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Pharmacological characterization of 2-methyl-N-((2'-(pyrrolidin-1-ylsulfonyl)biphenyl-4-yl)methyl)propan-1-amine (PF-04455242), a high-affinity antagonist selective for kappa opioid receptors.

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Running title:

Characterization of a novel kappa opioid receptor antagonist

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Abbreviations: CHO, Chinese Hamster Ovary; CPP, conditioned place preference; DOR, delta opioid receptor, FST, forced swim test; JVC, jugular vein cannulation; KOR,

kappa opioid receptor; LC-MS/MS, Liquid Chromatography/ Mass Spectrometry/ Mass Spectrometry; MED, minimal effective dose; MOR, mu opioid receptor; nor-BNI, nor-binaltorphimine; NSB, non-specific binding; NAc, nucleus accumbens; PEI, polyethylenimine; PET, positron emission tomography; RO, receptor occupancy; RT, room temperature; SB, specific binding; SDS, social defeat stress.

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Abstract

PF-04455242 is a novel kappa opioid receptor (KOR) antagonist with high affinity for human (3 nM), rat (21 nM) and mouse (22 nM) KOR, a ~20-fold reduced affinity for human mu opioid receptors (MOR; K_i = 64 nM) and negligible affinity for delta opioid receptors (K_i > 4 μM). PF-04455242 also showed selectivity for KORs in vivo. In rats, PF-04455242 blocked KOR and MOR agonist-induced analgesia with ID_{50} values of 1.5 and 9.8 mg/kg, respectively, and inhibited ex vivo [^{3}H]CI977 and [^{3}H]DAMGO binding to KOR and MOR receptors with ID_{50} values of 2.0 and 8.6 mg/kg, respectively. An in vivo binding assay was developed using [^{3}H]PF-04767135, a tritiated version of the KOR positron emission tomography ligand GR103545, in which PF-04455242 had an ID_{50} of 5.2 mg/kg. PF-04455242 demonstrated antidepressant-like efficacy (mouse forced swim test), attenuated the behavioral effects of stress (mouse social defeat stress assay) and showed therapeutic potential in treating reinstatement of extinguished cocaine-seeking behavior (mouse conditioned place preference). KOR agonist-induced plasma prolactin was investigated as a translatable mechanism biomarker. Spiradoline (0.32 mg/kg) significantly increased rat plasma prolactin levels from 1.9 ± 0.4 ng/mL to 41.9 ± 4.9 ng/mL. PF-04455242 dose-dependently reduced the elevation of spiradoline-induced plasma prolactin with an ID_{50} = 2.3 ± 0.1 mg/kg, which aligned well with the ED_{50} values obtained from the rat in vivo binding and efficacy assays. These data provide further evidence that KOR antagonists have potential for the treatment of depression and addiction disorders.

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Introduction

The mu, kappa and delta opioid receptors are members of the G protein-coupled receptor (GPCR) superfamily (Gutstein and Akil, 2006; Law et al., 2000). Mu opioid receptor (MOR) agonists, with morphine as the prototype, provide a gold standard for the treatment of pain. However, their therapeutic utility is limited by serious side effects including respiratory depression and dependence liability. Delta opioid receptor (DOR) agonists can also produce analgesia, but with the notable side effects of convulsions, which have limited their development. The endogenous kappa opioid receptor (KOR) system is involved in a number of physiological processes, rendering it opportunistic for drug development. KOR agonists are being pursued for the treatment of pain disorders without the risk of addiction and KOR antagonists have the potential for treatment of stress-related disorders including drug addiction and depression (Aldrich and McLaughlin, 2009; Wang et al., 2010).

Although it has been proposed that there are several KOR subtypes, only one has been cloned (KOR-1; see Bruijnzeel, 2009 for a recent review). KORs are widely expressed throughout the brain, spinal cord and peripheral tissues (Bian et al., 2010; Mansour et al., 1995) and are located on presynaptic terminals (Mansour et al., 1995). Agonist-induced activation of KORs results in the inhibition of adenylyl cyclase and calcium channel activity, and is regulated by the activity of kinases, most notably mitogen-activated protein kinase signal transduction cascades, thereby influencing action potential generation and neurotransmitter release (Fukuda et al., 1996; Law et al., 2000; Wang et al., 2010). KORs and their endogenous ligands, which primarily include dynorphin peptides, are co-localized within the A10 mesolimbic dopamine pathway which is associated with the perception of reward and the addictive effects of

psychostimulants, including cocaine. In addition, the endogenous KOR system plays a key role in the response to stress, which potentiates the rewarding properties of drugs of abuse and contributes to the likelihood of reinstatement of drug-seeking behavior in abstinent subjects (Bruchas et al., 2010).

Selective non-peptide antagonists, for example nor-binaltorphimine (nor-BNI), 5'guanidinonaltrindole and JDTic, have been used to explore the effect of kappa antagonists in animal models (see Aldrich and McLaughlin, 2009 for review), as well as the cyclic peptide zyklophin (Aldrich et al., 2009). These selective KOR antagonists have been shown to be effective in animal models representing anti-depressant-like activity and attenuation of addictive behaviors (Aldrich and McLaughlin, 2009; Aldrich et al., 2009; Metcalf and Coop, 2005).

This manuscript describes the pharmacological characterization of a novel KOR antagonist, 2-methyl-N-((2'-(pyrrolidin-1-ylsulfonyl)biphenyl-4-yl)methyl)propan-1-amine (PF-04455242; Fig. 1; Verhoest et al., 2011), for which in vitro and in vivo experiments have been performed to determine its pharmacological and behavioral properties. In addition, with a view to transitioning PF-04455242 to clinical development, receptor occupancy studies have been used to demonstrate target engagement, as well as establishing KOR-agonist induced prolactin challenge as a mechanistic biomarker, both of which can be translated to human studies. These data provide further evidence in support of the development of KOR antagonists for the potential treatment of depression and addictive disorders.

Methods

Opioid Receptor Binding Assays

Radioligand binding assays were performed using membranes prepared from Chinese Hamster Ovary (CHO) cells expressing either human KOR, MOR or DOR (Table 1 and Table 3), from CHO cells expressing rat KOR (Table 2) or from mouse forebrain (Table 2).

Binding Affinity

Frozen cell paste and rat forebrain tissue were homogenized in 50 mM Tris HCl buffer (pH 7.4 @ 4°C) containing 2.0 mM $MgCl_2$ using a Polytron (setting 5) and centrifuged at 40,000 x g for 10 min. The rat forebrain pellet was resuspended and the homogenate placed in a 37°C water bath for 45 minutes, in order to break down any endogenous opioids that may be present, followed by a second centrifugation at 40,000 x g for 10 min. Final pellets were resuspended in assay buffer, 50 mM Tris HCl buffer (pH 7.4 @ room temperature (RT), containing 1 mM EDTA and 5 mM $MgCl_2$. Incubations were carried out in 96-well v-bottom polypropylene plates. Cell membrane (human opioid receptors and rat KOR) and mouse forebrain (KOR) assays were initiated by the addition of approximately 40 µg or 200 µg protein, respectively, to test drugs and either 0.6 nM [^{3}H]diprenorphine or 5 nM [^{3}H]U69593, respectively, in a final volume of 250 µL. Non-specific binding was determined by radioligand binding in the presence of a saturating concentration of the non-selective opioid receptor antagonist naltrexone (10 µM). After a 60 min incubation period at RT, assay samples were rapidly filtered through Whatman GF/B filters (Brandel), presoaked in 0.5% polyethylenimine (PEI), using a Skatron cell harvester, and washed with ice-cold 50 mM Tris buffer (pH 7.2 at 4°C). Membrane bound [^{3}H]diprenorphine and [^{3}H]U69593 levels were determined by liquid scintillation

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counting of the filters in BetaScint on a Betaplate liquid scintillation counter (Wallac-Perkin Elmer). Protein concentrations were determined using the BCA assay (Pierce) with bovine serum albumin as the standard.

Functional activity

Frozen cell paste was homogenized in 50 mM Tris HCl buffer (pH 7.4 @ 4°C) containing 2.0 mM $MgCl_2$. Membranes were spun in a centrifuge at 40,000 x g for 10 min. The supernatant was discarded and the pellet was resuspended in assay buffer containing 50 mM Tris HCl (pH 7.4 @ 25°C), 5 mM $MgCl_2$, 1 mM EDTA, 100 mM NaCl and 30 μM GDP (final assay concentration). The tissue homogenate was allowed to sit on ice for 20 min while test compounds were dissolved and diluted. To test for antagonism, compounds were tested in the presence of an EC_{50} to EC_{80} concentration (10 or 32 nM) of agonists (U50488H or morphine for KOR and MOR, respectively). A dose response of the agonist was performed for each assay in order to determine an EC_{50} for every experiment. Membrane homogenate was added to 96-well FlashPlates® (PerkinElmer, Waltham, MA) containing test drugs and agonist. FlashPlates® were then incubated for 20 min at 30°C. Following incubation, 0.1 nM [^{35}S]GTPγS was added to each well and the plate was again incubated at 30°C for 30 min. The FlashPlates® were centrifuged at 1000 x g for 5 min and read in a Microbeta® TriLux counter (Wallac-PerkinElmer, Waltham, MA) to measure G-protein bound [^{35}S]GTPγS levels. Radioligand binding in the presence of a saturating concentration of cold GTPγS (10 μM) defined non-specific binding in the assay. Protein concentrations were determined using the BCA assay (Pierce Research Products, Rockford, IL) with bovine serum albumin as the standard.

Animals

Male ICR mice were purchased from Taconic (Germantown, NY), male C57Bl/6J mice were purchased from Jackson Laboratories (Bar Harbor, ME) and male Sprague-Dawley rats were purchased from Charles River Laboratories (Kingston, NY). For in vivo binding and prolactin-challenge studies, rats were purchased with jugular vein cannulations (JVC). Mice, non-cannulated rats and JVC rats were housed 10, 4 or 1 per cage, respectively. Animals were allowed free access to food and water under a 12-hour light/dark cycle with temperature (21-23 ºC) and humidity (47-51%) controlled and were allowed to acclimate to the vivarium for a minimum of 3 days before use. All procedures were carried out in compliance with the NIH Guide for the Care and Use of Laboratory Animals (1996) under protocols approved by Institutional Animal Care and Use Committees.

