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Nalfurafine is a G-protein biased agonist having significantly greater bias at the human than rodent form of the kappa opioid receptor

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Abstract

Nalfurafine is a moderately selective kappa opioid receptor (KOR) analgesic with low incidence of dysphoric side effects in clinical development for the treatment of uremic pruritis. The basis for its reduced dysphoric effect compared to other KOR agonists is not clear, but prior studies suggest that the aversive properties of KOR agonists require p38 α MAPK activation through an arrestin-dependent mechanism. To determine whether nalfurafine is a functionally selective KOR agonist, we measured its potency to activate the G protein-dependent early phase of Extracellular Signal-Regulated Kinase (ERK1/2) phosphorylation and the arrestin-dependent late phase of p38 MAPK signaling. Nalfurafine was approximately 250 fold more potent for ERK1/2 activation as compared to p38 MAPK activation in human KOR (hKOR) expressing HEK293 cells, and approximately 20 fold more potent for ERK1/2 activation than p38 activation in rodent KOR (rKOR) expressing HEK293 cells. The 10-fold greater G-bias at the hKOR than rKOR was unexpected, however the G protein biased effect of nalfurafine is consistent with its reduced dysphoric effects in human and rodent models. Although nalfurafine is reported to have low receptor selectivity in radio ligand binding assays, its antinociceptive effect was blocked by the selective KOR antagonist norbinaltorphimine. Nalfurafine pretreatment also resulted in a KOR-dependent and mu opioid receptor-independent reduction in scratching induced by 5'-GNTI. These findings suggest that nalfurafine is a functionally selective KOR agonist and that KOR agonists able to selectively activate G protein signaling without activating p38 α MAPK may have therapeutic potential as non-dysphoric antipruritic analgesics.

Keywords

Kappa opioid receptor; Biased signaling; p38 MAPK; Dysphoria

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1. Introduction

Mu opioid agonists including morphine-like opiates remain the principal analgesics for the treatment severe pain, however the respiratory depression, constipating, and addiction risks of these drugs are well known liabilities and diversion of prescription opioids is currently a major public health crisis [1]. Kappa opioid analgesics have been developed as safer alternatives to mu opioids for control of pain, however the selective kappa opioid receptor (KOR) analgesics that were initially developed produce profound dysphoric and psychotomimetic actions [2]. Attempts to reduce the dysphoric properties of KOR agonists by restricting their brain penetrance have been made; however these peripherally restricted compounds have demonstrated only modest analgesic activity [3,4]. Recent studies have suggested that the analgesic effects of KOR are mediated by G protein regulation of ion channels, including increased G protein gated potassium channel and decreased calcium channel conductance resulting in reduced neuronal excitability; whereas the aversive effects of KOR agonists require G protein-coupled receptor kinase 3 (GRK3)-dependent arrestin activation [5,6]. The concept that functionally selective agonists that preferentially activate one signaling cascade can be developed has re-energized opioid drug development [7,8]. Furthermore the hypothesis that a centrally active G protein biased KOR agonist that does not induce arrestin-dependent signaling might be a safer analgesic has stimulated the field [8,9].

Functionally selective KOR agonists have been recently identified [10–12], however these compounds lack drug-like qualities. In contrast, the moderately receptor selective KOR agonist nalfurafine (TRK820) is currently in clinical trials for the treatment of uremic pruritis [13], and has pharmacological properties suggestive of functional selectivity (e.g. analgesic, low incidence of dysphoria) [14–16]. In the present study, we assessed the functional selectivity of nalfurafine. Identification of a drug-like lead compound in the development of functionally selective KOR agonists would be a significant advance in the generation of novel analgesics potentially lacking the addictive effects of mu opioids and the dysphoric effects of conventional KOR agonists.

