κ-opioid receptors, 1 nM [^{3}H]DAMGO was used to label the μ-opioid receptors, and 1 nM [^{3}H]pCl-DPDPE was used to label the δ-opioid receptors in the respective cell membranes preparations. For each competition assay, 10 concentrations (10^{-13} M to 10^{-4} M, in duplicate) of FE 200665 or FE 200666 were each incubated with membranes (ranging between 15–25 μg) and radioligand in a total volume of 0.5 ml for 3 h at 25 °C in a shaking water bath and 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. Non-specific binding of the radioligand was defined as the amount of radioactivity bound to the cell membranes in the presence of 10 μM naloxone. Radioactivity was determined by liquid scintillation counting. Data were fitted by non-linear least squares analysis using GraphPad Prism. All analyses were based on 3 independent experiments.

2.2.2. GTPγS binding

GTPγS binding was done using membranes from cells that express the human κ-opioid receptor as previously described (Vanderah et al., 2004). Briefly, reactions were initiated by adding 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 FE 200665 or FE 200666 (10^{-13} M to 10^{-4} M) 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 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.

2.3. In vivo assays

2.3.1. Animals

Male ICR mice (20–25 g) and male Sprague Dawley rats (200 to 250 g) were used for the *in vivo* experiments. Animals were purchased from Harlan (Indianapolis, IN), housed on a regular 12 h light/dark cycle (lights on at 06:00 h), in a climate-controlled room with food and water *ad libitum*. Groups of 4 to 43 animals were used in all experiments.

2.3.2. Injection techniques

For intravenous (i.v.) administration, animals were placed in restrainers and the tail of the animal dipped into warm-water for approximately 10 to 15 s to produce tail vein vasodilatation and hence better visibility of the tail vein. Intravenous injections were made using a disposable 1 ml syringe equipped with a 30 gauge disposable needle. The needle was inserted into the tail vein at a 45° angle and a small amount of blood drawn back into the syringe before injection to confirm the localization of the extremity of the needle. Injection of compounds or vehicle was performed over a 5 s period. 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 holding the animal in a supine position and inserting a 30 gauge disposable needle attached to a 1 ml disposable syringe between the abdominal skin and underlying muscular wall in the lower left quadrant of the abdomen. Injections of compounds were performed over a 5 s period and were considered successful if development of an out-pocketing of the skin at the site of injection.

Intraperitoneal (i.p.) injections were performed by holding the animals in a supine position and inserting the extremity of a 30 gauge disposable needle attached to a 1 ml disposable syringe into the peritoneal cavity in the lower left quadrant of the abdomen.

2.3.3. Acetic acid induced writhing

The acetic acid induced writhing assay can detect antinociceptive activity for opioid drugs acting at peripheral, spinal and supraspinal levels (Hardy et al., 1989; Porreca et al., 1987). Animals were fasted 12 to 16 h prior testing. The nociceptive response (abdominal contractions or 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 mouse experiments, compounds were given intravenously (i.v.) in the tail vein. To determine the antinociceptive potency and efficacy, full dose–response curves were constructed with compounds given 5 min prior to acetic acid administration. The duration of action was determined by using increasing pretreatment times (5, 60, and 120 min) prior to acetic acid administration for a sub-maximally effective dose defined during the dose–response curve study. In rat experiments, compounds were given subcutaneously (s.c.) 15 min prior to acetic acid administration. To determine the antinociceptive potency and efficacy, full dose–response curves were constructed with compounds given 5 min prior to acetic acid administration.

The number of writhes was counted over the 15 min period following acetic acid administration. The antinociceptive activity was expressed as a percentage of inhibition of writhes and calculated as follows:

$$\text{Antinociception } (\%) = 100 \times \frac{(\text{average \# writhes in vehicle group}) - (\text{\# writhes in each drug treated animal})}{(\text{average \# writhes in vehicle group})}.$$

2.3.4. Rotarod

The CNS mediated sedative activity in the rotarod assay was used as an index of brain penetration for peripherally administered κ-agonists (Barber et al., 1994).

Animals were tested for their ability to balance on a slow rotating rod (Rotamex 4/8, 1.5 cm and 6.5 cm diameter for mice and rats, respectively; rate of rotation, 10 revolutions per min, Columbus Instruments, Columbus, Ohio) after peripheral administration of test compounds or vehicle. Prior to drug administration, animals were trained through three consecutive sessions to stay on the rod and reach the cut off time of 180 s. Animals that did not remain on the rod for the 180 s were not used for experiments. Once trained, animals were injected by either the i.v. (mice) or s.c. (rats) route with test compounds or vehicle. Animals were tested at 5, 10, and 15 min after i.v. (mice) or 15, 20, 25 min after s.c. (rats) administration. The latency to fall off the rod was recorded at each time point. Results are reported at the time of peak effect for each compound. The sedative activity was expressed as a percentage of inhibition of time spent on the rod and calculated as follows:

$$\text{Sedation } (\%) = 100 \times \frac{(\text{latency in vehicle group, } 180\,\text{s}) - (\text{latency in each drug treated animal})}{(\text{latency in vehicle group, } 180\,\text{s})}.$$

2.3.5. Hot-water (52 °C) tail-flick

The spinally and supraspinally mediated antinociceptive activity in the mouse tail-flick assay was used as an index of blood–brain barrier brain penetration for peripherally administered κ-agonists (Heyman et al., 1987; Janssen et al., 1963).

