Duration of action of a broad range of selective kappa opioid receptor antagonists is positively correlated with c-Jun N-terminal Kinase-1 activation


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Short Title: Duration of kappa antagonism correlates with JNK activation

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Abstract

The kappa opioid receptor is a widely expressed G-Protein Coupled Receptor that has been implicated in biological responses to pain, stress, anxiety, and depression, and its potential as a therapeutic target in these syndromes is becoming increasingly apparent. However, the prototypical selective kappa opioid antagonists have very long durations of action that have been attributed to c-Jun N-terminal Kinase (JNK) 1 activation in vivo. To test generality of this proposed noncompetitive mechanism, we used C57Bl/6 wild type mice to determine the durations of antagonist action of novel kappa opioid receptor ligands and examined their efficacies for JNK1 activation as compared with conventional competitive antagonists. Of the 12 compounds tested, 5 had long durations of action that positively correlated with JNK activation (RTI-5989-97; RTI-5989-194; RTI-5989-241; norBNI; JDTic), and 7 had short durations of action and did not increase phospho-JNK-ir (RTI-5989-212; RTI-5989-240; JSPA0658; JSPA071B; PF4455242; FP3FBZ; naloxone). Following long acting antagonist treatment, pJNK-ir did not increase in mice lacking the kappa opioid receptor; increased pJNK-ir returned to baseline by 48 hr after treatment; and a second challenge with norBNI 72 hr following the first did not increase pJNK-ir. Long lasting antagonism and increased phospho-JNK-ir were not seen in animals lacking the JNK1 isoform. These results support the hypothesis that the duration of action of small molecule kappa opioid receptor antagonists in vivo is determined by their efficacy in activating JNK1 and that persistent inactivation of the kappa receptor does not require sustained JNK activation.
Introduction

The kappa opioid peptide receptor (KOPr) is a Gi/o coupled receptor activated by the endogenous dynorphin opioid peptides (Chavkin et al., 1982; Bruchas et al., 2010). Dynorphins are released in brain during painful and stressful stimuli and have been shown to mediate the dysphoric components of the stress-response (Land et al., 2008; Carlezon et al., 2009). Kappa opioid antagonists promote stress-resilience in animal models and might have therapeutic utility in the treatment of stress-induced mood disorders (e.g. anxiety and depression) and stress-induced increase in drug addiction risk in humans (McLaughlin et al., 2003a; Carlezon et al., 2009; Land et al., 2009). However, development of clinically useful kappa antagonists has been slow, partly because the initially synthesized kappa opioid antagonists that demonstrated a high degree of selectivity (e.g. norbinaltorphimine (norBNI) and JDTic) showed remarkably long durations of action in vivo (lasting more than 21 d following a single administration) despite having receptor interactions that are freely reversible in vitro (Portoghese et al., 1987; Carroll et al., 2004; Negus et al., 2002; Broadbear et al., 1994; Butelman et al., 1993; Horan et al., 1992). The basis for this long in vivo duration of action was not initially clear and might have been a consequence of slow clearance from the brain (pharmacokinetic), metabolic conversion in vivo to a product that covalently bound to the receptor (metabolic), or through a long-lasting inactivation of the receptor signaling complex through an undefined mechanism.

In a prior study, we reported that some of the long lasting kappa opioid antagonists were actually ‘collateral agonists’ able to activate c-Jun N terminal kinase (JNK) through a kappa opioid receptor-dependent mechanism (Bruchas et al., 2007). Inhibition of JNK by SP600125 blocked the long-lasting antagonism by norBNI on the analgesic effects of the kappa opioid agonist U50,488 without blocking the acute kappa antagonism by norBNI (Bruchas et al., 2007;
Melief et al., 2010). This finding was extended by subsequently showing that norBNI was a short-acting competitive kappa antagonist in mice genetically lacking the JNK-1 isoform (Melief et al., 2010). These results suggested that the long-duration of kappa opioid antagonists in vivo might be a consequence of JNK activation, rather than a pharmacokinetic or metabolic effect. Furthermore, results showing that norBNI was able to activate a signaling pathway through the kappa receptor without producing analgesia typical of kappa opioid agonists provided evidence supporting the novel concept of ligand-directed signaling (also known as ‘functional selectivity’ and ‘biased agonism’) that may be a general property of G-protein coupled receptors (Urban et al., 2007; Kenakin, 2007).

In addition to the original selective kappa antagonists, norBNI and JDTic, several other compounds have recently been synthesized (Diaz et al., 2009; Mitch et al., 2010; Grimwood et al., 2011; Carroll et al., 2004). Comparison of these compounds shows that the kappa antagonists have significant structural differences, considerable structural flexibility, and few overall common features (Figure 1). JDTic and its analogues have a phenol group that was found to be crucial for KOPr selectivity (Thomas et al., 2003; Carroll et al., 2006), but the other compounds lack this chemical feature. Thus, the substantial structural differences among the compounds having kappa receptor antagonist activity obscure any underlying pattern to explain or predict their activity. Presumably, differences in the receptor conformation induced by ligand binding can control JNK activation efficacies, but in the absence of crystal structural information for the receptor-ligand complex, JNK activation needed to be empirically determined.