In Vivo Pharmacology

Tail Flick Test

The tail flick test (D'Amour and Smith, 1941) is a test for analgesic action, where a heat stimulus produced by a light beam is applied to the rat tail. The tail flick latency was measured using a Tail Flick Analgesia Meter (IITC Life Science Inc., CA; sensitivity setting, 100; light intensity, 500). Nociceptive stimulation was induced in rats (180-210 g) by subcutaneous (s.c.) administration of the KOR and MOR agonists spiradoline (5.6 mg/kg) and morphine (3.2 mg/kg), respectively. One hour (h) prior to testing rats were administered either vehicle or PF-04455242, followed by either vehicle or opioid receptor agonist 30 mins later. A built-in sensor was used to detect the tail-flick. Latency to withdrawal was recorded in 0.01 second (s) increments, with an automatic cut-off time of 12 s.

Receptor Occupancy Studies

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For ex vivo binding studies, rats (175-200 g) were administered either vehicle or PF-04455242 (s.c.) and then euthanized 1 h later. The forebrain was collected, frozen on dry ice, and stored at -80°C until further analysis. Animals used for the time-course study were euthanized at 0.25, 0.5, 1, 2, 4, 8 and 24 h after PF-04455242 administration. The forebrain was weighed and homogenized in cold 50 mM Tris-HCl and 1 mM EDTA buffer, pH 7.4 using 1:6 weight:volume with a Polytron homogenizer at setting 6 for 20 seconds. KOR and MOR ex vivo binding experiments were run side by side using tissue from the same animals. 2 nM [^{3}H]CI977 (KOR agonist) or [^{3}H]DAMGO (MOR agonist) was incubated with 100 μL forebrain homogenate at RT for 1 h in a total assay volume of 150 μL. 10 μM naloxone was used to determine non-specific binding. The reaction was stopped by rapid filtration through 0.3-0.5% PEI-soaked GF/B filters using a 96-well Brandel harvester. The filters were washed 5 times with ice-cold buffer (50 mM Tris HCl, pH 7.4 at RT) and transferred to scintillation vials. DOR ex vivo binding was not performed as PF-04455242 demonstrated weak DOR affinity (Table 1).

For in vivo binding studies JVC rats were administered PF-04455242 (s.c.) and then 50 minutes later were intravenously (i.v.) administered [^{3}H]PF-04767135 (100 μCi/kg), a tritiated version of the KOR positron emission tomography (PET) ligand GR-10345 (Talbot et al., 2005), via the JVC line at 2 mL /kg body weight. Animals were euthanized 10 min after i.v. injection. The forebrain and cerebellum were collected, weighed, and homogenized in cold 50 mM Tris-HCl buffer, pH 7.4 in 1:10 weight:volume with a Polytron homogenizer at setting 6 for 20 seconds. 400 μL (40 mg) of forebrain or cerebellum homogenate were filtered separately through 0.3-0.5% PEI-soaked GF/B filters using a 10-place filtration manifold (Hoefer Inc. Holliston, MA) and washed twice with 5 mL ice-cold buffer.

For both ex vivo and in vivo binding studies each sample was run in triplicate. Filters were transferred to individual vials. 10 mL scintillation cocktail (Beckman Coulter Inc., Brea, CA) added to each vial and filters were allowed to soak overnight before counting in a LS6500 Scintillation Counter (Beckman Coulter Inc., Brea, CA).

In Vivo Efficacy

Forced Swim Test

Mice (ICR, 25-30 g) were weighed and then administered (s.c.) either vehicle or test compound. One hour later they were placed for a total of 7 minutes into a 1000 mL beaker that contained 800-900 mLs of 19-20ºC water. After a 2 min acclimation period, swim activity was recorded every 30 s for 5 min. Animals were given a score of 1 for swimming or 0 if immobile. A mouse was considered immobile if it made only those movements necessary to keep its head above water. Ten mice were run concurrently with the investigator blind to treatment condition. A total of 10 ratings were made for each animal by direct observation.

Additionally, a set of C57Bl/6J mice were exposed to a two-day forced swim stress protocol as previously described (Aldrich et al., 2009; Carey et al., 2007) to produce stress-induced reinstatement of cocaine conditioned place preference (CPP) (see “reinstatement”, below). Mice from this experiment were pretreated each day with vehicle or PF-04455242 30 min prior to exposure to forced swim stress (see Fig. 7A). The day after the final exposure to forced swim stress, the place preference responses of mice were tested as described below to determine possible reinstatement of extinguished CPP.

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Social Defeat Stress assay

Social defeat stress (SDS) was induced as described previously (McLaughlin et al., 2006). Briefly, test mice ("the intruder") were transiently placed in the home cage of a resident male mouse (the "aggressor"). SDS behaviors of the intruder mouse during a 20 min confrontation trial with the resident aggressor were recorded. The number of seconds an intruder mouse maintained a socially-defeated posture in the presence of an aggressor was recorded throughout six trials by observers blind to prior drug treatment. Defeat was identified as the display of a submissive (supine) posture for at least 4 s during an agonistic interaction consisting of: immobility (four paws on ground, orienting toward resident), escape (fleeing the resident), crouching (four paws on ground, not orienting toward resident), or defensive upright stance (standing still and erect with forepaws extended) (as in Kabbaj et al., 2001). Note that the time spent in socially-defeated behavior has been suggested as a model of depression-like behavior (Avgustinovich et al., 2005; Berton et al., 2006). Intruder mice were pretreated with vehicle (0.9% saline, s.c.) or PF-04455242 (1, 3 or 10 mg/kg s.c.) 30 min prior to each trial or nor-BNI (10 mg/kg, i.p.) 1 h prior to each day's first social defeat trial. Intruders were exposed to SDS from 1030 to 1330 in the initial daily trial, then again 3 h later in the second trial of the day.

Cocaine-conditioned place preference, extinction and reinstatement

Conditioned place preference: C57Bl/6 mice were place-conditioned using the previously established apparatus and biased cocaine CPP protocol (Aldrich et al., 2009; Carey et al., 2007; McLaughlin et al., 2003), which has also been demonstrated as an effective protocol for the study of extinction and reinstatement (Aldrich et al., 2009; Carey et al., 2007; Szumlinski et al., 2002).

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All CPP studies were performed in a three-compartment box (San Diego Instruments, San Diego, CA). The compartmentalized box was divided into two equal-sized outer sections (25 cm x 25 cm x 25 cm) with distinct cues, joined by a small central compartment (8.5 cm x 25 cm x 25 cm) accessed through a single doorway (3 cm high). The entire unit was fitted with infrared beams, the breaking of which allowed an automated measure of the time animals spend in each chamber. The compartments differed in wall striping (vertical vs. horizontal alternating black and white lines, 1.5 cm in width) and floor texture (lightly mottled vs. smooth).

Time spent in each compartment was measured by allowing individual mice to move freely between all three compartments over a 30 min testing period. Unconditioned animals on average initially demonstrated an equivalent amount of time in each of the two outer conditioning compartments (641 ± 16 and 594 ± 16 s in the left and right compartments, respectively, p=0.11, NS, Student's T-test). All mice were screened for initial preconditioning preferences to establish baseline responses on the first day of the assay.

Place conditioning began following administration of cocaine (10 mg/kg, s.c.), U50488H (10 mg/kg, i.p.) or a graded dose of PF-04455242 on day 2, whereupon mice were confined for 30 min in the outer compartment not initially preferred during preconditioning preference testing. Conditioning with assay vehicle (0.9% saline, 0.25 mL/25 g body weight, s.c.) followed 4 h later (6 h in the case of U50488H), but paired to the opposite (initially preferred) chamber. This conditioning cycle was repeated once each day on day 3 (see Fig. 6A), and animals then tested for CPP the day after the final cycle of conditioning (day 4). Alternatively, mice used for reinstatement testing (see

below) were place conditioned with cocaine (10 mg/kg, s.c.) once daily from days 2-5, with final preference testing performed on day 6 (see Fig. 7A).

Extinction: Place preference for the cocaine-paired compartment after four days of place conditioning was re-examined once a week (see Fig. 7A) to determine extinction. Placing mice repeatedly into the apparatus with free access to all compartments for 30 min produced extinction, defined as a statistically significant decrease in the time spent in the cocaine-paired compartment during the extinction trial as compared to the immediate post-conditioning response, while also demonstrating no significant difference from the initial, preconditioning preference response. As established previously with the C57Bl/6J strain of mice, CPP responses subsided with weekly testing over the three week period of the assay (Aldrich et al., 2009; Brabant et al., 2005; Carey et al., 2007; Szumlinski et al., 2002).

Reinstatement: Reinstatement of drug preference was examined after either exposure to forced swim stress (see above) or an additional cycle of cocaine place conditioning (see Fig. 7A). Note that a single cycle of cocaine place-conditioning has been found to be insufficient to produce CPP alone in C57Bl/6J mice (Brabant et al., 2005). Mice were pretreated s.c. with vehicle or PF-04455242 daily for 2 days 20 min prior to either cocaine place conditioning (for one cycle as detailed above) or forced swimming (see above). The day after completion of stress exposure or cocaine place conditioning, animals were tested for place preference (see Fig. 7A).

Locomotor Activity

Locomotor activity of C57Bl/6J mice was examined using previously described methods (Reilley et al., 2010). Locomotor activity of mice was monitored for 60 min following a 5

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min delay after administration of vehicle (0.9% saline, s.c.), cocaine (10 mg/kg, s.c.), U50488H (10 mg/kg, i.p.) or PF-04455242 (1 or 10 mg/kg, s.c.). The doses of PF-04455242 used in the locomotor activity assay were selected on the basis of activity observed in the antinociceptive tail-flick testing experiments. Locomotor activity was captured and digitalized, with the distance traveled calculated by an Ethovision Pro locomotor tracking system (Noldus Information Technology, Leesburg, VA). Before testing, mice were initially administered vehicle and confined to the locomotor chambers for 60 min to habituate the animals to the apparatus.