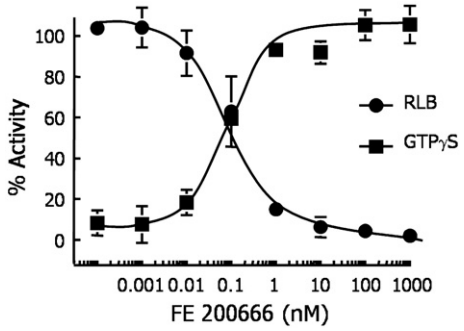

Fig. 2. Inhibition of ^{3}H-U69593 binding (radio ligand binding) and stimulation of [^{35}S]GTPγS binding by FE 200666 in hKOR stably transfected HN cell line.

The nociceptive stimulus consisted in dipping the distal third of the mouse tail into a 52 °C water bath. The latency to tail-flick or withdrawal of the tail from the water was taken as the endpoint with a cutoff of 10 s in order to avoid tissue damage. The stimulus was applied prior (e.g., baseline latency) to and after (e.g., test latency) compound administration. Animals with a baseline latency of over 5 s were discarded. The measurement was performed at 5, 10, and 15 min following drug administration. Results are reported at peak effect time for each compound.

The antinociceptive activity was expressed as a percentage of increase in tail-flick latency and calculated as follows:

$$\text{Antinociception}\ (\%) = 100 \times \frac{(\text{test latency, after drug treatment}) - (\text{baseline latency, before drug treatment})}{(\text{Cut off latency}) - (\text{baseline latency})}.$$

2.4. Statistical analysis

Radioligand binding and [^{35}S]GTPγS binding data were analyzed by non-linear regression analysis using GraphPad Inplot. For binding affinity, the K_i value(s) for each ligand was calculated from the IC_{50} value(s) based on the Cheng and Prusoff equation from at least three independent experiments. For GTPγS binding, potency was expressed as log EC_{50} ± S.E.M. Maximal effect was expressed as E_{max} ± S.E.M.

Statistical analysis on *in vivo* data were performed on raw score data instead of calculated activity data. For data sets that passed both normality and equal variance tests, the data were further analyzed using an ANOVA followed, when appropriate, by a multiple comparisons test (Dunnett's test). Data sets that did not pass either normality or equal variance tests, were further analyzed using ANOVA on ranks followed, when appropriate, by a non-parametric multiple comparisons test (Dunn's test). Statistical analyses were performed using SigmaStat® 2.0 (Jandel Scientific Software).

When made possible, potencies (or A_{50}) were determined by regression analysis of dose–response curve (log dose [x] vs response [y]) according to the method of analysis of the Graded Dose–Response (Tallarida and Murray, 1987). For the calculations of A_{50}'s, the minimal possible response was set to 0% (all negative values were replaced by 0).

Table 1
Affinity (Ki, nM) of FE 200665 and FE 200666 and reference compounds for cloned human opioid receptor types, (hKOR1: human kappa opioid receptor; hMOR1: human mu opioid receptor; hDOR1: human delta opioid receptor)

Compound	K_i (nM)			Receptor selectivity hKOR/hMOR/hDOR
	hKOR1	hMOR1	hDOR1	
FE 200665	0.24	4050	20,300	1/16,875/84,583
FE 200666	0.08	7090	>100,000	1/88,625/>1,250,000
(−) U50488	1.12	2400	7,100	1/2,142/6,339
Enadoline	1.25	272	707	1/218/566
Asimadoline	0.17	581	322	1/3,418/1,894
TRK-820	0.36	0.71	49.9	1/2/139
Morphine	14.7	4.4	150	3/1/34.1

3. Results

3.1. Affinity for opioid receptor subtypes

FE 200665 and FE 200666 competed for the specific binding of [^{3}H]-U69, 593 at the cloned κ-opioid receptor from human (hKOR1) in a concentration dependent manner (Fig. 2) with a K_i value of 0.24 nM and 0.08 nM, respectively (Table 1). In comparison, FE 200665 and FE 200666 had very low affinity for the μ-opioid receptor from human (hMOR1) (K_i=4.0 μM and 7.1 μM, respectively) and for the δ-opioid receptor from human (hDOR1) (K_i=20.3 μM and >100 μM, respectively). The selectivity of these two peptides for the hKOR1 over hMOR1 and hDOR1 are exceedingly high, showing a selectivity ratio of 1/16,900/84,600 (hKOR1/hMOR1/hDOR1) for FE 200665 and 1/88,600/>1,250,000 for FE 200666. The selectivity ratio exhibited by enadoline (1/218/566) and by asimadoline (1/3,420/1,900) for hKOR1 is low compared with the two peptides.

3.2. Agonist activity at the human κ-opioid receptor 1

FE 200665, FE 200666, and enadoline dose dependently induced [^{35}S]GTPγS binding in membranes that expressed the human κ-opioid receptor 1, confirming that like enadoline, FE 200665 and FE 200666 act functionally as agonists at the κ-opioid receptors (Table 2). The κ-agonist activity for both peptides had EC_{50} values that were similar to the affinity of the peptides for the human κ-opioid receptor 1, as determined in radioligand competition analysis (Table 1). The B_{max} values within Table 2 are expressed as a percentage of maximum [^{35}S]GTPγS binding within the membranes for comparison across compounds tested. Enadoline, FE 200665, and FE 200666 resulted in the maximum amount of such binding at concentrations of 1.8 nM, 0.8 nM, and 1.2 nM, respectively.