To assess the validity and generality of the hypothesis that long duration of kappa antagonism was a consequence of JNK activation, we examined the properties of this wide range of available ligands reported to produce selective kappa opioid antagonism (Portoghese et al., 1987; Carroll et al., 2004; Carroll et al., 2006; Cueva et al., 2009; Grimwood et al. 2011). In this analysis we find that for 12 structurally different kappa antagonists, stimulation of
phospho-JNK-immunoreactivity (ir), in spinal cords of antagonist-injected mice and in KOPr-
transfected HEK293 cells treated with antagonist in vitro, is strongly correlated with in vivo
duration of antagonism of U50,488 induced analgesia.

**Methods**

**Reagents:** norBNI and U50,488H, a selective kappa opioid agonist, were provided by the
National Institute of Drug Abuse (Bethesda, MD). JDTic and the RTI-5989-compounds were
synthesized at the Research Triangle Institute (Research Triangle Park, NC). FP3FBZ was
synthesized at Lilly Research Laboratories (Indianapolis, IN). Structurally related analogues
JSPA0658 (also known as LY2456302 (Diaz et al., 2009)) and JSPA071B were synthesized by
McLean Hospital and isolated as HCl salts (Belmont, MA). PF-4455242 was synthesized by
Pfizer (Groton, CT). All compounds used in this study had confirmed purities >95%. FP3FBZ,
JSPA0658, and JSPA071B were dissolved in 5% DMSO and 20% cremaphor, a vehicle that we
found to have no effects in the analgesia or pJNK assays used (Bruchas et al., 2007; Melief et
al., 2010). All other compounds were dissolved in 0.9% NaCl. All drugs were administered at a
volume of 10mL/kg. Other reagents were from Sigma-Aldrich (St Louis, MO).

*Kappa opioids used in this study:* Naloxone, a non-selective opioid receptor antagonist,
and norBNI (Portoghese et al., 1987). The compounds provided by the Research Triangle
Institute (RTI) include JDTic (Carroll et al., 2004) and the methylated analogues of JDTic, RTI-
5989-97: (3S)-7-Hydroxy-N-[(1S)-1-{(3R,4R)-4-(3-hydroxyphenyl)-3,4-dimethyl-1-
piperidinyl}methyl]-2-methylpropyl]-2-methyl-1,2,3,4-tetrahydro-3-isoquinolinecarboxamide,
RTI-5989-194: 3R)-7-Hydroxy-N-[(1S)-1-{(3R,4R)-4-(3-hydroxyphenyl)-3,4-dimethyl-1-
piperidinyl}methyl]-2-methylbutyl]-1,2,3,4-tetrahydro-3-isoquinolinecarboxamide, RTI-5989-212:
(3R)-N-[(1S)-1-{(3R,4R)-4-(3-hydroxyphenyl)-3,4-dimethyl-1-piperidinyl}methyl]-2-
 methylpropyl]-7-methoxy-1,2,3,4-tetrahydroisoquinoline-3-carboxamide, RTI-5989-240: (3R)-7-
Hydroxy-N-[(1S)-1-[(3R,4R)-4-(3-hydroxyphenyl)-3,4-dimethylpiperidin-1-yl]methyl]-2-methylpropyl]-3-methyl-1,2,3,4-tetrahydroisoquinoline-3-carboxamide, RTI-5989-241: (3R)-7-Hydroxy-N-[(1S)-1-[(3R,4R)-4-(3-methoxyphenyl)-3,4-dimethyl-1-piperidinyl]methyl]-2-methylpropyl]-1,2,3,4-tetrahydroisoquinoline-3-carboxamide (Thomas et al., 2004; Beardsley et al., 2010; Cueva et al., 2009). The diaryl ethers FP3FBZ (Mitch et al., 2010): [(S)-3-fluoro-4-((2-(3-fluorophenyl)pyrrolidin-1-yl)methyl)phenoxy]benzamide, JSPA0658 (previously referred to as LY-DMPF, (Peters et al., 2011); also known as LY2456302 (Diaz et al., 2009)): [(S)-3-fluoro-4-((2-(3,5-dimethylphenyl)pyrrolidin-1-yl)methyl)phenoxy]benzamide, and JSPA071B: [(S)-3-fluoro-4-((2-(3,5-bis(trifluoromethyl)phenyl)pyrrolidin-1-yl)methyl)phenoxy]benzamide. PF-4455242 [2-methyl-N-((2'-(pyrrolidin-1-ylsulfonyl)biphenyl-4-yl)methyl)propan-1-amine] (Grimwood et al. 2011).