2. Materials and methods

2.1. Chemicals/reagents

For cell culture experiments KOR agonists (–)U50,488 (Tocris) and nalfurafine hydrochloride ((2*E*)-*N*-[(5 α ,6 β)-17-(cyclopropylmethyl)-3,14-dihydroxy-4,5-epoxymorphinan-6-yl]-3-(3-furyl)-*N*-methylacrylamide) (NIDA Drug Supply) and the MOR agonist DAMGO ([D-Ala2, N-Me-Phe4, Gly5-ol]-enkephalin) (Sigma-Aldrich) were dissolved in water. For in vivo studies, the KOR agonists nalfurafine, (+/–)U50,488, and the KOR antagonists 5'-GNTI, and norBNI were obtained from the NIDA Drug Supply and were freshly dissolved in sterile saline on the day of use.

2.2. Animals

Male (antinociceptive and antipruritic testing) C57BL/6 mice (20–30 g) were group-housed, kept on a 12 h light/dark cycle, and provided with food and water ad libitum. Animal

procedures were approved by the Animal Care and Use Committee of the University of Washington and conform to the guidelines on the care and use of animals promulgated by the National Institutes of Health. Homozygous KOR^{-/-} and MOR^{-/-} mice were generated by homologous recombination as previously described [17,18] and backcrossed on C57BL/6 backgrounds for >10 generations. Knockout KOR^{-/-}, MOR^{-/-}, and wild-type (WT) littermate mice for behavioral studies were generated by heterozygote (+/-) crosses.

2.3. Antinociceptive testing

Antinociceptive responses were measured using the warm-water tail-withdrawal assay [19], with the experimenter blinded to treatment conditions. The response latency for an animal to withdraw its tail after being immersed in 52.5 °C water was measured before treatment with (-/+)U50,488 or nalfurafine. After drug administration, responses were measured 30min post drug administration. For the dose response, the same cohort of mice was used for all doses with tests administered 48–72 h apart and the order in which drug doses were testing was randomized for each mouse. After dose response testing was complete, animals were injected with saline or norBNI and retested 3–5 days later before and 30 min after (-/+)U50,488 or nalfurafine.

For tolerance studies, a separate cohort of animals were injected with saline or 1 mg/kg nalfurafine. 3 h later, baseline analgesia was recorded followed by administration of U50,488. A second response post-drug was recorded 30 min most U50,488. The same mice were treated in a similar fashion 24 h following the initial treatment with saline or nalfurafine. A 15 s maximal immersion duration was used as a cut off to prevent tissue damage.

2.4. Antipruritic testing

Antipruritic activity of nalfurafine was assayed as previously described [20]. WT, KOR^{-/-}, and MOR^{-/-} mice were placed individually in square observation boxes with white opaque sides (16" × 16" × 12") with 1600 ml of bed-o-cobb bedding, and were given 1 h to acclimate. Mice were injected subcutaneously in the flank with either saline or nalfurafine (0.05 mg/kg). Twenty minutes later, they were injected subcutaneously on the midline of the back of the neck with saline or 5'-GNTI (0.03 mg/kg). The mice were then video-recorded in 1080 p resolution with 60i frames a second, and the number of hind leg scratches directed to the back of their neck was counted for 30 min. All experiments were carried out between 2:00 PM and 7:00 PM. Testing and data analysis were done by an investigator blind to genotype and drug treatment. Locomotor activity of a subset of mice was analyzed using Ethovision (v.3.0, Noldus, Wageningen, The Netherlands); scores represent total distance moved (cm) during the 30 min session.

2.5. Cell culture

HEK293 cells stably expressing rKOR-GFP or flag-hKOR were generated as described previously [21]. HEK293 cells were maintained in Dulbecco's modified medium/F12 with 10% fetal bovine serum. HEK293 cells expressing KOR were grown in media supplemented with G418 (200 µg/ml). Cell lines expressed KOR at similar levels, with similar dose-responses for p38 and ERK1/2 activation by U50,488 [21].