3.3. Antinociceptive activity in the mouse writhing test after peripheral administration

All the κ-agonists that were tested in the mouse writhing assay produced a dose-related inhibition of writhing after i.v. administration (Fig. 3). All compounds were equally efficacious but differed in potency. The A_{50} values for FE 200665 and FE 200666 were 0.007 and 0.013 mg/kg, i.v., respectively. The A_{50} values for asimadoline and morphine were 0.342 and 0.156 mg/kg, i.v., respectively. FE 200665 and FE 200666 were, respectively, 49- and 26-fold more potent than asimadoline and 22- and 12-fold more potent than morphine. The A_{50} value for (−) U50,488 was 0.102 mg/kg, i.v. with FE 200665 being 15-fold more potent and FE 20066 being 8-fold more potent than (−) U50,488, respectively. Only enadoline (A_{50}: 0.002 mg/kg, i.v.) was more potent than the two peptides in this mouse model of peripheral pain.

Table 2
Stimulation of [^{35}S]GTPγS binding (EC_{50}, nM) by FE 200665, FE 200666 and enadoline in hKOR stably transfected HN cell line

Compound	EC_{50} (nM)	E_{max} (%)
FE 200665	0.03	130
FE 200666	0.08	93
Enadoline	0.50	84

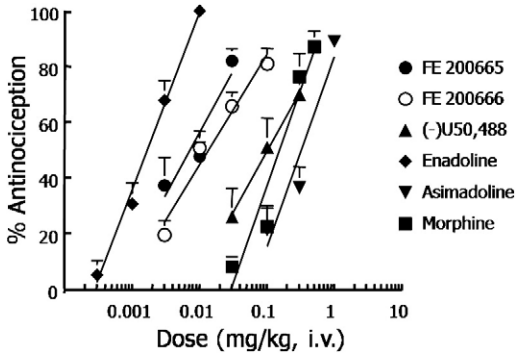

Fig. 3. Dose–response antinociceptive activity in the mouse writhing test after peripheral administration for FE 200665, FE 200666, (−) U50, 488, enadoline, asimadoline and morphine. All test compounds were administered i.v. 5 min prior to 0.6% acetic acid given i.p. The number of writhes where counted over a 15 min period. The A_{50} values were calculated for all dose–response curves with the following order of potency enadoline>FE200665>FE200666>(−) U50, 488>morphine>asimadoline.

The duration of action was evaluated in the mouse writhing test for FE 200665, FE 200666, enadoline, asimadoline and morphine using increasing pretreatment times (5, 60, and 120 min) before acetic acid administration to determine a submaximal and equieffective dose (a dose that produces about 80% antinociception, which was different for each compound due to their difference in potency at 5 min pretreatment time). Under these conditions, morphine (0.5 mg/kg, i.v.) and asimadoline (1.0 mg/kg, i.v.) had durations of action of less than one hour (Table 3). At even lower doses, both FE 200665 (0.030 mg/kg, i.v.) and FE 200666 (0.100 mg/kg, i.v.) maintained significant antinociceptive activities for at least one hour after administration, which was equivalent to that of enadoline.

The dose–response curves for FE 200665 and FE 200666 in the mouse writhing test were displaced to the right in the presence of naloxone (1 mg/kg, s.c.), substantiating an opioid-receptor mediated effect (Table 4); this was likely to be due to the activation of κ-opioid receptors because of the peptides' lack of affinity and functional activity at the μ-opioid receptor and the δ-opioid receptors.

3.4. Motor impairment/sedative activity in the mouse rotarod after peripheral administration

All the above κ-agonists were evaluated also for their ability to induce motor impairment/sedation in the mouse rotarod assay after peripheral (i.v.) administration. As expected, the brain penetrating κ-agonist, enadoline, produced a dose-related inhibition of time spent on the rotating rod (Fig. 4A). The potency in the rotarod assay was not significantly different from the antinociceptive potency in the mouse writhing test for enadoline

Table 3
Duration of the antinociceptive activity of a sub-maximally effective dose of FE 200665 (0.030 mg/kg, i.v.) and FE 200666 (0.100 mg/kg, i.v.) compared to enadoline (0.006 mg/kg, i.v.), asimadoline (1 mg/kg, i.v.) and morphine (0.5 mg/kg, i.v.) in the mouse writhing test

Pre-treatment time (min)	% Antinociception, mean±s.e.m. (*n*) [activity normalized to 100% at 5 min]				
	FE 200665 (0.030 mg/kg, i.v.)	FE 200666 (0.100 mg/kg, i.v.)	Enadoline (0.006 mg/kg, i.v.)	Asimadoline (1 mg/kg, i.v.)	Morphine (0.5 mg/kg, i.v.)
−5 min	81.31±3.74 (*n*=9)[100]	74.13±7.02 (*n*=12) [100]	85.19±7.50 (*n*=9) [100]	89.06±2.59 (*n*=10) [100]	87.11±5.48 (*n*=15) [100]
−60 min	62.71±2.48 (*n*=10) [77]	67.69±6.60 (*n*=22) [91]	63.03±10.91 (*n*=10) [74]	18.16±9.03 (*n*=9) [20]	25.21±6.40 (*n*=12) [29]
−120 min	22.17±7.80 (*n*=9) [27]	25.14±6.91 (*n*=13) [34]	37.15±8.27 (*n*=15) [44]	6.16±4.20 (*n*=8) [7]	–

(A_{50} values of 0.004 and 0.002 mg/kg, i.v.; [95% confidence interval: 0.003–0.006 and 0.001–0.002], respectively). Thus, for this compound it was not possible to dissociate the antinociceptive activity from the motor impairment/sedative activity. Asimadoline, the purported peripheral κ-agonist, showed a tendency to a greater dissociation between antinociception and motor impairment/sedation (A_{50} values of 1.58 and 0.34 mg/kg, i.v.; [95% C.I.: 0.820–3.027 and 0.249–0.470] respectively, Fig. 4B). Nevertheless, the potency ratio between antinociception and motor impairment/sedation remained low for asimadoline (mR/mWT; Table 5). In contrast, both FE 200665 and FE 20066 were able to induce antinociception within a dose range that was 548- and 182- fold lower than that required to produce motor impairment/sedation (Table 5; Figs. 4C and 5). As motor impairment/sedation as measured on the rotarod is a CNS mediated response, and because the antinociceptive activity in the mouse writhing test for the two peptides occurred at doses that were several hundred fold lower than the brain penetrating doses, the antinociception observed for the two peptides in the writhing test was likely to be mediated by action at the peripheral nervous system. The ratios of potency between peripheral antinociceptive and central motor impairment/sedative effects ranked the peripheral selectivity of the tested compounds in the following order: FE 200665 [548]>FE 200666 [182]>TRK-820 [8]>asimadoline [5]>enadoline [2].