Animals: Male C57Bl/6 mice (20-25g) were purchased from Charles River Laboratories (Wilmington, MA). Kappa opioid receptor knockout mice (KOPr⁻/⁻) were generated by homologous recombination as described (Clarke et al., 2002). Breeding pairs of JNK1⁻/⁻ mice were initially purchased from Jackson Laboratories (Sacramento, CA) on a C57Bl/6 background. KOPr⁻/⁻ and JNK1⁻/⁻ mice were bred by heterozygous crossing within the University of Washington vivarium under specific pathogen free (SPF) conditions. Mice were group housed and kept on a 12-hour light/dark cycle with food and water available ad libitum. Animal procedures were approved by the Animal Care and Use Committee of the University of Washington and conform to the guidelines of the NIH on the care and use of animals.

Cell Culture: HEK293 cells were grown as previously described (McLaughlin et al 2003b; Bruchas et al, 2007) in Dulbecco’s modified Eagle’s medium/nutrient mixture F-12 with L-glutamine and 15 mM HEPES (Invitrogen) with 10% fetal bovine serum, 50 units/ml penicillin, and 50 µg/ml streptomycin at 37 °C and 5% CO₂. HEK293 cells transfected with green fluorescent protein tagged KOR-GFP were maintained in the above media with an additional
200 µg/ml G418 to maintain selective pressure. Untransfected HEK293 cells for control experiments were grown in the absence of G418. Prior studies have established that the GFP tag on the C-terminal domain of the kappa opioid receptor does not block agonist-induced activation of G-protein signaling, ERK1/2 or p38 MAPK activation, agonist-induced receptor internalization or JNK activation (McLaughlin et al 2003b; Bruchas et al, 2007).

**Analgesia:** Antinociceptive responses were measured using the warm-water tail-withdrawal assay as previously described (Vaught and Takemori, 1979; Melief et al., 2010). Briefly, the latency to tail withdrawal following immersion in a 52.0°C water bath was measured before 15 mg/kg i.p. U50,488H administration and 30 min after. Animals were tested for normal U50,488H induced analgesic responses one day before administration of kappa opioid antagonists (10 mg/kg i.p., except diaryl esters which were also given at 50 mg/kg i.p.) and were then retested for U50,488H-induced analgesia 60 min, 1d, 3d, 7d, 14d, 21d, and 28d after administration of kappa opioid antagonists. Repeated administration of U50,488 by this schedule did not result in analgesic tolerance detected at time of assay. Animals administered naloxone (10 mg/kg i.p.) were injected with U50,488H 15 min after naloxone administration instead of 60 min because of the short duration of naloxone action.

**Immunoblotting:** For spinal cord samples, mice were injected i.p. with drug as doses and times indicated, tissue was dissected 60 min after injection and was homogenized in lysis buffer (50 mM Tris-HCl, 300 mM NaCl, 1 mM EDTA, 1 mM Na3VO4, 1 mM NaF, 10% glycerol, 1:100 phosphatase inhibitor mixture set 1 (Calbiochem), and 1:100 protease inhibitor mixture set 1 (Calbiochem) using a 2-ml Dounce homogenizer. For HEK293 lysate samples, KOPr-GFP expressing cells were treated with drug indicated for 5 min (pERK experiment), 15 min (naloxone samples) or 60 min and then homogenized in lysis buffer. Homogenates were sonicated for 5 s (Fisher Sonic Dismembrator, setting 4). All lysates were centrifuged (14,000 x g, 20 min, 4°C) (Eppendorf, model 5418), and protein concentration was determined using
bicinichonic colorimetric assay (Pierce, Rockford, IL) with bovine serum albumin (BSA) standards. Totals of 15 µg (spinal cord samples) or 25 µg (cell lysates) protein for each sample were heated at 100°C for 5 min in Laemmli buffer before loading onto non-denaturing 10% bisacrylamide precast gels (Invitrogen) and running at 120V for 2 h. Blots were transferred to nitrocellulose (Whatman, Middlesex, UK) for 2 h at 30V. The nitrocellulose was then blocked with 5% BSA/Tris buffered saline (TBS) (60 min), and incubated overnight at 4°C in phospho-JNK (Thr-183/Tyr-185) rabbit antibody or phospho-ERK1/2 (Thr-202/Tyr-204) diluted 1:1000 in 5% BSA/TBS (Cell Signaling, Beverly, MA). After overnight incubation, the blots were washed with TBS-0.1%Triton-X100 (TBST) and incubated for 60 min at room temperature in anti-rabbit IRDye800 diluted 1:10,000 in a 1:1 mixture of 5% milk/TBST and Li-Cor blocking buffer (Li-Cor Biosciences, Lincoln, NE). The blots were washed with TBST and then scanned and relative intensities of fluorescent bands were quantified using the Odyssey Infrared Imaging System (Li-Cor Biosciences, Lincoln, NE).

Data Analysis. Statistical significance for behavioral assays was determined by two-way ANOVA (time x treatment) followed by Bonferroni multiple comparison post hoc tests. Statistical significance for immunoblot assays was determined by one-way ANOVA with Bonferroni post hoc tests.