2.6. Immunoblotting

HEK293 cells were incubated in serum-free media 6 h and then treated as described in Results. Cells were lysed and cytosolic proteins were analyzed for phospho-ERK1/2, phospho-p38, ERK2, or actin by western blot as previously described [21]. Blots were scanned on the Odyssey Infrared Imaging System (Li-Cor Biosciences). Band intensity was measured using the Odyssey software and expressed as phospho-ERK1/2 over ERK2 or phospho-p38 MAPK over actin band intensity. Data were calculated as a fraction of increase over basal, relative to the increase induced by 1 μ M U50,488 and plotted using GraphPad Prism 6 software. Statistical significance ($p < 0.05$) was determined by analysis of variance followed by Bonferroni's post-hoc test (GraphPad Prism 6 software).

2.7. Dose response curves

Dose response curves for p38 and ERK1/2 phosphorylation were calculated in GraphPad Prism6, using a three parameter least squares non-linear regression with no weighting and a bottom constrained at 0 for each replicate and these values averaged. Significant differences between EC_{50} and E_{max} were determined by t -test with Holm-Sidak correction for multiple analyses. Signaling bias was calculated by $-\log(\text{relative activity, RA})$ [22–25]. Values were calculated from dose response data in Fig. 1A–B and previously published data [21].

3. Results

3.1. Does nalfurafine differentially activate p38 and ERK1/2 MAPK pathways by rKOR?

KOR agonists have previously been shown to promote ERK1/2 phosphorylation at 5 min and p38 phosphorylation at 30 min via different signaling cascades [21,26]. We first sought to establish whether nalfurafine activates these cascades with similar efficacy and potency. HEK293 cells stably expressing rKOR were treated for 5 or 30 min with nalfurafine (10 pM–100 nM) or U50,488 (1 μ M) prior to lysis and phospho-ERK1/2 (5 min) or phospho-p38 (30 min) was quantified by western blot (Fig. 1A–B). Nalfurafine activated ERK1/2 at 5 min with an EC_{50} of 500 pM (confidence interval 380 pM to 1.6 nM, $n = 5$) and an efficacy of 1.0 relative to U50,488. Nalfurafine induced p38 activation at 30 min with an EC_{50} of 5.2 nM (confidence interval 4.4 nM to 160 nM, $n=4$) and an efficacy of 1.1 relative to U50,488. Nalfurafine had full agonist activity for rKOR at both pathways but was approximately 10 fold more potent for activation of ERK1/2 as compared to phospho-p38 ($p < 0.05$).

3.2. Does nalfurafine differentially activate p38 and ERK1/2 MAPK pathways by hKOR?

Since differences in MAPK activation by hKOR and rKOR have previously been reported [21], we sought to determine if p38 and ERK1/2 MAPK are also differentially activated by hKOR. HEK293 cells stably expressing hKOR were treated for 5 or 30 min with nalfurafine (100 pM–10 μ M) or U50,488 (1 μ M) prior to lysis and phospho-ERK1/2 (5 min) or phospho-p38 (30 min) was quantified by western blot (Fig. 1C–D). Nalfurafine induced ERK1/2 activation at 5 min with an EC_{50} of 1.4 nM (confidence interval 1.1 to 3.8 nM, $n=5$) and an efficacy of 0.99 relative to U50,488. Nalfurafine induced p38 activation at 30 min with an EC_{50} of 110 nM (confidence interval 100 nM to 6 μ M, $n=4$) and an efficacy of 1.0 relative to U50,488. Nalfurafine had full agonist activity at hKOR for both pathways but was

approximately 80 fold more potent for activation of ERK1/2 as compared to p38 ($p < 0.05$), with only modest p38 phosphorylation at concentrations that maximally stimulated ERK1/2 phosphorylation. There was no significant difference between hKOR and rKOR expressing HEK293 cells in potency for ERK1/2 phosphorylation induced by nalfurafine, but nalfurafine was approximately 12 fold less potent at inducing p38 phosphorylation in hKOR expressing HEK293 cells as compared to rKOR expressing cells ($p < 0.05$; Table 1). It is unlikely that this resulted from the difference in tagging strategies as the higher potency at p38 activation by rKOR is in contrast to prior studies with these receptors examining other opioids [21].