Prolactin Challenge Assay

Blood samples (0.44 mL) were collected through a JVC catheter using a DiLab AccuSampler (North Chelmsford, MA) at 30, 60 and 120 min post spiradoline administration. Baseline blood samples were collected before the first injection. A spiradoline dose-response study using 0.1, 0.32, 1.0 and 3.2 mg/kg spiradoline (s.c.) previously demonstrated that a dose of 0.32 mg/kg was the approximate ED_{50} value (Chang et al., in press) and this was therefore used for experiments investigating blockade of the prolactin response. PF-04455242 was s.c. administered to rats (300-400 g) 30 min prior to spiradoline administration. Blood samples were collected into BD Microtainer tubes (Franklin Lakes, NJ) containing lithium heparin and kept at 4^{o}C. The blood samples were then spun down at 10,000 rpm, 4^{o}C and plasma was transferred into new tubes and stored either at -80^{o}C for prolactin analysis by radioimmunoassay, or at -20^{o}C for drug exposure analyses by Liquid Chromatography/ Mass Spectrometry/ Mass Spectrometry (LC-MS/MS). 50 μL aliquots of plasma were analyzed in duplicate with a rat prolactin radioimmunoassay kit (Amersham Biosciences, Piscataway, NJ). The sensitivity of the prolactin assay was 0.07 ng/tube. The intra- and inter-assay

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coefficients of variation (CV) were 3.2% and 10%. Each individual kit was calibrated with standard prolactin, in the range of 160 pg/tube to 10 ng/tube.

Drug Exposure Analyses

Concentrations of PF-04455242 and spiradoline in rat plasma were determined using a model 4000 LC-MS/MS triple quadrupole mass spectrometer (Applied Biosystems/ MDS Sciex Inc., Ontario, Canada). Plasma samples were treated with 4 times the sample volume of acetonitrile containing a suitable internal standard, centrifuged (4000 × g) and the 10 µL of clear supernatant was injected into the LC-MS/MS system. Analytes were chromatographically separated followed by data dependent multiple reaction monitoring of ions in a triple quadrupole mass spectrometer. An autosampler was programmed to inject 10 µL on a Phenomenex Synergi Polar-RP, 30 x 2.0 mM 4 micron column using a mobile phase consisting of 0.1% formic acid in 10 mM ammonium formate and acetonitrile at a flow rate of 0.4 mL/min. Ionization was conducted in the positive ion mode at the ionspray interface temperature of 500°C, using nitrogen as the nebulizing and heating gas. The ion spray voltage was 4500 kV. PF-04455242 and spiradoline were analyzed in the MRM mode using the transitions *m/z* 373→ *m/z* 230 and *m/z* 425 → *m/z* 354, respectively. Calibration curves were prepared by plotting the appropriate peak area ratios against the concentrations of drug in plasma using 1/*x2* weighting of PF-04455242- or spiradoline- /internal standard peak height ratios. The concentration of the analytes in the plasma samples was determined by interpolation from the standard curve, and the dynamic range of the assay was 0.5-2000 ng/mL.

Data Analyses

Radioligand binding studies: K_i values were calculated according to the Cheng-Prusoff equation. For [^{3}H]diprenorphine binding, $K_i = IC_{50}/(1 + (L/K_d))$, where L is the

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concentration of the radioligand used in the experiment and the K_d value is the dissociation constant for the radioligand (determined previously in separate saturation experiments). For [^{35}S]GTPγS binding, $K_i = IC_{50}/[1 + (C/ EC_{50})]$, where C is the concentration of agonist challenge used in the experiment and EC_{50} is the concentration of the agonist challenge which produces a half-maximal stimulation of specific binding. The EC_{50} for each agonist was determined in every experiment and used in the K_i calculation for data collected on that day. Geometric means of the K_i values are expressed as pK_i values ± S.E.M.

Tail-flick: Data analysis was performed using LabStats software (Computer Lab Solutions LLC).

Receptor occupancy: To determine ex vivo specific binding levels (SB) the number of non-specific binding counts (NSB; defined using 10 μM naloxone in the reaction tube), was subtracted from the number of total binding counts (TB; defined using assay buffer in the reaction tube): TB-NSB = SB. The SB for in vivo [^{3}H]PF-04767135 binding was calculated as the number of counts from cerebellar homogenate (CB) subtracted from the number of counts from forebrain homogenate (FB): FB-CB = SB. The mean SB value of the vehicle group (SBv) and the mean specific binding value of each PF-04455242 treated group (SBt) were calculated as mean ± standard error of the mean (S.E.M.). The degree of inhibition of SB (%) resulting from PF-04455242 treatment was calculated as: [(SBv-SB)/SBv]*100.

Forced swim test: Statistical analyses were performed using the Kruskall-Wallis non-parametric analysis of variance test with the Mann-Whitney U test for post-hoc comparisons (LabStats).

Conditioned place preference: Data for conditioned place preference experiments were analyzed with ANOVA using the SPSS 14.0 statistical package (Chicago, IL). Analyses

examined the main effect of CPP phase (e.g. post-conditioning, week of preference test, reinstatement) and the interaction of drug pretreatment (PF-04455242 or vehicle) X reinstatement condition (stress or cocaine exposure), as appropriate. Significant effects were further analyzed using Tukey HSD and Neuman-Keuls Multiple Comparison post hoc testing as appropriate. All data are presented as mean ± S.E.M., with significance set at $p<0.05$.

Locomotor activity: Data for locomotor effects were analyzed with one-way ANOVA using SPSS 14.0, with significant effects further analyzed by Tukey's HSD post hoc testing.

Prolactin assay: Data are presented as mean ± S.E.M.. Statistical analyses were performed using the two samples unpaired *t*-test (LabStats).

Drug exposure analyses: PF-04455242 free drug exposure in plasma was calculated from total plasma (Tp) exposure values using protein binding values (fu,p) determined via equilibrium dialysis (rat fu,p = 0.03; mouse fu,p = 0.122; (Verhoest et al., 2011). and drug molecular weight (372.53) as follows:

Free drug (nM) = (Tp x 1000 x fu,p)/ 372.53

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Drugs

PF-04455242 and spiradoline (2-(3,4-dichlorophenyl)-N-methyl-N-[(5R,7S,8S)-7-pyrrolidin-1-yl-1-oxaspiro[4.5]decan-8-yl] acetamide) were synthesized by the Pfizer Medicinal Chemistry Department. Naltrexone (17-(cyclopropylmethyl)-4,5α-epoxy- 3,14-dihydroxymorphinan-6-one), naloxone (1*S*,5*R*,13*R*,17*S*)-10,17-dihydroxy-4-(prop-2-en-1-yl)-12-oxa-4-azapentacyclo[9.6.1.$0^{1,13}$.$0^{5,17}$.$0^{7,18}$]octadeca-7(18),8,10-trien-14-one),U50488H (2-(3,4-dichlorophenyl)-N-methyl-N-[(1R,2R)-2-pyrrolidin-1-ylcyclohexyl]acetamide), cocaine (methyl (1R,2R,3S,5S)-3-(benzoyloxy)-8-methyl-8-azabicyclo[3.2.1] octane-2-carboxylate), morphine (5α,6α)-7,8-didehydro-4,5-epoxy-17-methylmorphinan-3,6-diol) and amitriptyline HCl (3-(10,11-dihydro-5H-dibenzo[a,d]cycloheptene-5-ylidene)-N,N-dimethylpropan-1-amine) were purchased from Sigma-Aldrich (St Louis, MO). Test compounds were prepared in sterile water or sterile saline (0.9%) to a dose volume of 2 mL/kg or 10 mL/kg for rat and mouse studies, respectively, and s.c. administered. [^{3}H]PF-04767135 (specific activity = 83.1 Ci/mmol) was synthesized by the Pfizer Radiochemistry Group (Groton, CT, USA). [^{3}H]CI977 (2-(benzofuran-4-yl)-N-methyl-N-((5S,7R,8R)-7-(pyrrolidin-1-yl)-1-oxaspiro[4.5]decan-8-yl)acetamide; 41 Ci/mmol) and [^{3}H]DAMGO ((2S)-2-[[2-[[(2R)-2-[[(2S)-2-amino-3-(4-hydroxyphenyl) propanoyl]amino]propanoyl]amino]acetyl]-methylamino]-N-(2-hydroxyethyl)-3 phenylpropanamide; 67 Ci/mmol) were purchased from Amersham Biosciences (Piscataway, NJ) and [^{3}H]U69593 (N-methyl-2-phenyl-N-[(5R,7S,8S)-7-(pyrrolidin-1-yl)-1-oxaspiro[4.5]dec-8-yl]acetamide; 50 Ci/mmol) from PerkinElmer (Waltham, MA).

Results

In Vitro Pharmacology

Receptor Binding Studies

The receptor binding data for PF-04455242 and two potent non-selective opioid receptor antagonists (naltrexone or naloxone), is summarized in Tables 1 & 2. PF-04455242 bound with high affinity to human KOR stably expressed in CHO cells with an average K_i value of 3.0 nM (n=7). PF-04455242 bound with moderate affinity to human MOR (average K_i value of 65 nM, n=7) and with little or no affinity to human DOR (average K_i value >4,000 nM, n=7). PF-04455242 also bound with high affinity to rat KOR stably expressed in CHO cells with an average K_i value of 21 nM (n=10), and to native mouse forebrain KOR with an average K_i value of 22 nM (n=5), representing a ~7-fold decrease in rodent potency compared to human KOR affinity. Additional selectivity data are provided as supplementary information.

As PF-04455242 did not demonstrate affinity for the DOR, the functional profile of PF-04455242 at human KOR and MOR alone was determined using a [^{35}S]GTPγS binding assay. PF-04455242 was shown to be an antagonist at both KOR and MOR stably expressed in CHO cells (Table 3) and showed no agonist effect when tested alone (100 nM; data not shown). PF-04455242 was a more potent antagonist at human KOR with an average K_i value of 1.2 nM (n=8), compared to human MOR, with an average K_i value of 10 nM (n=4).