Results

Activation of JNK by Kappa opioid antagonists

To determine the efficacy of these compounds in increasing pJNK-ir in vitro, we applied compounds at different concentrations to KOPr-GFP expressing HEK293 cells (McLaughlin et al., 2003b; Bruchas et al., 2007). Cells were harvested and lysed after 60 min and pJNK-ir intensities were examined by western blotting. Dose response curves were generated for the RTI compounds using JDTic and norBNI as positive controls (Fig 2A, B). RTI-5989-97, RTI-
5989-194, and RTI-5989-241 significantly increased phospho-JNK-ir at 10 μM (one-way ANOVA with Bonferroni post hoc comparisons), whereas RTI-5989-212 and RTI-5989-240 did not (Fig 2A, B). Treatment with JSPA0658, JSPA071B, and PF-4455242 at 10 μM also did not significantly increase pJNK-ir, whereas FP3FBZ (at 10 μM) produced robust JNK activation under these conditions (Fig 2B). In comparison, none of the antagonist compounds studied effectively increased pERK1/2-ir in KOPr-GFP expressing HEK293 cells, whereas U50,488H produced robust ERK activation (Fig 2C). This finding suggested that these antagonist compounds did not have partial agonist activities in this system.

**Long lasting effects of kappa opioid antagonists**

We previously showed that kappa opioid antagonists norBNI and JDTic increase pJNK-ir and produce persistent antagonist effects for weeks following a single in vivo administration (Bruchas et al., 2007). In this study, we next examined the duration of action for each compound using the warm water tail-withdrawal assay (Vaught and Takemori, 1979) (Fig 3 and Table 1). Animals were initially challenged with U50,488H (15 mg/kg i.p.) 24 hr prior to antagonist (Day -1), and latency of tail withdrawal from a 52°C water bath was measured. On Day 0, animals received the indicated kappa opioid antagonist (10 mg/kg i.p., FP3FBZ 10 mg/kg and 50 mg/kg i.p.) 60 min (15 min for naloxone) prior to the second U50,488H administration and tail-withdrawal testing. The efficacy of U50,488H to induce analgesia was then repeatedly measured up to 28 days afterward. All of the antagonist compounds significantly reduced the U50,488H-induced analgesia to close to baseline responses (dashed line) when challenged the same day (one-way ANOVA with Bonferroni post hoc comparisons) (Fig 3 and Table 1). As previously documented (Bruchas et al., 2007), norBNI blocked U50,488H induced analgesia for 21 days as compared with saline controls, whereas naloxone was only effective acutely on Day 0 (Fig 3A). The effects of RTI-5989-97 persisted for 1 day before returning to control levels (Table 1). RTI-5989-194 and RTI-5989-241 showed long lasting antagonistic effects at 21 and 7
days of duration, respectively, whereas RTI-5989-212 and RTI-5989-240 were only effective acutely (Fig 3B and Table 1). FP3FBZ administered at 10 mg/kg i.p. was only effective acutely, but showed long-lasting effects persisting up to 14 days when administered at 50 mg/kg i.p. (Fig 3C). Because JSPA0658 and JSPA071B are analogues of FP3FBZ, we also tested these compounds at both doses (10 mg/kg and 50 mg/kg i.p.). Consistent with the absence of JNK activation induced by these compounds in HEK293 cells and with previous results (Peters et al., 2011), both JSPA0658 and JSPA071B were short acting at both doses tested (Table 1). PF-4455242 was also short acting (Table 1). These results correlate well with the efficacies of each compound at activating JNK in vitro (slope = 9.84 ± 1.64 p=0.0093, r² = 0.92) (Fig 5A).

**Activation of JNK by long-lasting kappa opioid antagonists**

To determine if pJNK-ir responses observed in vitro were also evident in the animal model, we examined the efficacy of each compound in activating JNK in C57Bl/6 mice by isolating the spinal cords of mice 60 min (15 min for naloxone) after drug administration. norBNI, FP3FBZ (50 mg/kg), RTI-5989-194, RTI-5989-241, and JDTic each significantly increased pJNK-ir compared to saline-injected controls (one-way ANOVA with Bonferroni post hoc comparisons) (Fig 4A). In contrast, RTI-5989-97, RTI-5989-212, RTI-5989-240, FP3FBZ (10 mg/kg), JSPA0658, JSPA071B, and PF-4455242 did not significantly increase pJNK-ir in spinal cord. Comparing the relative activities of the 12 kappa compounds, we observed a highly significant correlation between the duration of antagonist action in the analgesia assay and the increase in pJNK-ir induced in the mouse spinal cord (slope = 2.94 ± 0.44 p=0.0069, r² = 0.94) (Fig 5B).

We have previously shown that the long-lasting effects of norBNI are not due to sequestration of the compound in the lipid bilayer and sustained receptor occupancy in vivo (Bruchas et al., 2007). To determine if these effects were caused by persistent activation of JNK
in the absence of continued receptor occupancy, we measured pJNK-ir in spinal cord homogenates 1, 24, and 48 hr after treatment with norBNI (10 mg/kg i.p.). Expression of pJNK-ir was significantly increased over saline controls 1 and 24 hr after administration, but returned to baseline by 48 hr (one-way ANOVA with Bonferroni post hoc comparisons) (Fig 4B). These data indicate that persistent JNK activation is not responsible for long durations of action of these compounds (which can persist for 21 days).