3.3. What is the selectivity of nalfurafine for KOR compared to MOR based on ERK1/2 activation?

Nalfurafine is also a lower affinity partial agonist for the mu opioid receptor (MOR), and has some activity at the nociceptin (ORL1) and delta opioid receptors [27]. The reported selectivity of nalfurafine for KOR over other receptors depends on the assay used. To determine the selectivity of nalfurafine for KOR over MOR in MAPK activation, HEK293 cells stably expressing rMOR were treated for 5 min with nalfurafine (100 pM–100 nM) or DAMGO (1 μ M) prior to lysis and phospho-ERK1/2 was quantified by western blot (Fig. 2A–B). Nalfurafine induced ERK1/2 activation at 5 min with an EC_{50} of 7.9 nM (confidence interval 4.9 nM to 13 nM, $n=3$) and an efficacy of 0.84 relative to DAMGO. Nalfurafine was significantly less potent at inducing ERK1/2 phosphorylation in rMOR expressing cells as compared to rKOR (approximately 16 fold, $p < 0.05$) or hKOR (approximately 5 fold, $p < 0.05$) expressing cells. No significant increase in ERK1/2 or p38 phosphorylation was observed in untransfected HEK293 cells treated with 1 μ M nalfurafine (Fig. 2C–D, $n=3$), indicating that nalfurafine-induced ERK1/2 and p38 phosphorylation were receptor dependent.

3.4. Signaling bias quantification

We previously found that U50,488 induced ERK1/2 and p38 phosphorylation with similar potency and pentazocine was more potent for inducing p38 phosphorylation than ERK1/2 phosphorylation [21]. To quantify the bias of nalfurafine, bias for ERK1/2 activation as compared to p38 activation, was calculated by $-\log(RA)$, with U50,488 [21] used as the reference compound. Nalfurafine was identified as a G protein biased ligand for rKOR (bias factor of 7), and a strongly G protein biased ligand for hKOR (bias factor of 300; Table 2).

3.5. Does nalfurafine produce KOR-mediated thermal analgesia in C57Bl/6 mice?

While the analgesic effects of nalfurafine have been demonstrated to be KOR-dependent in the acetic acid writhing [28] and rat paw pressure test [29], KOR-dependence has not been tested for nalfurafine analgesia in the warm water tail withdrawal assay in mice. The warm water tail withdrawal assay was used to assess the antinociceptive properties of nalfurafine and to determine if nalfurafine analgesia is KOR-mediated in this test. Treatment with U50,488 resulted in a dose dependent increase in tail withdrawal latency, indicative of analgesia ($p < 0.001$; Fig. 3A), and an effect of drug compared to baseline ($p < 0.01$; interaction $p < 0.05$). Similarly with nalfurafine, drug treatment significantly increased latency to withdrawal (Fig. 3B; $p < 0.001$; interaction $p < 0.01$). Specifically, at 15 and 30

mg/kg U50,488, animals showed a significant analgesic response to drug (2.9 ± 1.1 s and 2.8 ± 0.9 s, respectively; Bonferroni $p < 0.001$) and at 50 and 150 μ g/kg nalfurafine (1.5 ± 0.7 s and 1.7 ± 0.4 s, respectively; Bonferroni $p < 0.01$ and $p < 0.001$, respectively).