In Vivo Pharmacology

Tail Flick Test

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Administration of either the KOR-selective agonist spiradoline (5.6 mg/kg, s.c.) or the MOR-preferring agonist morphine (3.2 mg/kg, s.c.) were shown to significantly increase latency to tail withdrawal in rats (Fig. 2). PF-04455242 was shown to attenuate these effects in a dose-dependent manner, with mean EC_{50} values of 1.5 and 9.8 mg/kg for attenuation of KOR and MOR effects, respectively (n=7-8 animals per dosing group; Fig. 2). These data were similar to those previously obtained for PF-04455242 in the mouse tail-flick assay, where EC_{50} values of 0.67 and 12.0 mg/kg were obtained for blockade of KOR- and MOR-mediated effects, respectively (Verhoest et al., 2011). PF-04455242 (32 mg/kg) did not exhibit any agonist activity *per se* in mice (data not shown).

Receptor Occupancy Studies

PF-04455242 (1 h, s.c.) inhibited ex vivo [^{3}H]CI977 and [^{3}H]DAMGO binding to KOR and MOR receptors, respectively, in a dose-dependent manner (Fig. 3A). PF-04455242 was shown to have significantly higher KOR occupancy, compared to MOR, over the dose range 1 mg/kg to 32 mg/kg PF-04455242, with ID_{50}s of 2.0 mg/kg and 8.6 mg/kg, respectively (Fig. 3A). 10 mg/kg PF-04455242 was used for a time course study as this dose produced 95% inhibition of [^{3}H]CI977 binding in the dose-response study. A time-dependent inhibition of [^{3}H]CI977 binding to rat brain was observed (Fig. 3B). At fifteen minutes post-administration, PF-04455242 inhibited [^{3}H]CI977 binding by 60% (p=0.004), reaching a maximal level of 82% (p=0.003) 30 min after PF-04455242 administration, which was maintained for 2 h; this inhibition was no longer present 8 h postdose. The selection of 1 h as the single time point for the ex vivo binding dose-response study matched with that of the tail flick assay. This time point also showed the highest apparent receptor occupancy (RO) in the time-course study (Fig. 3B).

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Ex vivo binding measurements can potentially result in an under-estimation of RO determinations due to dissociation of test ligand during homogenization and radioligand binding assay procedures (Grimwood and Hartig, 2009). [^{3}H]PF-04767135 (a radiolabeled version of the KOR ligand GR103545) was therefore synthesized as an in vivo binding tool to determine preclinical RO levels with potential to translating to the clinic using [^{11}C]PF-04767135 PET to ascertain RO in humans. To establish optimal in vivo binding conditions, [^{3}H]PF-04767135 concentration response (7.5-200 μCi/kg, i.v., 10 min) and time course (200 μCi/kg for 5, 15, and 30 min) studies were performed. Binding levels in rat forebrain increased with increasing doses of [^{3}H]PF-04767135. Binding levels were ~1000 counts per minute (cpm) in 40 mg of brain homogenates when 60 μCi/kg of [^{3}H]PF-04767135 was administered, reaching 5000 cpm at 200 μCi/kg [^{3}H]PF-04767135. Binding levels in rat forebrain were at their highest level 5 min after 200 μCi/kg administration and were reduced by half at 15 min and to one third at 30 min, with the ratio of binding counts between forebrain and cerebellum being highest at the 15 min time point. Intravenous administration of 100 μCi/kg [^{3}H]PF-04767135 for 10 min was selected for PF-04455242 in vivo binding studies. Ten minutes after i.v. administration of 100 μCi/kg [^{3}H]PF-04767135, the binding counts in rat forebrain were 2273 ± 114 cpm (mean ± S.E.M., n = 9) in 40 mg of crude homogenate, which was 260% higher than the counts in cerebellum (859 ± 76 cpm, mean ± S.E.M., n = 9). Rats predosed with 10 mg/kg PF-04455242 for 50 min reduced the binding of [^{3}H]PF-04767135 in forebrain to 1477 ± 86 cpm (mean ± S.E.M., n = 11). However, the binding in cerebellum remained at the same level (807 ± 55 cpm, mean ± S.E.M., n = 11), indicating that the binding in cerebellum was not KOR-specific and that the cerebellum could be used to determine non-specific binding levels.

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PF-04455242 inhibited in vivo [^{3}H]PF-04767135 binding in a dose-dependent manner with an ID_{50} of 5.2 mg/kg (Fig. 3C). To determine whether differences observed between ID_{50} values determined using ex vivo [^{3}H]CI977 agonist binding (2.0 mg/kg) and in vivo [^{3}H]PF-04767135 antagonist binding (5.2 mg/kg) were due to differences in binding technique or the use of agonist/antagonist radioligands, ex vivo [^{3}H]PF-04767135 binding was also performed. Inhibition of ex vivo [^{3}H]PF-04767135 and [^{3}H]CI977 binding by PF-04455242 was determined side by side using homogenates prepared from the same animals. PF-04455242 inhibited ex vivo binding to rat brain KOR receptors using [^{3}H]PF-04767135 (ID_{50} = 9.8 mg/kg) and [^{3}H]CI977 (ID_{50} = 1.7 mg/kg), in a dose-dependent manner (Fig. 3C). The ID_{50} of PF-04455242 obtained using the in vivo binding technique was less than 2-fold lower than that obtained by ex vivo binding (5.2 mg/kg vs. 9.8 mg/kg); which was not a significant difference (Fig. 3C). The ex vivo binding methodology therefore did not appear to have underestimated RO determinations for PF-04455242. The ID_{50} for [^{3}H]CI977 binding in this experiment was similar to the ID_{50} shown above (1.7 mg/kg vs. 2.0 mg/kg) and the ID_{50} for ex vivo [^{3}H]PF-04767135 binding was 5-fold higher than the ID_{50} of [^{3}H]CI977 (9.8 mg/kg vs. 1.7-2.0 mg/kg; Fig. 3C). The observation that PF-04455242 inhibited [^{3}H]CI977 and [^{3}H]PF-04767135 binding with significantly different affinities was confirmed using an in vitro binding displacement study. PF-04455242 spiked into crude rat forebrain homogenates displaced [^{3}H]CI977 and [^{3}H]PF-04767135 binding in a concentration-dependent manner with K_i values of 8 nM and 34 nM, respectively (n=3; data not shown).

In Vivo Efficacy

Forced Swim Test

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Pretreatment with PF-04455242 (1h, s.c.) reduced immobility in mice with a minimal effective dose (MED) of 3.2 mg/kg (p<0.01; Fig. 4). The tricyclic antidepressant amitriptyline (10 mg/kg; 1h s.c.), which was used as a positive control, also showed a significant effect in this assay (p<0.01; Fig. 4).

Social Defeat Stress assay

The time vehicle-treated mice spent in characteristic socially-defeated postures increased daily upon repeated exposure to social defeat stress (SDS; Fig. 5), consistent with previous reports (Kabbaj et al., 2001). Pretreatment with the KOR antagonist nor-BNI significantly reduced the time mice spent in socially defeated immobile postures (factor trial and factor nor-BNI, $F_{trial/4,174}$= 4.15, p=0.017; $F_{nor\text{-}BNI/4,174}$= 42.5, p<0.0001), although these effects were not specific to interaction ($F_{trial\ x\ nor\text{-}BNI/4,174}$=1.65, p=0.195). Notably, when mice were repeatedly exposed to SDS over the next two days, nor-BNI pretreatment significantly reduced the time spent in socially defeated, immobile postures (p<0.01; Tukey HSD post hoc test, Fig. 5 days 2 and 3). Likewise, mice pretreated with PF-04455242 showed a similar day-dependent reduction in time spent in socially defeated immobile postures as compared to the responses of vehicle-pretreated animals. Two-way ANOVA (factor trial and factor dose PF-04455242, $F_{trial/10,163}$= 1.21, p=0.31; $F_{dose\ PF\text{-}04455242/10,163}$= 14.7, p<0.0001; $F_{trial\ x\ dose\ PF\text{-}04455242/10,163}$=2.47, p=0.026) showed significant effects of PF-04455242 and interaction. While there were no significant differences from vehicle during the day 1 trial regardless of the pretreatment dose of PF-04455242 used, time spent immobile was significantly reduced on day 3 by pretreatment with any of the three doses tested (p<0.01; Tukey HSD post hoc test, Fig. 5 day 3) following repeated exposure to SDS. Curiously, only pretreatment with 3 mg/kg PF-04455242 significantly reduced time spent in socially defeated postures on the second day of testing (p<0.01; Tukey HSD post hoc test).

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Conditioned Place Preference

The acutely rewarding or aversive effects of PF-04455242 were determined by place conditioning mice with doses of 1 and 10 mg/kg (Fig. 6A). Place conditioning for two days with either dose of PF-04455242 resembled the results produced with saline alone, inducing no significant change in CPP response from either initial, pre-conditioning place preference responses or when compared to saline place-conditioning (Two-way ANOVA; factor pre/post CPP response and factor treatment, $F_{pre\text{-}post/2,84}$= 1.02, p=0.32; $F_{treatment/2,84}$= 0.06, p=0.94; $F_{pre\text{-}post\ x\ treatment/2,84}$=0.57, p=0.57; Fig. 6B). These results contrast with the place-conditioned aversion produced by U50488H (10 mg/kg, i.p.), which significantly decreased time spent on the drug-paired side (One-way ANOVA, F(4,72) = 9.01; p<0.01; Neuman-Keuls post-hoc test; Fig. 6B), and the place conditioned preference response demonstrated following conditioning with cocaine (10 mg/kg, s.c.) which significantly increased time spent on the cocaine-paired side (p<0.01; Fig 6B, central bars).