3.5. Antinociceptive activity in the mouse tail-flick after peripheral administration

Since opioid antinociception in the mouse tail-flick can be mediated by the activation of the spinal and/or supraspinal opioid receptors but not by activating peripheral opioid receptors, this assay was used here as a surrogate model to determine blood–brain barrier penetration after peripheral administration of the drugs. After intravenous administration, enadoline produced a dose-related antinociception in the mouse tail-flick test. The antinociceptive potency of enadoline in the mouse tail-flick (A_{50}: 0.007 mg/kg, i.v. [95%C.I.: 0.006–0.009]) was consistent with the potency of this compound in the mouse rotarod assay and the writhing test (A_{50}: 0.004 and 0.002 mg/kg, i.v., respectively), resulting in an mTF/mWT potency ratio of 4 (Table 5, Fig. 5). The mTF/mWT potency ratio for asimadoline was slightly higher than its mR/mWT potency ratio (13 vs. 5, respectively). Nevertheless, this index of peripheral selectivity for asimadoline remained lower than that of the brain penetrating agonist, morphine (mTF/mWT ratio: 25) (Table 5, Fig. 5). According to this parameter, both FE 200665 and FE 200666 exhibited an extremely low tendency to cross the blood–brain barrier. The rank order of potency of the compounds to show peripheral selectivity was: FE 200665 [>1429]>FE 200666 [242]>TRK-820 [32]>morphine [25]>asimadoline [13]>enadoline [4].

Table 4
Antinociceptive potency (A_{50}, mg/kg) in the mouse writhing test after peripheral (i.v.) administration of FE 200665 and FE 200666 in the absence or presence of the opioid antagonist naloxone (1 mg/kg, s.c.)

Compound	Antinociceptive potency in mouse writhing test (A_{50} mg/kg, i.v., [95% confidence interval])	
	Control	Naloxone (1 mg/kg, s.c.)
FE 200665	0.009 [0.007–0.011] (*n*=30)	0.021 [0.016–0.026] (*n*=18)
FE 200666	0.013 [0.010–0.018] (*n*=55)	0.055 [0.041–0.073] (*n*=20)

3.6. Activity in the rat writhing test and rotarod assays after peripheral administration

FE 200665 was also active at very low doses (0.003–0.030 mg/kg, s.c.) after peripheral administration in the rat writhing test (A_{50}: 0.010 mg/kg, s.c., [95%C.I.: 0.008–0.012] Table 6; Fig. 6A). FE 200665 was totally inactive in the rat

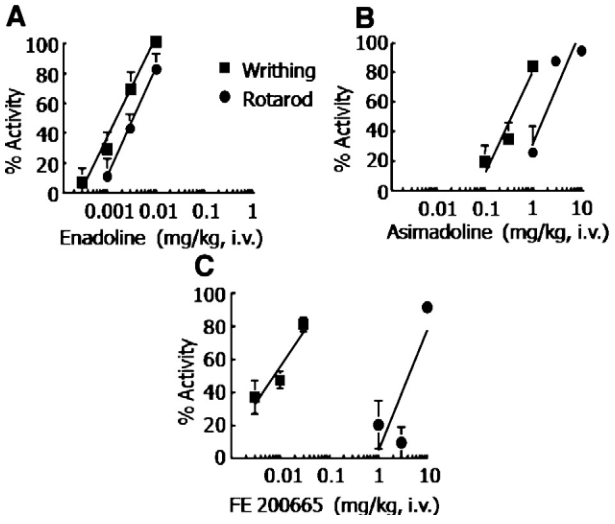

Fig. 4. Dose–response antinociceptive activity in the mouse writhing test compared to the dose-response sedative activity in the mouse rotarod after peripheral (i.v.) administration of (A) enadoline (B) asimadoline (C) FE 200665.

Table 5
Potencies for peripheral (mouse writhing test) vs centrally-mediated activities (mouse rotarod and tail-flick assays) after peripheral (i.v.) administration and peripheral selectivity indexes for FE 200665, FE 200666 and reference compounds

Compound	Potencies (A_{50}, mg/kg, i.v. [95% confidence interval])			Peripheral selectivity indexes (potency ratios)	
	Mouse writhing test (mWT)	Mouse rotarod (mR)	Mouse tail-flick (mTF)	mR/mWT	mTF/mWT
Enadoline	0.002[0.001–0.002] (n=40)	0.004[0.003–0.006] (n=24)	0.007[0.006–0.009] (n=22)	2	4
Asimadoline	0.342[0.249–0.470] (n=19)	1.578[0.820–3.027] (n=16)	4.321[3.117–5.990] (n=23)	5	13
TRK-820	0.001[0.001–0.002] (n=27)	0.008[0.004–0.014] (n=22)	0.032[0.025–0.043] (n=23)	8	32
Morphine	0.156[0.124–0.198] (n=39)	n.d.	3.889[3.072–4.924] (n=21)	n.d.	25
FE 200666	0.013[0.010–0.018] (n=55)	2.372[1.722–3.268] (n=24)	3.151[1.801–5.515] (n=24)	182	242
FE 200665	0.007[0.005–0.012] (n=20)	3.835[2.666–5.517] (n=37)	>10 (n=23)	548	>1429

rotarod at much higher doses, up to 10 mg/kg, s.c., suggesting a preference for peripheral activity in rats when compared to that seen in mice. In contrast, as previously observed in mice, it was impossible to dissociate the antinociceptive activity from the motor impairment/sedative activities for (−) U50, 488 in rat (Fig. 6B).