Following long-acting antagonist treatment, kappa receptor agonists did not evoke a behavioral or \[^{35}\text{S}\text{GTP}_\gamma\text{S}\] response (Melief et al., 2010), but whether the JNK activation by subsequent antagonist treatment was also blocked was not clear. To determine if receptor inactivation by norBNI precludes further antagonist stimulated JNK responses, animals were pretreated with saline or norBNI (10 mg/kg i.p.) 72 hr before a second challenge with saline or norBNI (10 mg/kg i.p.). Spinal cord homogenates dissected 1 hr after the second challenge showed that norBNI significantly increased pJNK-ir in saline pretreated animals, but had no effect in norBNI pretreated animals (two-way ANOVA with Bonferroni post hoc comparisons) (Fig 4C). These data indicate that the persistent inactivation of the kappa receptor does not require sustained JNK activation and that both agonist and collateral agonist responses were persistently blocked.

**Long duration of action requires the JNK1 isoform**

We have previously shown norBNI does not have long-lasting effects in mice lacking the JNK1 isoform (JNK1\(^{-/-}\)) (Melief et al., 2010). To determine if the other long-acting antagonists also required activation of the JNK1 isoform, RTI-5989-97, RTI-5989-194, RTI-5989-241, FP3FBZ (50 mg/kg i.p.) and JDTic were administered to JNK1\(^{-/-}\) mice as described for previous experiments. RTI-5989-194 was ineffective as an antagonist in mice lacking JNK1 (Fig 6A). The other compounds remained effective competitive antagonists (one-way ANOVA with Bonferroni
post hoc comparisons), but their effects did not persist longer than 24 hr. We measured pJNK-ir in response to norBNI in the spinal cords of JNK1^-/- mice and found that knockout of the JNK1 isoform completely prevented the increase pJNK-ir induced by norBNI as compared with wild type littermate controls (Fig 6B). Together these results indicate that JNK1 activation has a critical role in the persistent inactivation of the KOPr produced by long-lasting antagonists.

**JNK activation requires KOPr**

To ensure that the JNK activation we observed was dependent on KOPr mediated effects, we determined the efficacy of JNK activation in untransfected HEK293 cells. JDTic, FP3FBZ, and norBNI, examples of each family of compounds that elicited robust JNK activation in KOPr-GFP expressing cells, were applied to untransfected HEK293 cells (10 μM) for 60 min. All were unable to significantly activate JNK in the untransfected cells (Fig 7A). Additionally, to determine the necessity of KOPr in JNK activation in vivo, mice lacking functional KOPr (KOPr^-/-) were administered saline, JDTic (10 mg/kg i.p.), or FP3FBZ (50 mg/kg i.p.) and spinal cords were dissected 60 min later. As has been previously seen with norBNI (Bruchas et al., 2007), neither JDTic nor FP3FBZ significantly increased pJNK-ir in KOPr^-/- mice (Fig 7B).

**Discussion**

In this study we determined the durations of action of several novel kappa opioid antagonists and compared them with the efficacy of each compound at activating JNK. We found that kappa opioid antagonists that effectively activate JNK in KOR-GFP transfected HEK293 cells and mouse spinal cord also have longer durations of analgesic antagonist action than those that are classical competitive antagonists, and that JNK1 is the isoform specifically required for these long-lasting effects. The **in vitro** data suggest that activation of JNK is a direct consequence of KOPr activation by long-acting antagonists **in vivo**, but how JNK activation results in kappa receptor inactivation is not yet clear. The long duration was not caused by
persistent effect as JNK activation in spinal cord was not evident 48 hr after norBNI treatment. In addition, residual antagonist would not be able to produce sustained JNK activation since a second challenge with norBNI 72 hr following the first did not increase pJNK-ir.

Although this will require further molecular characterization, the presumed actions of long-acting kappa antagonists are better described as ‘collateral’ agonism than ‘inverse’ agonism (Bruchas et al., 2007). While both collateral and inverse agonists would produce effects that oppose the actions of the conventional agonist, they would do this by distinctly different molecular mechanisms. Unlike neutral antagonists that competitively block the agonist binding site, collateral agonists are thought to activate an alternative signaling pathway resulting in a distinctly different molecular action (e.g. JNK-dependent receptor inactivation) than conventional agonists (e.g. Gβγ dependent effector activation); whereas inverse agonists are thought to act by reducing constitutive receptor activity (e.g. inhibition of basal G-protein activation in the absence of agonist) (Kenakin, 2004; Cotecchia, 2007). In addition, like neutral antagonists, inverse agonist effects would presumably be terminated by drug dissociation from the receptor, whereas JNK-dependent receptor inactivation would presumably persist after ligand dissociation. While these proposed distinctions require additional experimental definition, based on this conceptual scheme, the actions of long-duration kappa antagonists are more consistent with the emerging concepts of ‘ligand-directed signaling’ as recently reviewed (Urban et al 2007).