KOR antagonists have been demonstrated to suppress stress-induced reinstatement of cocaine-seeking behavior (Aldrich et al., 2009; Beardsley et al., 2005; Carey et al., 2007), prompting the present examination of the effects of PF-04455242. C57Bl/6J mice were first place conditioned over four days with cocaine (Fig. 7A), demonstrating a significantly greater cocaine CPP response over their initial preferences (Fig. 7B, left bars; $F(_{3,230})$=24.77, p<0.0001; one-way ANOVA with Tukey HSD post hoc test) that demonstrated extinction after 3 weeks with a place preference response similar to initial preferences, yet statistically less than the place-preference immediately following place conditioning (p<0.05). Following extinction of cocaine place preference, mice were administered vehicle or PF-04455242 (1 or 10 mg/kg, s.c.) daily and exposed 30 min later to repeated forced swim stress. After forced swimming on the second day, mice

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were tested for place preference to examine reinstatement of drug seeking behavior. Consistent with the reported activity of KOR antagonists, stress-exposed vehicle-pretreated mice subsequently demonstrated a reinstatement of CPP (Fig. 7B, striped center-left bar, $F_{(4,157)}$=24.98, p<0.0001; one-way ANOVA with Tukey HSD post hoc test.) Notably, pretreatment with 1, but not 10, mg/kg s.c. PF-04455242 prevented stress-induced reinstatement of cocaine-CPP. Additional tests exposed mice demonstrating extinction of CPP instead to an additional single cycle of cocaine conditioning prior to place preference testing. Cocaine-exposed mice pretreated with either vehicle (Fig. 7B, dark grey thatched right bar) or PF-04455242 (10 mg/kg s.c.; Fig. 7B, rightmost bar) exhibited reinstatement of place preference ($F_{(3,133)}$=32.58, p<0.0001; one-way ANOVA with Tukey HSD post hoc test). Consistent with the actions of established KOR antagonists, PF-04455242 prevented stress-, but not cocaine-induced reinstatement of place preference.

Locomotor Activity

The effect of PF-04455242 on locomotor activity was assessed in C57Bl/6J mice over a 60 min period (Fig. 8). Mice were s.c. administered PF-04455242 at either 1 or 10 mg/kg. Additional mice were administered vehicle (saline, 0.9%), cocaine (10 mg/kg, s.c.) or the KOR agonist U50488H (1 mg/kg, i.p.) for comparison. As expected of a psychostimulant, this dose of cocaine significantly increased the distance traveled ($F_{(4,38)}$= 17.79; p<0.0001; one-way ANOVA with Tukey HSD post hoc test), doubling the distance traveled by saline-treated mice (12.8 ± 1.0 m vs. 6.03 ± 1.16 m, respectively). Notably, PF-04455242 treatment did not significantly alter locomotor activity from the values observed by saline pretreatment, at either 1 mg/kg (6.12 ± 0.82 m) or 10 mg/kg pretreatment doses (6.46 ± 0.64 m; Fig. 8, triangles). However, the low dose of

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U50488H used reduced locomotor activity (3.21 ± 0.31 m), but this effect was brief, and was not significantly different from either saline or the PF-04455242 responses (Fig. 8).

Biomarker Assay: spiradoline-induced rat plasma prolactin levels

Spiradoline (0.32 mg/kg; s.c.; 30 min administration) significantly increased rat plasma prolactin levels from 1.9 ± 0.4 ng/mL to 41.9 ± 4.9 ng/mL (22-fold elevation; Fig. 9). PF-04455242, when administered at 3.2 or 10 mg/kg s.c., was shown to have no effect on prolactin levels *per se* (data not shown). 30 min pretreatment of PF-04455242 reduced the elevation of plasma prolactin in a dose-dependent manner with ED_{50} = 2.3 ± 0.1 mg/kg (95% confidence 1.9-2.8 mg/kg) (Fig. 9). Plasma samples taken from the dose response study were analyzed for PF-04455242 and spiradoline concentrations. Free plasma PF-04455242 concentrations (mean ± standard deviation of the mean, n=3) 1 h post PF-04455242 (0.32, 1 or 3.2 mg/kg) administration were calculated to be 3.35 ± 13.1, 13.2 ± 17.2 and 35.0 ± 85 nM, respectively. The presence of PF-04455242 in these plasma samples had no significant effect on the concentration of plasma spiradoline (data not shown). Plasma samples from a mouse tail-flick experiment (Verhoest et al., 2011) were also analyzed for PF-04455242 concentrations. Free plasma PF-04455242 concentrations from mice administered either 0.32, 1 or 3.2 mg/kg PF-04455242 (s.c.) were calculated to be 0.644 ± 0.244, 3.28 ± 1.22 and 23.7 ± 13.3 nM, respectively.

Discussion

The “opium cure” has been recommended for centuries for the treatment of melancholia. Evidence has since been accumulating to suggest that kappa opioid receptors may be a viable target for the development of a novel antidepressant. KOR agonists, such as butorphanol and enadoline, have been reported to increase dysphoria, confusion, sedation and to produce feelings of depersonalisation in humans (Greenwald and Stitzer, 1998; Walsh et al., 2001), whilst the naturally occurring selective KOR agonist salvinorin A induces hallucinations (Roth et al., 2002). Additional evidence is provided by the partial MOR agonist/weak partial KOR agonist buprenorphine, which can be effective in the pharmacological treatment of affective disorders (Gerra et al., 2006).

Here we describe the pharmacological characterization of a novel KOR ligand with antagonist properties, PF-04455242. PF-04455242 was shown to possess high affinity for the KOR, with affinities of 3.0, 21 and 22 nM for human, rat and mouse receptors, respectively. In vitro selectivity data obtained using in vitro binding assays showed 21- and >1330-fold selectivity for KOR over MOR and DOR, respectively. The rat tail flick assay demonstrated that PF-04455242 was systemically active while being devoid of agonist activity in vivo, with a 6.5-fold selectivity for KOR compared to MOR. These data compared well with those obtained for mouse tail-flick, with ED_{50} values of 0.67 and 12.0 mg/kg for KOR and MOR, respectively (Verhoest et al., 2011). In vivo selectivity of PF-04455242 was also demonstrated using ex vivo and in vivo binding paradigms, with a 4.3-fold selectivity for KOR vs. MOR using agonist radioligand binding ex vivo. A number of selective KOR antagonists, including Nor-BNI and JDTic, have been shown to have an exceptionally long duration of action in vivo, which might constrain their therapeutic value (see Metcalf and Coop, 2005). Receptor occupancy time course data

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showed that PF-04455242 was no longer bound to KORs after 8 hours, suggesting a relatively short duration of action, which has subsequently been confirmed using a warm water tail-withdrawal assay (Melief EJ, Miyatake M, Carroll, F.I., Béguin, C., Carlezon Jr, W.A., Cohen, B.M., Grimwood, S., Mitch, C.H., Rorick-Kehn, L. and Chavkin' C, submitted).

The ID_{50} value for PF-04455242 inhibiting KOR-mediated analgesia measured using the rat tail-flick assay (1.8 mg/kg) was similar to the ID_{50} value in the rat prolactin challenge assay (2.3 mg/kg). In the mouse assays PF-04455242 was effective at similar MEDs in the FST (3.2 mg/kg), SDS assay (1 mg/kg), CPP (1 mg/kg) and stress-induced placement of cocaine-seeking behavior (1 mg/kg), aligning well with the mouse tail-flick assay ID_{50} (0.67 mg/kg; Verhoest et al., 2011). Extrapolation of a compound's in vitro binding affinity to efficacy in vivo is dependent on a number of factors including route of administration, plasma protein binding, brain penetration, target engagement and metabolism, all of which might be species dependent (Shaffer 2010). PF-04455242 has been shown to have excellent brain penetration, to lack the capacity to serve as a P-gp substrate, and although highly bound to plasma protein, to be well equilibrated between plasma and brain, with free brain exposures correlating strongly with KOR binding affinities (Verhoest et al., 2011). Free plasma PF-04455242 exposures following PF-04455242 administration at doses required for efficacy, for example at 3.2 mg/kg, in rat (35 nM) and mouse (23.7 nM) were demonstrated to be similar to the measured Ki value for rat (21 nM) or mouse (22 nM). PF-04455242 doses required for efficacy in rat and mouse were also generally similar to or below those required for 50% KOR occupancy (2 - 5.2 mg/kg for rat; data not shown for mouse). All of these data sets for PF-04455242 therefore align very well across in vitro affinity values and in vivo

pharmacological and behavioral assays in rat and mouse, providing high confidence for future dose projections in humans.

GPCR antagonists for a wide array of different receptor targets, therapeutic applications, and physiological responses have been shown to consistently require a similar range of target site occupancy (50–87%) for optimal activation of physiological or therapeutic actions (Grimwood and Hartig, 2009). It's been suggested that the degree of target site occupancy required within this range may be dependent on the amount of receptor reserve, with the release of moderate to high levels of endogenous agonists driving the system requiring very high levels of antagonists to significantly inhibit the response and unusually low antagonist target occupancy expected when a physiological system is weakly activated by endogenous agonists (Grimwood and Hartig, 2009). PET studies have demonstrated that a clinical dose of the opioid receptor ligand naloxone, used to reverse opiate overdose, produced 50% receptor occupancy in healthy volunteers (Melichar et al., 2003), suggesting that opioid receptors require a degree of target occupancy at the lower range of that demonstrated for GPCR antagonists. The observation that PF-04455242 required ~50% KOR occupancy for preclinical efficacy aligns with the clinical data for naloxone, again providing confidence in the level of dosing that would be required for clinical efficacy. Preclinical receptor occupancy studies were translated to humans using [^{11}C]GR103545 PET (Jacobsen et al., 2010; manuscript in preparation).

Multiple lines of evidence developed using KOR antagonists in preclinical models has also contributed to establishing confidence in rationale for this mechanism in the treatment of depression. Immobilization stress, forced swim, or induction of learned

helplessness has been shown to increase dynorphin immunoreactivity in specific subregions of the hippocampus, as well as the nucleus accumbens (NAc), and blockade of KORs in these regions produces an antidepressant response in the learned helplessness model of depression (Shirayama et al., 2004). In addition, reward is mediated by the ventral tegmental-NAc dopaminergic pathway which is modulated by KORs located directly on dopamine containing cells that project to the NAc (Hyman et al., 2006). Here we have shown that PF-04455242 was effective in the FST in a manner similar to other KOR antagonists (Mague et al., 2003). The SDS assay was used as a second animal model for assessing depression-like behavior. While not as well established as the FST or often used as such, the SDS model has been validated for the testing of antidepressants (Avgustinovich et al., 2005). Bolstering this use is the finding that mice subjected to chronic social defeat demonstrate long-lasting socially-defeated behavior that is reversed by treatment with established antidepressant, but not anxiolytic, agents (Berton et al., 2006). Testing in the SDS assay may better reflect the psychological aspects of depression that are not measured in the FST (Berton et al., 2006), emphasizing its value as a battery of behavioral tests for depression-like behavior. SDS results obtained with PF-04455242 were consistent with earlier findings using nor-BNI in this assay (McLaughlin et al., 2006). Moreover, results consistent with other demonstrations of KOR antagonists proving effective as antidepressants in the subsequent (but not first) day of depression-like testing (McLaughlin et al., 2003).