4. Discussion

We have previously reported the discovery of a new class of κ-agonists by screening positional scanning format mixtures of a tetrapeptide combinatorial library using κ- and μ-opioid receptor radioligand binding assays. The best κ-agonist tetrapeptide identified was an all D-amino-acid tetrapeptide (H-D-Phe-D-Phe-D-Nle-D-Arg-NH_2). This initial lead showed high affinity for [^{3}H]U69,593 binding sites in guinea-pig cerebellum and low affinity for [^{3}H]DAMGO binding sites in rat brain (Dooley et al., 1998). Moreover, this tetrapeptide elicited potent antinociception in the mouse writhing test after peripheral administration within a dose-range that did not induce sedation in the mouse rotarod assay (Vanderah et al., 2004). However, the compound had a rather short duration of action (less than 30 min). These results are supportive of a highly peripheral selective κ-agonist, which is a critical feature for the development of safe κ-agonist drugs. A subsequent lead optimization program aiming at identifying novel peripherally selective and long-lasting κ-agonists was initiated. The pharmacological characterization of two representatives of this class of compounds, FE 200665 (H-D-Phe-D-Phe-D-Nle-D-Arg-NH-4-Picolyl) and FE 200666 (H-D-Phe-D-Phe-D-Leu-D-Orn-Morpholine amide), are reported here. The two compounds are compared to reference opioid agonists, including enadoline, asimadoline, and morphine. These two compounds were first reported to have κ- selective anti-inflammatory effects *in vivo* using a model of Freund's complete adjuvant (CFA) injection in the hind paw of rats (Binder et al., 2001). Furthermore, the two novel compounds reduced paw inflammation as measured by a reduction in CFA-induced paw volume displacement and resulted in significant antinociception as measured by paw withdrawal from a noxious force (Binder et al., 2001). These studies identified local, peripheral activity of FE 200665 and FE 200666 by either giving the compounds locally into the paw and measuring significant antinociception or by systemically administering an opioid antagonist, naloxone methiodide, prior to the compounds and demonstrating an antagonism of the antinociception (Binder et al., 2001). These studies first identified the peripheral activity of such selective κ- agonist,

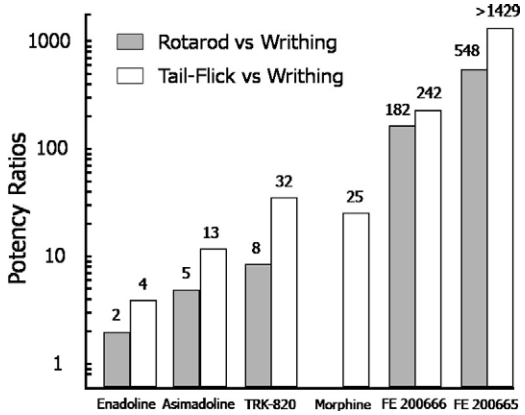

Fig. 5. Peripheral selectivity indexes in mouse, obtained by calculation of the potency ratios between activities in the mouse rotarod and writhing assays or between activities in the mouse tail-flick and writhing assays. Ratios are calculated from the A_{50} i.v. dose–response curves. [95% confidence intervals] for each A_{50} value are shown in Table 5.

Table 6
Potencies for peripheral (rat writhing test) vs. centrally-mediated activities (rat rotarod assay) after peripheral (s.c.) administration for FE 200665 and (−) U-50,488

Compound	Potencies (A_{50}, mg/kg, i.v. [95% confidence interval])		Peripheral selectivity index (potency ratios)
	Rat writhing test (rWT)	Rat Rotarod (rR)	rR/rWT
(−) U50, 488	0.298 [0.256–0.347] (n=12)	0.814 [0.434–1.525] (n=28)	2.7
FE 200666	0.031 [0.024–0.041] (n=29)	1.620 [0.853–3.083] (n=16)	52
FE 200665	0.010 [0.008–0.012] (n=25)	>10.0 (n=28)	>1000
Morphine	0.269 [0.193–0.376] (n=12)	n.d.	N/A

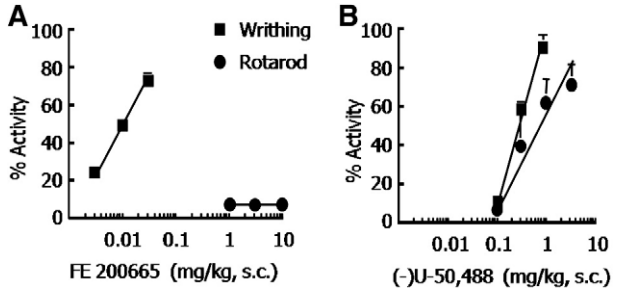

Fig. 6. Dose–response antinociceptive activity in the rat writhing test compared to the sedative activity in the rat rotarod after peripheral (s.c.) administration of (A) FE 200665 and (B) (−) U50, 488. In the writhing assay, compounds were administered 15 min prior to acetic acid. In the rotarod, animals were tested 15, 20, and 25 min after compound administration.

however resulted in many more inquiries including their comparison to other κ-agonists.