To determine if this analgesia was KOR dependent, mice were injected with norBNI (10 mg/kg) 3–5 days prior to treatment with U50,488 or nalfurafine. As expected, norBNI blocked the U50,488-mediated increase in withdrawal latency ($p < 0.01$, Fig. 3C), with a change in tail withdrawal latency of 0.7 ± 0.2 s and 0.76 ± 0.2 s after 15 and 30 mg/kg U50,488. norBNI also blocked the nalfurafine-mediated increase in withdrawal latency ($p < 0.05$, Fig. 3D), with a change in tail withdrawal latency of 0.6 ± 0.1 s and 0.7 ± 0.2 s after 50 and 150 μ g/kg nalfurafine. These results demonstrate a KOR-dependent analgesic response to 50 and 150 μ g/kg nalfurafine in this assay.

3.6. Does nalfurafine produce acute tolerance?

Prior studies have found nalfurafine to produce minimal analgesic tolerance in the acetic acid writhing and herpetic pain assays [30,31]. To determine if high doses of nalfurafine are capable of producing analgesic tolerance in the warm water tail withdrawal assay, U50,488-induced increases in tail withdrawal latency were used to measure cross-tolerance at KOR following a pre-injection of nalfurafine. Mice were pretreated with saline or nalfurafine (1 mg/kg, i.p.). After 3 h, tail withdrawal latency was measured prior to and 30 min following a U50,488 injection (15 mg/kg, i.p.) (Fig. 4A). Twenty-four hours after the initial pretreatment, tail flick withdrawal latency was measured again prior to and after U50,488 treatment (Fig. 4B). Three hours after pretreatment, nalfurafine pretreatment significantly decreased U50,488-induced analgesia that was not observed with saline pretreated mice (effect of U50,488 $p < 0.05$; interaction between nalfurafine pretreatment and U50,488 pretreatment $p < 0.05$; two-way ANOVA with repeated measures, $n = 13$ for saline, $n = 12$ for nalfurafine). This was not a consequence of elevated baseline following nalfurafine pretreatment, as no difference in response latency was observed between saline and nalfurafine pretreated mice prior to U50,488 injection ($p > 0.05$; Student's t -test), indicating that high doses of nalfurafine are capable of producing an acute cross-tolerance in this assay. Twenty-four hours after pretreatment, U50,488 stimulated an increase in response latency regardless of pretreatment (effect of U50,488 $p < 0.05$; interaction $p > 0.05$; two-way ANOVA with repeated measures), demonstrating a lack of residual analgesic tolerance at 24 h. Together, these results indicate that high doses of nalfurafine (1 mg/kg) produce acute analgesic cross-tolerance, but that tolerance is resolved rapidly, with normal KOR function one day later.

3.7. Does nalfurafine produce KOR mediated antipruritic effects in C57BL/6 mice?

To assess the antipruritic effects of nalfurafine in C57BL/6 mice, subcutaneous injection of 5'-GNTI (30 μ g/kg) was used to induce scratching [32]. 5'-GNTI induced a significant increase in scratching as compared to saline in wild type ($n=10$) and KOR^{-/-} ($n=8$) mice (Fig. 5A; effect of GNTI, $p < 0.0001$; genotype $p > 0.05$; two-way ANOVA with repeated measures). 5'-GNTI treatment produced a significant increase in scratching in both wild type and KOR^{-/-} mice as compared to saline injection ($p < 0.0001$, Bonferroni's post-hoc). Wild type and KOR^{-/-} mice scratched 63 ± 23 times and 76 ± 20 times in 30 min following

saline, but 566 ± 60 times and 550 ± 84 times in 30 min following 5'-GNTI. These results indicate that 5'-GNTI-induced scratching is not KOR-mediated, in agreement with prior literature, which found no effect of norBNI on 5'-GNTI-induced scratching [32].