Place conditioning experiments with PF-04455242 did not demonstrate rewarding or aversive effects directly, but PF-04455242 pretreatment attenuated stress-induced reinstatement of cocaine CPP in mice. KOR antagonists have previously been shown to suppress stress-induced reinstatement of cocaine-seeking behavior (Aldrich et al., 2009;

Beardsley et al., 2005; Carey et al., 2007). Importantly, pretreatment with 1 mg/kg (but not 10 mg/kg) PF-04455242 resulted in the prevention of stress-induced reinstatement of cocaine-CPP. It is possible that this was due to the onset of MOR agonist activity in addition to KOR agonism produced by PF-04455242 at a dose of 10 mg/kg.

KOR agonists increase plasma prolactin levels in both preclinical and clinical models (Butelman et al., 2007; Ur et al., 1997). These effects are thought to be mediated by hypothalamic opioid receptors, which modulate the dopaminergic tuberoinfundibular system (Manzanares et al., 1991). PF-04455242 had no effect on basal plasma prolactin levels and inhibited spiradoline-induced rat plasma prolactin levels at concentrations consistent with KOR activity. PK/PD modeling of these data was performed (Chang et al., in press), demonstrating successful prediction of clinical response in a proof of mechanism study (Jacobsen et al., 2010; manuscript in preparation).

In conclusion, here we describe the pharmacological characterization of a novel KOR antagonist, PF-04455242, which provides further evidence that KOR antagonists may provide an effective therapy for the treatment of depression and addiction disorders. Preclinical biomarker data demonstrating target engagement and attentuation of a prolactin challenge assay were subsequently shown to translate to humans, demonstrating proof of mechanism for PF-04455242 in early clinical development (Jacobsen et al., 2010; manuscript in preparation).

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Authorship Contributions

Participated in research design: SG, JPM, SM, YL, PRV, SW, AWS, MAV-F, AS-B

Conducted experiments: JPM, YL, JF, MAV-F, EM

Contributed new reagents or analytical : PRV

Performed data analysis: JPM, SM, YL, JF, MAV-F, EM

Wrote or contributed to the writing of the manuscript: SG, JPM, YL, AWS

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Legends for Figures

Figure 1

Structure of PF-04455242.

Figure 2

Rat Tail Flick Test: PF-04455242 attenuated opioid receptor agonist induced latency to withdrawal, with a higher affinity for KOR. Latency to withdrawal (seconds, s) was measured 30 minutes after subcutaneous (s.c.) administration of either vehicle or an agonist for (A) KOR (spiradoline; 5.6 mg/kg) or (B) MOR (morphine; 3.2 mg/kg), in the absence or presence of PF-04455242 (1 h administration, s.c.). Data shown are the mean ± SEM (n=7-8 animals per group). EC_{50} values were 1.5 and 9.8 mg/kg for KOR and MOR, respectively. Opioid receptor agonist treatment significantly increased latency to withdrawal ($^{\dagger\dagger\dagger}p<0001$, unpaired t-test, compared to vehicle treatment), and this was attenuated by PF-04455242 ($^{*}p<0.05$; $^{***}p<0.0001$, compared to agonist treatment alone).

Figure 3

Rat Receptor Occupancy: **(A)** Ex vivo binding dose response analysis. Kappa opioid receptors (KOR) were labeled with [^{3}H]CI977 (Δ) and mu opioid receptors (MOR) were labeled with [^{3}H]DAMGO (□). Data shown are the mean ± S.E.M. of n = 3 rats. Ex vivo binding was run side by side using forebrain homogenates (right and left side) prepared from the same animals. PF-04455242 showed significantly higher KOR occupancy, compared to MOR, with ID_{50}s of 2.0 mg/kg and 8.6 mg/kg, respectively. $^{*}p<0.05$,

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p<0.01, ***p<0.001; unpaired t-test. **(B) Time course analysis. Data shown are the mean ± S.E.M. of n = 3 rats. PF-04455242 (10 mg/kg) inhibited ex vivo [^{3}H]CI977 binding to rat forebrain KOR in a time-dependent manner. **p<0.001; ***p<0.0001, compared to vehicle treatment. **(C)** PF-04455242 inhibition of in vivo [^{3}H]PF-04767135 and ex vivo [^{3}H]CI977 and [^{3}H]PF-04767135 binding to rat brain KOR. Data shown are the mean ± SEM for in vivo [^{3}H]PF-04767135 binding (●; n = 8-10 rats) or ex vivo [^{3}H]PF-04767135 (○, n=4 rats) or [^{3}H]CI977 binding (Δ; n=4 rats). For in vivo binding, male Sprague Dawley rats with jugular vein catheters were pretreated with PF-04455242 or vehicle s.c. for 50 minutes and then administered [^{3}H]PF-04767135 i.v. for 10 minutes. For ex vivo binding studies brain homogenates (predosed with vehicle or PF-04455242) were incubated with [^{3}H]PF-04767135 or [^{3}H]PF-CI977 for 60 minutes in the absence or presence of cold naloxone to define total and non-specific binding, respectively. Ex vivo binding experiments were run side by side using homogenates prepared from the same animals. Significant differences were observed between ex vivo [^{3}H]PF-04767135 and [^{3}H]PF-CI977 binding determinations, *p<0.05,*** p<0.001.

Figure 4. Forced Swim Test. Data shown are the mean ± S.E.M. (n = 10 mice per group) from a representative experiment which was repeated once with similar results. Subcutaneous administration of amitriptyline (Amit; 10 mg/kg) and PF-04455242 significantly attenuated time (seconds, s) to immobility in the forced swim test an hour later. PF-04455242 showed a mimimal effective dose of 3.2 mg/kg. **p<0.01, Kruskall-Wallis and Mann-Whitney U tests.

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Figure 5. *PF-04455242 reduced time spent in socially defeated postures in a time and dose-dependent manner.* Data shown are the mean ± S.E.M. of mice repeatedly exposed to social defeat stress (SDS) in 6 trials over 3 days, with the time C57Bl/6J mice spent immobile and in socially defeated postures recorded daily during the first trial. Mice received daily injections of vehicle (circles) or PF-04455242 (triangles) 30 min prior to each social defeat stress trial (tr), or nor-BNI (squares) 1 h prior to the first SDS exposure. Nor-BNI pretreatment significantly reduced the time spent in socially defeated postures in each trial, while PF-04455242 pretreatment dose-dependently reduced the time spent in socially defeated postures on days 2 and 3. Points represent n = 24 (vehicle treated mice), n = 36 (nor-BNI treated mice) or n =11-12 (PF-04455242-treated mice). * = significant difference between matching response of vehicle-treated mice, $p<0.05$; †=significant difference in time spent in submissive postures as compared to matching response of first day; two-way ANOVA followed by Tukey's HSD post hoc test.

Figure 6. *PF-04455242 did not induce cocaine-like conditioned place preference or U50488H-like conditioned place aversion.* **(A)** Schematic of testing protocol. **(B)** Summary graph of place-conditioning results. Initial preference for either the left or right compartment of the CPP system was determined on Day 1 of testing for each group of mice (solid bars) and plotted as the difference in time spent on the eventual drug-paired side (s). On days 2 and 3, individual mice were place conditioned in the initially non-preferred chamber side with vehicle (0.9% saline, leftmost bars), U50488H (10 mg/kg, i.p.; second pair of bars from left), cocaine (10 mg/kg, s.c.; central pair of bars), or PF-04455242 (1 mg/kg, s.c., fourth pair of bars from left or 10 mg/kg, s.c.; rightmost pair of bars). Place-conditioning in the initially preferred chamber followed 4-6 h later with vehicle (0.9% saline). On the fourth day of testing mice were again tested to determine final place preference (striped bars). Final conditioned place preference did not

significantly differ from the initial preference for mice treated with vehicle or PF-04455242 (1 mg/kg, s.c.), but was significant for U50488H, cocaine and the higher dose of PF-04455242 (10 mg/kg, s.c.). Each bar represents 12 – 22 mice. * = significant difference from the initial preference $p<0.05$; † = significant difference from final preference of U50488 and cocaine place-conditioned mice $p<0.05$, Tukey's HSD post hoc test.

Figure 7. *Stress-induced reinstatement of cocaine CPP prevented by PF-04455242 pretreatment.* **(A)** Schematic of reinstatement and testing protocol. Vehicle (0.9% saline) or PF-04455242 were administered on days 28 and 29, 20 min prior to initial exposure to forced swim stress (diamonds) or cocaine place conditioning (square, day 29). **(B)** After 4 days of cocaine (10 mg/kg s.c. daily), mice exhibited significant preference for the cocaine paired environment, with extinction occurring by 3 weeks (left bars). Mice were exposed to forced swim stress (center bars) or an additional round of cocaine place conditioning (right bars), reinstating place preference. PF-04455242 pretreatment (1, but not 10, mg/kg s.c.) prevented stress-induced reinstatement of place preference (center bars). In contrast, PF-04455242 pretreatment (10 mg/kg s.c.) was ineffective at preventing cocaine-induced reinstatement of CPP (rightmost bar). Bars represent n = 8 -17 mice; cocaine place conditioning data on left represents combined responses of 81 mice. *=Significantly different from preconditioning place preference response (leftmost bar); †=significantly different from postconditioning place preference response (second bar on left); ‡=significantly different from stress-induced reinstatement of place preference response (striped grey bar, center), Tukey's HSD post hoc test.