FE 200665 and FE 200666 display high affinity in the subnanomolar concentration and unprecedented selectivity of greater than 5 orders of magnitude for the human κ-opioid receptor over the human μ-opioid receptor or the human δ-opioid receptor. These results are entirely consistent with our previous findings on the initial lead compound, FE 200041 (Vanderah et al., 2004). The virtual lack of affinity at both human μ-opioid receptor and human δ-opioid receptor for these κ-agonist tetrapeptides is of particular importance as it eliminates almost all risks of gastrointestinal transit inhibition and associated constipation, respiratory depression and the potential risks of activation of the reward pathways.

FE 200665 and FE 200666 show potent κ-agonist activity at the human κ-opioid receptor 1 using an *in vitro* functional assay. The tetrapeptides are more potent (17- and 6-fold for FE 200665 and FE 200666, respectively) and slightly more efficacious (E_{max}: 130% and 94%, respectively) than enadoline. In rodent pain models, i.e., the mouse and rat writhing test, FE 200665 and FE 200666 appear slightly less potent than enadoline. It is not clear whether the cloned κ-opioid receptor 1 from human or the rodent models is more predictive of the *in vivo* efficacy of this class of compounds in humans; however, FE 200665 and FE 200666 may be predicted to be active at lower doses than enadoline.

FE 200665 and FE 200666 were active in the mouse writhing test after peripheral (i.v.) administration. Both compounds were more potent than asimadoline (49- and 26-fold, respectively) and morphine (22- and 12-fold, respectively). The dose–response curves for FE 200665 and FE 200666 in the mouse writhing tests were sensitive to s.c. naloxone, confirming an opioid-mediated effect most likely at the κ-opioid receptors based on the receptor selectivity shown by the two peptides. Only enadoline (A_{50}: 0.002 mg/kg, i.v.) is more potent than the two tetrapeptides in this mouse pain model. In addition, FE 200665 and FE 200666 are longer acting (about an hour) than morphine or asimadoline (less than an hour) when compared at submaximal and equi-effective doses. Combination of higher potency as well as longer duration of action for FE 200665 and FE 200666 compared to asimadoline suggests that these κ-agonist tetrapeptides could possibly achieve antinociception at daily doses that are several hundred folds lower than that of asimadoline. The reasons for the long duration of action of these peptides remain to be elucidated. It is very likely that the all D-amino acid composition of these peptides renders them more resistant to degradation by peptidases; however, not all D-amino-acid agonist tetrapeptides in this class exhibit long duration of action *in vivo* (Wisniewski et al., 2001). Thus, other factors may contribute to their longer duration of action, e.g. decreased liver uptake or renal elimination, larger volume of distribution, etc.

There is also a clear separation between the antinociceptive activity based on the mouse writhing test and the motor impairment/sedative activity based on the rotarod test for the two peptides. This is in clear contrast to the centrally acting κ-agonist, enadoline, for which the antinociceptive dose and the motor impairment/sedative activity could not be dissociated. Interestingly, the proposed peripheral κ-agonist, asimadoline shows a higher antinociceptive/sedative separation than enadoline but the ratio is modest (5-fold). FE200665 and FE200666 are over a 100-fold more peripherally selective than asimadoline. The acetic acid induced writhing assay can detect antinociceptive activity for opioid drugs acting at peripheral, spinal and supraspinal levels, whereas motor impairment/sedation in the mouse rotarod test can be mediated at the supraspinal level only (Hardy et al., 1989; Porreca et al., 1987). Therefore, to be active after peripheral administration in the mouse rotarod assay, a compound must reach the CNS and cross the blood–brain barrier. For these reasons, the CNS mediated motor impairment/sedative activity in the rotarod assay has been used as a surrogate index of brain penetration for peripherally administered κ-agonists (Barber et al., 1994). Because neither FE 200665 nor FE 200666 are able to induce motor impairment/sedation within the antinociceptive dose-range in the mouse writhing test, it is very likely that antinociceptive doses in the writhing test did not elicit active brain concentrations, supporting a peripheral mode of action in the mouse writhing test and lack of brain penetration. The data show that this class of tetrapeptides confers an unprecedented level of peripheral selectivity as defined by the potency ratio between peripheral nociceptive and central motor impairment/sedative activities.

These results were further confirmed by using the mouse tail-flick. The mouse tail-flick can detect antinociceptive activity for opioid drugs acting at the spinal or supraspinal levels, but not in the periphery. Hence, to be active after peripheral administration, an opioid must reach the CNS and cross the blood–brain barrier. Thus, this assay was used as an index of blood–brain barrier brain penetration for peripherally administered κ-agonists. As previously shown in the mouse writhing test, it was not possible for the brain penetrating κ-agonists, asimadoline, to dissociate active dose ranges in the writhing (A_{50}: 0.002 mg/kg, i.v.) and tail-flick (A_{50}: 0.007 mg/kg, i.v.) assays in mice. Asimadoline achieves only a limited separation between the two activities leading to a modest potency ratio (mTF/mWT) of 13. Under these conditions, the brain penetrating agonist, morphine shows a higher ratio of 25. In contrast, and consistent with data in the mouse rotarod assay, FE 200665 and FE 200666 show a marked dissociation

between active dose range for peripherally- (writhing test) and centrally- (tail-flick) mediated antinociception after intravenous administration confirming an extremely low ability to cross the blood–brain barrier. The peripheral selectivity of FE 200665 was also confirmed in the rat writhing and rotarod assays and the data are consistent with that seen in mice.