In contrast to previously published studies using Swiss-Webster mice [20], 20 $\mu\text{g/kg}$, s.c., nalfurafine 20 min prior to 5'-GNTI did not produce significant antipruritic effects in C57BL/6 wild type mice (average 556 ± 67 scratches in 30 min, $n = 10$; data not shown). Therefore a dose of 50 $\mu\text{g/kg}$ nalfurafine, which produced significant analgesia in the warm water tail withdrawal assay, was tested for antipruritic activity (Fig. 5B). No impairment of general locomotor activity was observed following 50 $\mu\text{g/kg}$ nalfurafine (4097 ± 953 and 3531 ± 1215 cm moved over 30 min in saline and nalfurafine treated mice, respectively; $p=0.73$ with Student's *t*-test, $n=3$). Nalfurafine significantly reduced 5'-GNTI-induced scratching in wild type and MOR^{-/-}, but not KOR^{-/-} mice. A significant effect of nalfurafine pretreatment ($p < 0.0001$) and time ($p < 0.0001$) was observed in wild type mice (two-way ANOVA with repeated measure, $n = 9$). Wild type mice scratched a total of 696 ± 58 times in 30 min following saline and 241 ± 33 times following nalfurafine, with peak scratching at 5–15 min and a significant reduction in scratching following nalfurafine at all time points except at 20–25 min (Bonferroni's post-hoc, $p < 0.05$). A significant effect of nalfurafine pretreatment ($p < 0.0001$), time ($p < 0.0001$), and an interaction between time and nalfurafine pretreatment ($p < 0.001$) was observed in MOR^{-/-} (two-way ANOVA with repeated measures, $n = 8$). MOR^{-/-} mice scratched a total of 598 ± 46 times in 30 min following saline and 99 ± 40 times in 30 min following nalfurafine, with a peak of scratching at 10–20 min and a significant reduction following nalfurafine at all time points after 5 min. In KOR^{-/-} mice, a significant effect of time ($p < 0.0001$) was observed (two-way ANOVA with repeated measures, $n=8$) but no significant effect of nalfurafine pretreatment ($p > 0.05$). KOR^{-/-} mice scratched a total of 508 ± 67 times following saline and 580 ± 16 times in 30 min following nalfurafine, with peak scratching at 5–15 min. These results show that the antipruritic effects of nalfurafine are KOR-mediated and MOR-independent in C57BL/6 mice, although a higher dose of nalfurafine was required than in previously studied strains.

4. Discussion

The major finding of this study is that nalfurafine is a G protein biased ligand with greater bias at the human than rodent KOR. Nalfurafine is biased towards ERK1/2 activation (G protein-dependent), with 20 $\times$ and 250 $\times$ lower potency for p38 activation by rKOR and hKOR, respectively. These findings have implications for the development of KOR agonists with therapeutic use, as p38 contributes the aversive properties of KOR agonists [5,6,33]. We also found that nalfurafine-induced thermal analgesia and anti-pruritus in C57BL/6 mice is KOR mediated, but requires higher doses of nalfurafine than reported in other models. Additionally, we demonstrated that the KOR antagonist 5'-GNTI induces scratching in a KOR-independent manner.

5'-GNTI injected subcutaneously resulted in robust scratching in wild type and KOR^{-/-} mice. This is the first published study examining 5'-GNTI-induced scratching in KOR^{-/-} mice and corroborates prior research demonstrating that norBNI pretreatment does not affect

5'-GNTI-induced itching [20], and that the peptide KOR antagonist zyklophen induces scratching in KOR^{-/-} mice and wild type mice pretreated with norBNI [34]. Together, these studies demonstrate that although several KOR-selective antagonists produce an itching response when injected subcutaneously, this effect is due to an off-target site of action. The necessary dose of nalfurafine for antipruritic effects in this study (50 µg/kg, with no effect at 20 µg/kg) was higher than other published studies, presumably due to mouse strain differences [20]. Although higher concentrations were required, nalfurafine antipruritis was KOR-mediated.