Unexpectedly, there appears to be no obvious structural homology required for JNK activation by kappa opioid antagonists, as JDTic, RTI-5989-194, and RTI-5989-241 were long-lasting, while their closely related analogues RTI-5989-212 and RTI-5989-240 were not. Likewise, norBNI, which is structurally unrelated to the RTI compounds, also has a long duration of action. Long duration of action is also apparently unrelated to receptor binding affinity, potency, or clearance of the compounds, as the Kd values for long and short acting compounds
are similar (Peters et al., 2011), and the persistence of compounds in the brain is not correlated with duration of antagonist action (Beardsley et al., 2010).

Interestingly, while some compounds are incapable of activating JNK at the doses tested, for some ligands the activation of JNK and subsequent inactivation of the receptor appears to be dose-dependent. Our \textit{in vitro} data indicates that the novel kappa opioid antagonist FP3FBZ is capable of activating JNK at a very high concentration, as evidenced by a robust increase in pJNK-ir in HEK293 cells following a 10 \( \mu \text{M} \) dose for 60 min. \textit{In vivo}, however, we found that a moderate dose of FP3FBZ (10 mg/kg) functionally blocks KOPr-induced analgesia without activating JNK or showing persistent effects (Fig 3C), which is consistent with previous studies (Peters et al., 2011). Based on our \textit{in vitro} data, we examined the effects of this compound at a much higher dose. When administered at 50 mg/kg, FP3FBZ does activate JNK and is long-lasting, indicating a threshold or low efficacy for JNK activation. This property was unique for FP3FBZ, as the structurally related compounds JSPA0658 and JSPA071B do not have the same dose-dependent effects. It is possible that FP3FBZ is no longer selective for KOPr at the high dose, but as duration of action was measured as blockade of U50,488H induced analgesia (which requires KOPr), we consider the FP3FBZ effects to be KOPr mediated. The tight correlation between pJNK-ir in spinal cord homogenates and duration of antagonistic effects that we observed indicates that the relative efficacy of each compound at activating JNK determines how long the antagonist effect persists. For compounds that activate this system, these data point to a receptor occupancy model, in which compounds producing robust activation of JNK inactivate the KOPr system entirely. In this case receptor synthesis may be required for reinstatement of KOPr function (Bruchas et al., 2007). In comparison, compounds that weakly or moderately activate JNK, such as RTI-5989-97, impair only a subset of the available KOPrs, and recovery of the KOPr system is faster. This model of receptor
occupancy in determining receptor effects and MAPK activation by different ligands has been shown before in different systems (Nandagopal et al., 2001; Huwiler et al., 2009).

We did note an unexpected dissociation between RTI-5989-97 induced JNK activation observed in vitro and the results we observed in vivo. This compound produced significant JNK phosphorylation in KOPr-GFP expressing HEK293 cells, and was more potent than the other RTI compounds in doing so (Fig 2A and 2B). In contrast, phosphorylation of JNK in the spinal cord was not as robust as that induced by JDTic or RTI-5989-194, nor did the antagonist effects persist as long (1 day vs. 21 days). A possible explanation for this divergence is a difference in the cohort of signaling proteins anchored to the receptor in our cultured cells as compared with spinal neuronal cells. As previously stated, JNK associates with a number of different anchoring proteins, which in turn couple with different signaling targets (Bogoyevitch and Kobe, 2006). A possible explanation would include more rapid degradation of the compound in vivo, or poor penetration of the drug across the blood brain barrier, although its evident ability to acutely antagonize KOPr effects in vivo would seem to exclude this latter explanation. Nevertheless, the lack of robust JNK activation in spinal cord is consistent with the short duration of RTI-5989-97 antagonism in vivo.

Another unexpected result was the effect of RTI-5989-194 in JNK1−/− animals. Contrary to results found with the other long lasting compounds, RTI-5989-194 was not effective as an acute antagonist in mice lacking functional JNK1. This was surprising as our data as well as previous studies (Beardsley et al., 2010) clearly show that RTI-5989-194 is an antagonist of KOPr-induced behaviors in wild type animals. That the knockout of JNK1 could prevent antagonist effects indicates that RTI-5989-194 is not a simple competitive antagonist, but requires JNK activation to block activity at the KOPr.
Although this study does not address the mechanisms by which each of these compounds activate JNK or by which JNK inactivates the receptor, we have previously shown that the norBNI induced increases in JNK phosphorylation required KOPr expression and were blocked by Gö6976 (a PKC selective inhibitor), suggesting that norBNI binding to the kappa opioid receptor activates protein kinase C that subsequently increases JNK phosphorylation (Melief et al., 2010). The details of the pathways have not yet been resolved, and how JNK activation by long-acting kappa antagonists results in prolonged receptor inactivation is not yet clear. The drug does not persist in the tissue (Bruchas et al., 2007), and JNK activation is not evident 48 hrs after norBNI treatment (the present study). Kappa receptor binding sites are not reduced in number or agonist affinity by norBNI (Horan et al, 1992; Bruchas et al 2007), yet JNK activation by norBNI blocks agonist stimulation of $^{35}$S$\text{GTP}^\gamma \text{S}$ binding to spinal cord membranes isolated from mice administered norBNI 7 d prior to tissue harvest (Melief et al., 2010). A parsimonious explanation is that JNK activation phosphorylates a substrate that tightly binds to the kappa receptor and sterically blocks G-protein association, although the hypothetical JNK-substrate has not been identified and alternative mechanisms have not yet been excluded.