Taken together, these data indicate that this novel class of κ-agonist tetrapeptides has unprecedented affinity and selectivity for the human κ-opioid receptor 1 and exhibit peripheral selectivity and long-lasting antinociception activity in multiple pain models in rodents. Condition place preference studies using FE 200665 resulted in no significant difference from baselines at 3.0 mg/kg confirming a lack of CNS κ-opioid receptor induced condition placed aversion (personal communication with Larry Reid and Pierre J-M. Rivière). Simultaneous studies demonstrated aversive behavior with the κ-agonist (−) U50, 488 (1.0 mg/kg and 3.0 mg/kg) and condition placed preference with the μ-opioid agonist, morphine, at 4.0 mg/kg. These studies confirm such antinociceptive doses of FE 200665 and FE 200666 as lacking CNS κ-receptor-induced dysphoric effects. Their predicted therapeutic window is expected to be 109- and 357-fold wider than that of asimadoline and enadoline, respectively. Due to advances in drug delivery including compounds that are absorbed through patches, compounds reaching the circulation rapidly by inhalation or by injection via implantable pumps, these types of stable peptides that lack side effects are perfect candidates as novel medications (Wisniewski et al., 2001; Eisenach et al., 2003).

On this basis, these compounds appear suitable for assessment as novel, peripherally-acting κ-agonists as new analgesics in humans. FE 200665, a.k.a. CR 665, is now under clinical development by Cara Therapeutics. Preliminary disclosure by the company reported that CR 665 was safe and well tolerated in volunteers, hence being devoid of CNS side-effects associated with brain penetrating compounds.

References

Barber, A., Bartoszyk, G.D., Bender, H.M., Gottschlich, R., Greiner, H.E., Harting, J., Mauler, F., Minck, K.O., Murray, R.D., Simon, M., 1994. A pharmacological profile of the novel, peripherally-selective kappa-opioid receptor agonist, EMD 61753. Br. J. Pharmacol. 113, 1317–1327.

Bickel, A., Dorfs, S., Schmelz, M., Forster, C., Uhl, W., Handwerker, H.O., 1998. Effects of antihyperalgesic drugs on experimentally induced hyperalgesia in man. Pain 76, 317–325.

Binder, W., Macelska, H., Mousa, S., Schmidtt, T., Riviere, P.J., Junien, J.-L., Stein, C., Schafer, M., 2001. Analgesic and antiinflammatory effects of 2 novel kappa-opioid peptides. Anesthesiology 94, 1034–1044.

Carr, D.J., DeCosta, B.R., Kim, C.H., Jacobson, A.E., Guarcello, V., Rice, K.C., Blalock, J.E., 1989. Opioid receptors on cells of the immune system: evidence for delta- and kappa-classes. J. Endocrinol. 122, 161–168.

Chavkin, C., James, I.F., Goldstein, A., 1982. Dynorphin is a specific engogenous ligand for the kappa opioid receptor. Science 215, 413–415.

Di Chiara, G., Imperato, A., 1988. Drugs abused by humans preferentially increase synaptic dopamine concentrations in the mesolimbic system of freely moving rats. Proc. Nat'l. Acad. Sci., (USA) 85, 5274–5278.

Donzanti, B.A., Althaus, J.S., Payson, M.M., Von Voigtlander, P.F., 1992. Kappa agonist-induced reduction in dopamine release: site of action and tolerance. Res. Commun. Chem. Pathol. Pharmacol. 78, 193–210.

Dooley, C.T., Ny, P., Bidlack, J.M., Houghten, R.A., 1998. Selective ligands for the mu, delta, and kappa opioid receptors identified from a single mixture based tetrapeptide positional scanning combinatorial library. J. Biolog. Chem. 273, 18848–18856.

Eisenach, J.C., Carpenter, R., Curry, R., 2003. Analgesia from a peripherally active κ-opioid receptor agonist in patients with chronic pancreatitis. Pain 101, 89–95.

Endoh, T., Matsuura, H., Tajima, A., Izumimoto, N., Tajima, C., Suzuki, T., Saitoh, A., Narita, M., Tseng, L., Nagase, H., 1999. Potent antinociceptive effects of TRK-820, a novel kappa-opioid receptor agonist. Life Sci. 65, 1685–1694.

Field, M.J., Carnell, A.J., Gonzalez, M.I., McCleary, S., Oles, R.J., Smith, R., Hughes, J., Singh, L., 1999. Enadoline, a selective kappa-opioid receptor agonist shows potent antihyperalgesic and antiallodynic actions in a rat model of surgical pain. Pain 80, 383–389.

Freye, E., Hartung, E., Schenk, G.K., 1983. Bremazocine: an opiate that induces sedation and analgesia without respiratory depression. Anesthesiol. Analg. 62, 483–488.

Goldstein, A., Tachibana, S., Lowney, L.I., Hunkapillar, M., Hood, L., 1979. Dynorphin- (1-13), an extraordinarily potent opioid peptide. Proc. Nat'l. Acad. Sci. (USA) 76, 6666–6670.

Gottschlich, R., Barber, A., Bartoszyk, G.D., Seyfried, C.A., 1995. The peripherally acting kappa-opiate agonist EMD 61753 and analogues: opioid activity versus peripheral selectivity. Drugs under Exp. Clin. Res. 21, 171–174.

Hardy, G.W., Lowe, L.A., Mills, G., Sang, P.Y., Simpkin, D.S., Follenfant, R.L., Shankley, C., Smith, T.W., 1989. Peripherally acting enkephalin analogues. 2. Polar tri- and tetrapeptides. J. Med. Chem. 32, 1108–1118.

Heyman, J.S., Mulvaney, S.A., Mosberg, H.I., Porreca, F., 1987. Opioid δ receptor involvement in supraspinal and spinal antinociception in mice. Brain Res. 420, 100–108.