Figure 8. *PF-04455242 treatment did not significantly alter locomotor activity.* Mice were administered vehicle (0.9% saline; circles), cocaine (10 mg/kg; squares), U50488H

(10 mg/kg; diamonds) or PF-04455242 (1 mg/kg, triangles; 10 mg/kg, inverted triangles) and locomotor activity determined for 60 minutes. PF-04455242-treated mice did not show significant total changes in locomotor activity (cm/5 min) or when compared to vehicle-treated mice, as produced by U50488 (hypolocomotion) or cocaine (hyperlocomotion). Groups represents 7 - 8 mice/treatment.

Figure 9. *Effect of PF-04455242 on spiradoline-induced rat plasma prolactin levels.* Rat plasma prolactin data are the mean ± S.E.M. of n = 4-11 animals from 3 experiments. Animals were pretreated with PF-04455242 s.c., 30 minutes before spiradoline dosing. Rat blood samples were collected 30 minutes after spiradoline (0.32 mg/kg) dosing. PF-04455242 reduced the elevation of spiradoline induced plasma prolactin in a dose-dependent manner. ***p<0.001.

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Table 1: Affinity of PF-04455242 at Human Opioid Receptors

Human receptor	Kappa Receptor			Mu Receptor			Delta Receptor		
	pKi ± S.E.M.	Ki, nM	n	pKi ± S.E.M.	Ki, nM	n	pKi ± S.E.M.	Ki, nM	n
PF-04455242	8.52 ± 0.10	3.02	7	7.19 ± 0.09	65.1	7	<5.40	>4,000	7
Naltrexone	9.04 ± 0.05	0.92	4	9.20 ± 0.07	0.63	4	7.53 ± 0.04	30	4

[^{3}H]Diprenorphine binding to CHO cell membranes expressing human opioid receptors was performed in 96-well plates using naltrexone to define non-specific binding. Data shown are the mean ± S.E.M. of n determinations.

Table 2: Affinity of PF-04455242 at Rodent Kappa Opioid Receptors

	Rat			Mouse		
	pKi ± S.E.M.	Ki nM	n	pKi ± S.E.M.	Ki nM	n
PF-04455242	7.68 ± 0.13	21	10	7.66 ± 0.14	22	5
Naloxone				8.22 ± 0.14	6	4
Naltrexone	8.32 ± 0.17	3.9	3			

[^{3}H]Diprenorphine binding to CHO cell membranes expressing rat kappa opioid receptors were performed in 96-well plates using naltrexone to define non-specific binding. [^{3}H]U69593 binding to mouse forebrain receptors were performed in 96-well plates using naloxone to defone non-specific binding. Data shown are the mean ± S.E.M. of n determinations.

Table 3: Antagonist Activity of PF-04455242 at Human Kappa and Mu Opioid Receptors

Human receptor	Kappa Receptor			Mu Receptor		
	pKi ± S.E.M.	Ki, nM	n	pKi ± S.E.M.	Ki, nM	n
PF-04455242	8.91 ± 0.05	1.23	8	8.00 ± 0.18	10	4
Naltrexone	8.18 ± 0.10	6.5	4	8.59 ± 0.11	2.5	4

[^{35}S]GTPγS binding to CHO cell membranes expressing human opioid receptors was performed in 96-well plates using cold GTPγS to define non-specific binding. Data shown are the mean ± S.E.M. of n determinations.

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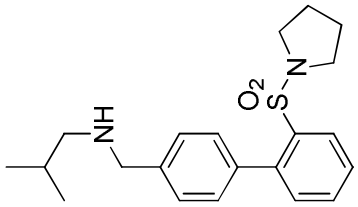

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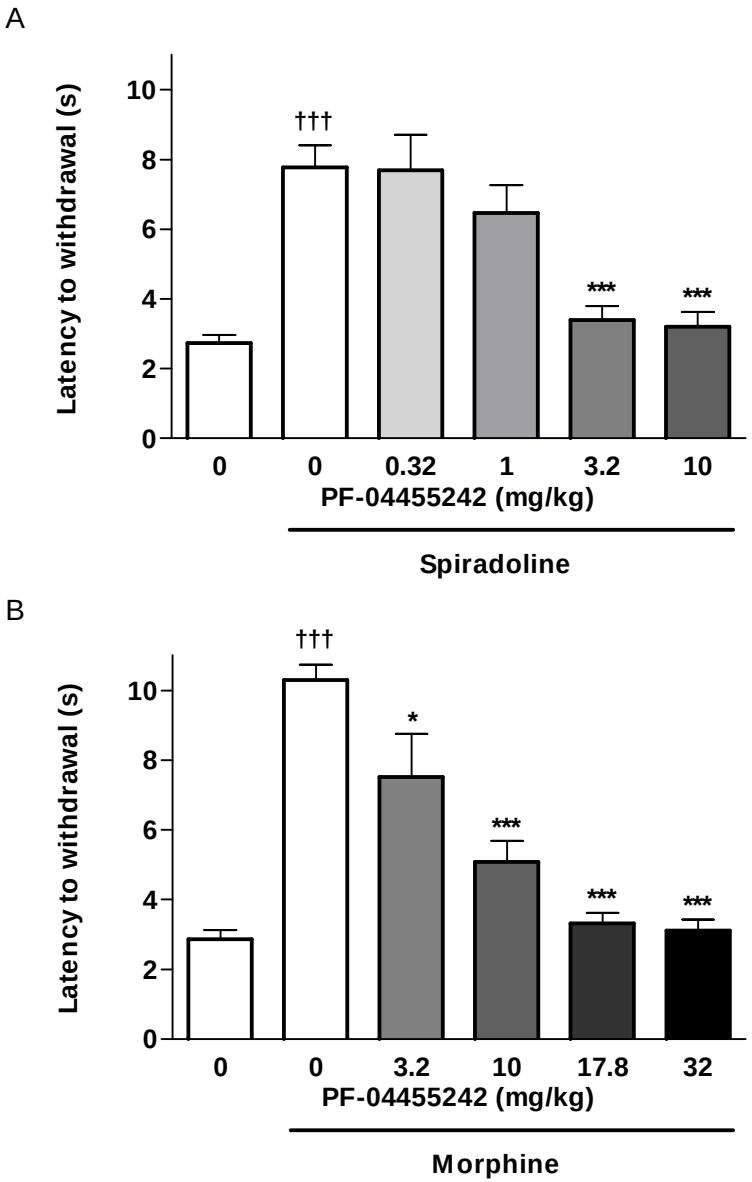

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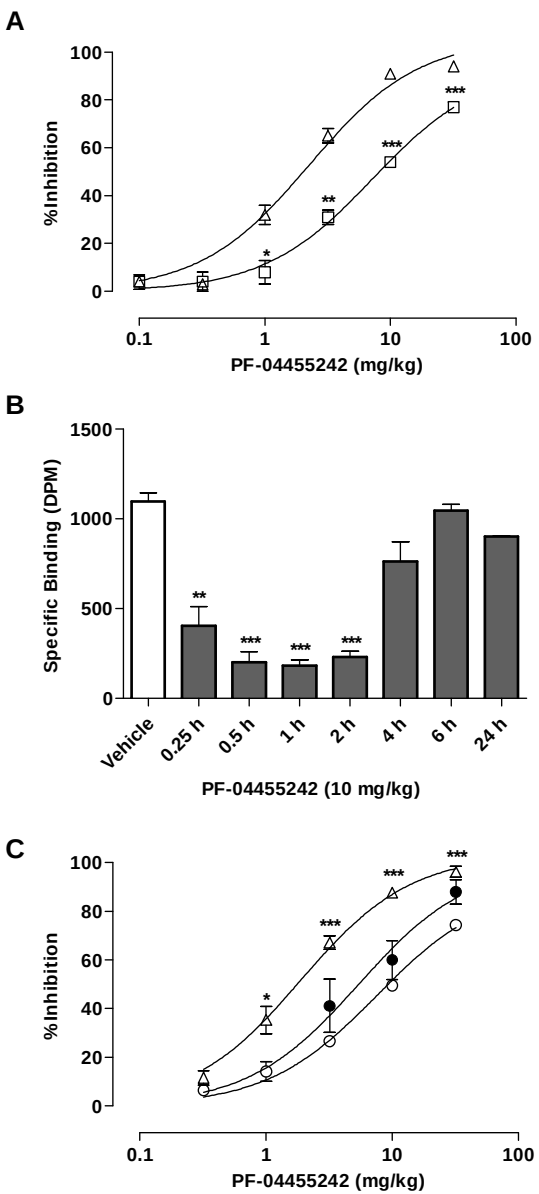

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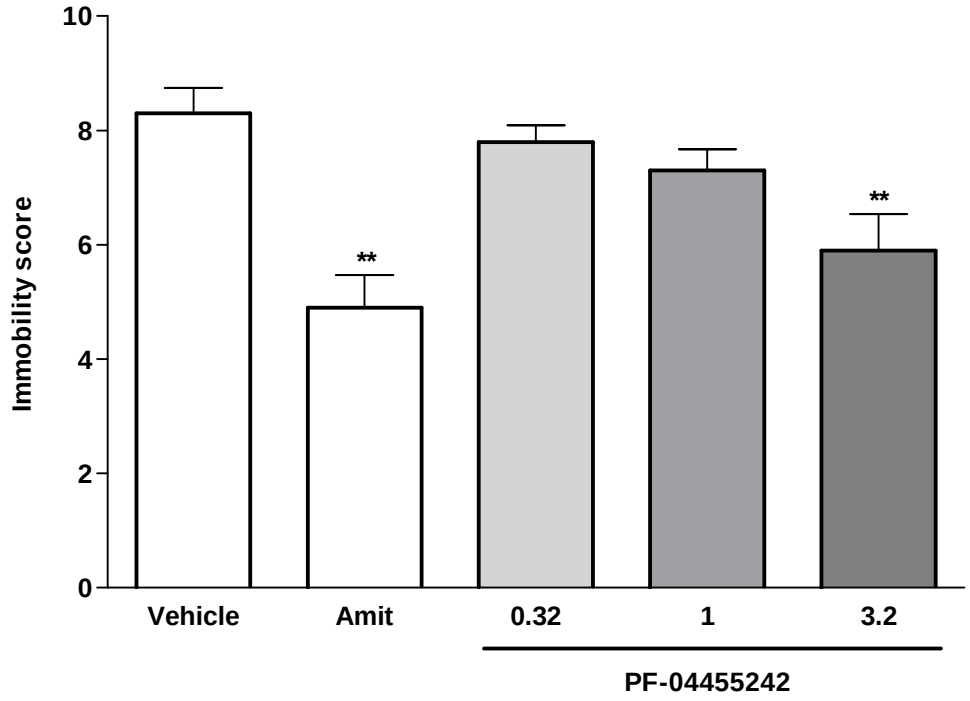