There are a large number of known JNK targets that couple to each of the individual anchoring proteins that bind JNK and have a variety of downstream effects (Bogoyevitch and Kobe, 2006; Davis, 2000; Engström et al., 2010). Alternatively, there are numerous regulatory proteins that are known to sequester or inactivate G-proteins, a number of which have been identified as regulating morphine signaling in the mu opioid receptor system, in some cases in a PKC dependent manner (Ajit et al., 2007; Garzón et al., 2005a, Garzón et al., 2005b; Rodriguez-Munoz et al., 2006).

Kappa opioid antagonists are becoming recognized as potentially important therapeutic tools in managing disorders of stress, anxiety, and depression (Land et al., 2008; Carlezon et al., 2009). In animal models, such compounds have been shown to be efficacious at preventing
dysphoria and relapse to drug seeking in animals exposed to drugs of abuse (Beardsley et al., 2010; Land et al., 2009). As the clinical development of these compounds advances, understanding the mechanism of action by which they exert their effects will become increasingly important. At present, only two kappa opioid antagonists, JDTic and RTI-5989-194, are known to be effective when administered orally (Beardsley et al., 2010), and both of these are long-lasting in our studies. Therapeutic agents that have long durations of action are often undesirable for use in humans as some patients may not tolerate the effects well. In such cases a short acting compound may be preferable as its use would allows substitution to a different therapeutic quickly. On the other hand, in patients that do respond well to treatment, long duration of action may be desirable, especially for compounds that are not effective orally and may need to be parenterally administered. Of course, the effects of JNK activation on measures not tested in studies thus far may also have implications for the behavioral and clinical effects of long-lasting kappa opioid antagonists.

In conclusion, we have demonstrated that a subset of kappa opioid antagonists that are able to activate JNK1 also have long durations of action. The limited structure activity analysis described here did not reveal the key features distinguishing short from long lasting ligands. Further characterization of the mechanisms of long duration kappa opioid antagonism are necessary to better understand the structure and regulation of the signaling complex.
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Authorship Contributions.

Participated in research design: EJM, MM, CC.

Conducted experiments: EJM, MM.

Contributed new reagents or analytic tools: FIC, CB, WAC, BMC, SG, CHM, LRK.

Performed data analysis: EJM, MM, CC.

Wrote or contributed to the writing of the manuscript: EJM, MM, CC.
References


Cueva JP, Cai TB, Mascarella SW, Thomas JB, Navarro HA, and Carroll FI (2009) Synthesis and in vitro opioid receptor functional antagonism of methyl-substituted analogues of (3r)-7-hydroxy-n-[(1s)-1-[(3r,4r)-4-(3-hydroxyphenyl)-3,4-dimethyl-1-piperidinyl[methyl]-2-methylpropyl]-1,2,3,4-tetrahydro-3-isoquinolinecarboxamide (JDTic) Journal of Medicinal Chemistry 52: 7463-7472.


Footnotes:

This study was supported by a USPHS grant from the National Institute on Drug Abuse [DA11672].
Legends

Figure 1. Compound names and chemical structures of kappa opioid antagonists. The ‘A-L’ code is also used to designate the compounds presented in Figure 5.

Figure 2. Activation of JNK in HEK293 cells by kappa opioid antagonists. A) Dose response curves generated from pJNK-ir quantification of western blots for JDTic, RTI-5989-97, RTI-5989-1 RTI-5989-94, RTI-5989-212, RTI-5989-240, RTI-5989-241, and norBNI (western blot not shown). All compounds were administered for 60 min prior to lysing the cells. B) Quantification of pJNK-ir from western blots of cell lysates from cells treated with 10 μM of each compound for 60 min. C) Quantification of pERK-ir from western blots of cell lysates from cells treated with 10 μM of each compound or U50,488H for 5 min. Results were compared with vehicle controls and analyzed by one-way ANOVA with Bonferroni post-hoc comparisons (*p<0.05, **p<0.01, ***p<0.001).