Horan, P.J., Porreca, F., 1993. Lack of cross-tolerance between U69,593 and bremazocine suggests kappa-opioid receptor multiplicity in mice. Eur. J. Pharmacol. 239, 93–98.

Hunter, J.C., Leighton, G.E., Meecham, K.G., Boyle, S.J., Horwell, D.C., Rees, D.C., Hughes, J., 1990. CI-977, a novel and selective agonist for the kappa-opioid receptor. Br. J. Pharmacol. 101, 183–189.

Janssen, P.A., Niemgeers, C.J.E., Dony, J.G.H., 1963. The inhibitory effect of fentanyl and other morphine-like analgesics on the warm water-induced tail withdrawal reflex in rats. Arzneim. Forsch. 13, 502–507.

Lai, J., Ma, S.W., Zhu, R.H., Rothman, R.B., Lentes, K.U., Porreca, F., 1994. Pharmacological characterization of the cloned kappa opioid receptor as a kappa 1b subtype. NeuroReport 5, 2161–2164.

Machelska, H., Pfluger, M., Weber, W., Piranvisseh-Volk, M., Daubert, J.D., Dehaven, R., Stein, C., 1999. Peripheral effects of the kappa-opioid agonist EMD 61753 on pain and inflammation in rats and humans. J. Pharmacol. Exp. Ther. 290, 354–361.

Mansour, A., Fox, C.A., Akil, H., Watson, S., 1995. Opioid-receptor mRNA expression in the rat CNS: anatomical and functional implications. Trends Neurosci. 18, 22–29.

Millan, M.J., Czlonkowski, A., Morris, B., Stein, C., Arendt, R., Huber, A., Hollt, V., Herz, A., 1988. Inflammation of the hind limb as a model of unilateral, localized pain: influence on multiple opioid systems in the spinal cord of the rat. Pain 35, 299–312.

Pande, A.C., Pyke, R.E., Greiner, M., Wideman, G.L., Benjamin, R., Pierce, M.W., 1996. Analgesic efficacy of enadoline versus placebo or morphine in postsurgical pain. Clin. Neuropharmacol. 19, 451–456.

Porreca, F., Burks, T.F., 1983. The spinal cord as a site of opioid effects on gastrointestinal transit in the mouse. J. Pharmacol. Exp. Ther. 227, 22–27.

Porreca, F., Mosberg, H.I., Hurst, R., Hruby, V.J., Burks, T.F., 1984. Roles of mu, delta and kappa opioid receptors in spinal and supraspinal mediation of gastrointestinal transit effects and hot-plate analgesia in the mouse. J. Pharmacol. Exp. Ther. 230, 341–348.

Porreca, F., Mosberg, H.I., Omnaas, J.R., Burks, T.F., Cowan, A., 1987. Supraspinal and spinal potency of selective opioid agonists in the mouse writhing test. J. Pharmacol. Exp. Ther. 240, 890–894.

Reece, P.A., Sedman, A.J., Rose, S., Wright, D.S., Dawkins, R., Rajagopalan, R., 1994. Diuretic effects, pharmacokinetics, and safety of a new centrally acting kappa-opioid agonist (CI-977) in humans. J. Clin. Pharmacol. 34, 1126–1132.

Shippenberg, T.S., Stein, C., Huber, A., Millan, M.J., Herz, A., 1988. Motivational effects of opioids in an animal model of prolonged inflammatory pain: alteration in the effects of kappa- but not of mu-receptor agonists. Pain 35, 179–186.

Stein, C., 1991. Peripheral analgesic actions of opioids. J. Pain Symptom Manage. 6, 119–124.

Stein, C., Gramsch, C., Herz, A., 1990. Intrinsic mechanisms of antinociception in inflammation: local opioid receptors and beta-endorphin. J. Neurosci. 10, 1292–1298.

Stein, C., Millan, M.J., Shippenberg, T.S., Peter, K., Herz, A., 1989. Peripheral opioid receptors mediating antinociception in inflammation. Evidence for involvement of mu, delta and kappa receptors. J. Pharmacol. Exp. Ther. 248, 1269–1275.

Tallarida, R.J., Murray, R.B., 1987. Manual of Pharmacological Calculations with Computer Programs, Second Edition. Springer-Verlag Publishers, New York.

Taub, D.D., Eisenstein, T.K., Geller, E.B., Adler, M.W., Rogers, T.J., 1991. Immunomodulatory activity of mu- and kappa-selective opioid agonists. Proc. Nat'l. Acad. Sci. (USA) 88, 360–364.

Unterwald, E., Sasson, S., Kornetsky, C., 1987. Evaluation of the supraspinal analgesic activity and abuse liability of ethylketocyclazocine. Eur. J. Pharmacol. 133, 275–281.

Vanderah, T.W., Schteingart, C.D., Trojnar, J., Junien, J.-L., Lai, J., Riviere, PJ.-M., 2004. FE 200041 (D-Phe-D-Phe-D-Nle-D-Arg-NH_2): a peripheral efficacious κ-opioid agonist with unprecedented selectivity. J. Pharmacol. Exp. Ther. 310, 326–333.

Von Voigtlander, P.F., Lewis, R.A., 1988. Analgesic and mechanistic evaluation ospiradoline, a potent kappa opioid. J. Pharmacol. Exp. Ther. 246, 259–262.

Wisniewski, K., Sueiras-Diaz, J., Schteingart, C., Galyean, R., Houghten, R., Vanderah, T.W., Porreca, F., Riviere, P.J.M., Junien, J.L., Trojnar, J., 2001. Long acting, selective, peripheral kappa agonists. In: Martinez, J., Fehrentz, J.-A. (Eds.), Peptides 2000, Proc. 26th European Peptide Symposium, pp. 775–776.