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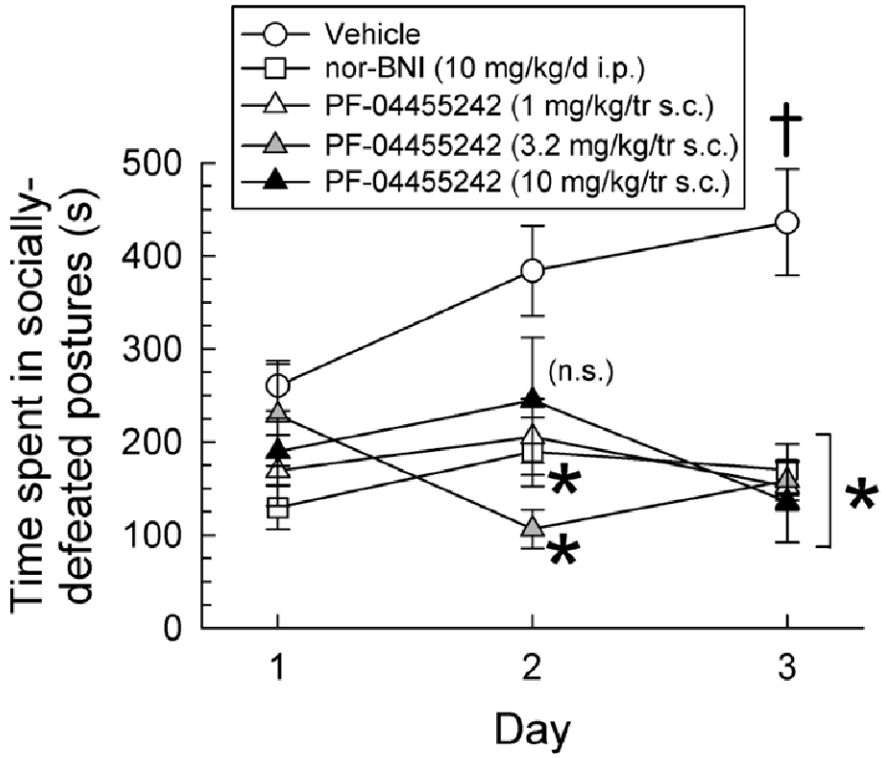

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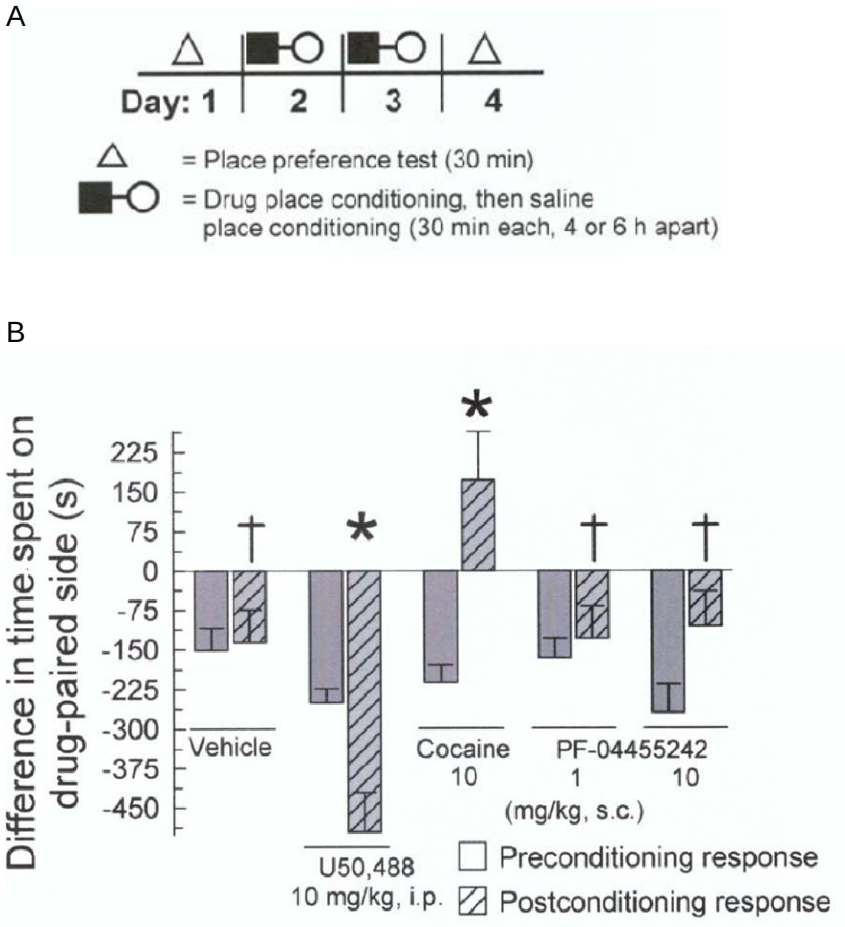

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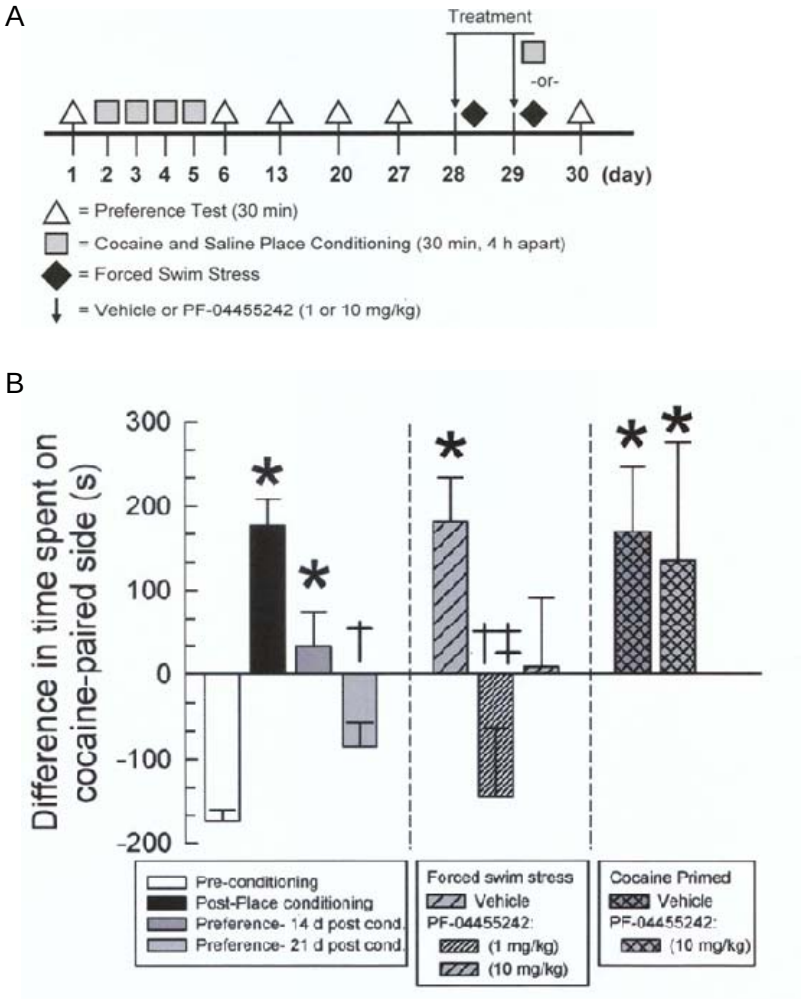

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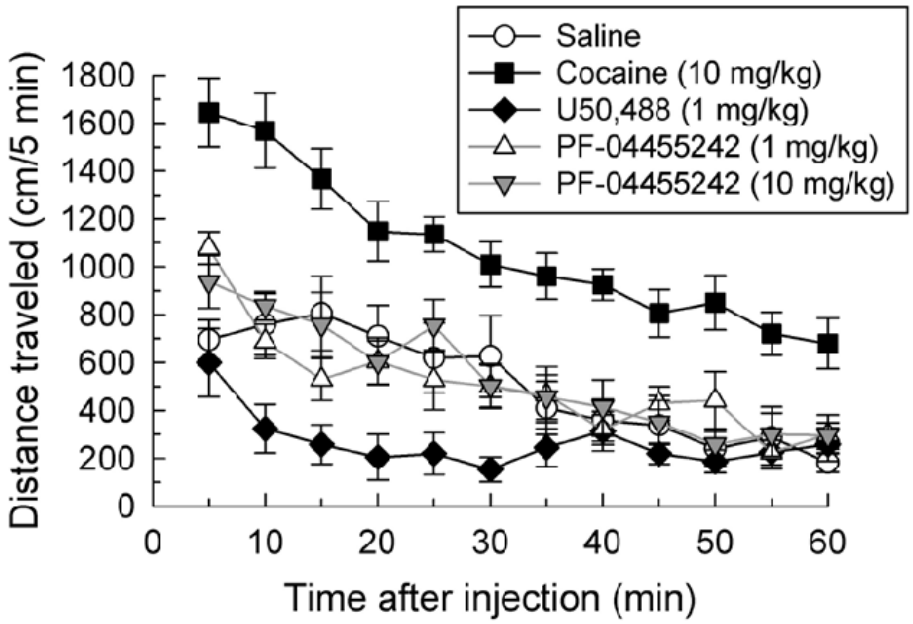

Figure 9.