Figure 3. Duration of action of kappa opioid antagonists. Duration of action was measured by antagonism of U50,488H induced analgesia at different times following a single antagonist injection on Day 0 (10 mg/kg i.p., except for FP3FBZ which was also given at 50 mg/kg i.p.). The dashed line represents baseline (pre-U50,488) tail withdrawal latency responses. Control mice received saline (10 mL/kg, i.p.). Animals were challenged by 15 mg/kg U50,488 on 1d, 3d, 7d, 14d, 21d, and 28d after injection. Compounds shown are A) naloxone (NLX) and norBNI, B) RTI-5989-212 and RTI-5989-194, and C) FP3FBZ (10 mg/kg) and FP3FBZ (50 mg/kg). All
compounds were statistically compared with saline controls. Data analyzed by two-way ANOVA with Bonferroni post-hoc comparisons (*p<0.05, **p<0.01, ***p<0.001).

Figure 4. Activation of JNK in spinal cord by kappa opioid antagonists. A) Quantification of pJNK-ir from western blots of spinal cord homogenates taken from animals treated with each compound for 60 min (15 min for NLX). Each compound was administered at 10 mg/kg i.p., except FP3FBZ which was administered at 10 mg/kg i.p. (FP3FBZ 10) and 50 mg/kg i.p. (FP3FBZ 50). B) Quantification of pJNK from spinal cords of animals treated with a norBNI time course. Animals were administered saline or norBNI and tissue was harvested at 1hr, 24hr, or 48hr time points. Data were compared with saline controls and analyzed by one-way ANOVA with Bonferroni post-hoc comparisons (*p<0.05, **p<0.01). C) Quantification of pJNK from spinal cords of animals pretreated with saline or norBNI (10mg/kg i.p.) 72 hours before challenge with saline (S) or norBNI (10mg/kg i.p.) (N) 1hr prior to dissection. Data are presented as percent change over the saline/saline group and is analyzed by two-way ANOVA with Bonferroni post-hoc comparisons (***p<0.001).

Figure 5. Efficacy of JNK activation correlates with duration of action. Quantification of pJNK-ir from western blots was plotted as a function of the natural log of duration of antagonism for each compound. Natural log was used to transform the x values because the recovery of response following receptor inactivation follows the asymptotic kinetics of receptor expression approach to equilibrium. Letters correspond to labels in Fig 1 (G10 = FP3FBZ 10mg/kg, G50 = FP3FBZ 50mg/kg). Results were analyzed by linear regression. Duration of action was correlated with JNK activation as determined in A) HEK293 cells (slope = 9.84 ± 1.64 p=0.009, r² = 0.92) and B) spinal cord homogenates (slope = 2.94 ± 0.44 p=0.0069, r² = 0.94).
Figure 6. Long duration of action is dependent on the JNK1 isoform. A) Long-lasting Kappa opioid antagonists (JDTic, RTI-5989-97, RTI-5989-194, RTI-5989-241, and FP3FBZ (50mg/kg)) were administered to JNK1−/− mice as in Figure 3. Duration of antagonism was determined by blockade of U50-488H induced analgesia. Results were analyzed by two-way ANOVA with Bonferroni post-hoc comparisons (*p<0.05, **p<0.01, ***p<0.001). B) Quantification of pJNK-ir from spinal cord homogenates of JNK1+/+ and JNK1−/− mice treated with norBNI for 60 min as compared with saline controls. Data analyzed by one-way ANOVA with Bonferroni post-hoc comparisons (*p<0.05).

Figure 7. JNK activation requires functional KOPr. A) Untransfected HEK293 cells were treated with norBNI, JDTic, or FP3FBZ (10 μM) for 60 min. pJNK-ir was determined by western blot of cell lysates. Results were analyzed by one-way ANOVA and was insignificant. B) KOPr+/+ and KOPr−/− mice were administered saline, JDTic (10 mg/kg i.p.), or FP3FBZ (50 mg/kg i.p.) and spinal cord homogenates were prepared 60 min later. pJNK-ir was determined by western blot. Results were analyzed by two-way ANOVA with Bonferroni post hoc comparisons. (**p<0.01, ***p<0.001 as compared with saline controls, ###p<0.001 as compared with KOPr+/+ controls).
Table 1. Duration of action of kappa opioid antagonists. Data were measured as in Figure 3 and is presented as latency to tail withdrawal (seconds ± standard error of the mean) for each time point measured. Animals were given each compound at 10 mg/kg i.p. (or 50 mg/kg i.p. as indicated) 60 min prior to U50,488H challenge on Day 0 (15 min for naloxone). Duration is defined as the last time point at which treated animals differed significantly from saline controls.

<table>
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<th>Compound</th>
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<th>0</th>
<th>1</th>
<th>3</th>
<th>7</th>
<th>14</th>
<th>21</th>
<th>28</th>
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<td>Saline</td>
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<td>2.02 ± 0.15**</td>
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<td>1.87 ± 0.09***</td>
<td>3.04 ± 0.16</td>
<td>3.46 ± 0.47</td>
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<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
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</table>
Figure 1

Research Triangle Institute Compounds

A. JDTic
B. RTI-5989-97
C. RTI-5989-194
D. RTI-5989-212
E. RTI-5989-240
F. RTI-5989-241

Eli Lilly and McLean Compounds

G. FP3FBZ
H. JSPA0658
I. JSPA071B

J. norBNI
K. Naloxone
L. PF-4455242