We found that nalfurafine (50 µg/kg and 150 µg/kg) increased tail withdrawal latency in the warm water tail withdrawal assay, with 150 µg/kg producing the same analgesic response as 50 µg/kg (1.5 s increase). Antinociception was blocked by norBNI, indicating that nalfurafine analgesia in this assay is KOR-mediated. While nalfurafine analgesia in the rat paw pressure test and acetic acid writhing assay had previously been shown to be blocked by pretreatment with norBNI, [28,29], KOR-dependence had not previously been demonstrated for thermal pain assays. Further, this study found that high doses of nalfurafine are capable of inducing acute cross-tolerance, but the tolerance is resolved within one day.

The KOR ligand 6'-GNTI has been reported to be G protein biased, but is a peripherally restricted partial agonist [10]. In contrast, nalfurafine is a full KOR agonist for both pathways examined in this study, indicating that partial agonism is not required for signaling bias. The identification of nalfurafine as a G protein biased agonist with low potency for induction of p38 phosphorylation by both hKOR and rKOR is in contrast to our findings with butorphanol and pentazocine [21]. Butorphanol and pentazocine were found to be p38-biased in hKOR-expressing HEK293 cells, but were identified as G protein biased or unbiased, respectively, in rKOR expressing HEK293.

As p38 MAPK has been previously shown to play an important role in the aversive effects of KOR agonists, the G protein bias of nalfurafine may help explain the reported lack of aversive effects to nalfurafine in clinical trials and the minimal aversive responses at analgesic and antipruritic doses in animal models. While the behaviors measured in this study were KOR-dependent, nalfurafine lacks the desired receptor selectivity for a KOR agonist. Binding affinity for KOR is only between 2 and 15 fold higher than for MOR and has partial agonist to full agonist activity at MOR [27,35,36]. As a consequence of this lack of selectivity, it is difficult to interpret data on the rewarding or aversive properties of nalfurafine.

5. Conclusions

Despite lacking optimal receptor selectivity, nalfurafine is a centrally-active prototype G protein biased agonist and therefore provides a base structure for future analgesic drug development. An ideal KOR agonist would be highly efficacious for G protein-mediated pathways with minimal efficacy or greatly reduced potency for GRK3 mediated-p38 activation. Additional qualities would be low affinity and minimal efficacy for other opioid receptors, notably MOR, and being centrally active. This compound would be predicted to be an effective analgesic with reduced tolerance and a large therapeutic window before

encountering side effects such as dysphoria or euphoria. Testing of nalfurafine analogs could provide key insights into the structural basis of signaling bias by KOR ligands ultimately leading to development of more selective KOR ligands with reduced side effects.

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Abbreviations

ERK1/2	extracellular signal-regulated kinase
GRK3	G protein-coupled receptor kinase 3
hKOR	human kappa opioid receptor
MAPK	mitogen activated protein kinase
norBNI	norbinaltorphimine
rKOR	rodent kappa opioid receptor

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Fig. 1.

Nalfurafine is less potent in KOR-expressing HEK293 for activation of p38MAPK as compared to ERK1/2. HEK293 cells expressing rKOR (A, B) or hKOR (C, D) were treated for 5 min (pERK) or 30 min (pp38) with indicated concentrations of nalfurafine or with 1 μ M U50,488 prior to lysis and immunoblotted for phospho-ERK1/2 or phospho-p38 (n= 4–5).

Fig. 2.

Nalfurafine activates p38 in rMOR expressing HEK293 with lower potency than rKOR. (A, B) To determine the potency of nalfurafine for ERK1/2 activation by MOR, HEK293 cells expressing rMOR were treated for 5 min with the indicated concentrations of nalfurafine or with 1 μ M DAMGO prior to lysis (n=3). Data are presented as an increase in phosphorylation over basal, normalized to the level of phosphorylation induced by U50,488 or DAMGO in the same experiment. (C, D) Selectivity was determined by treating untransfected HEK293 cells with 1 μ M nalfurafine for 5 min (pERK) or 30 min (pp38) prior to lysis (n= 3).

Fig. 3.

Nalfurafine has KOR-mediated analgesic effects in the warm-water tail-withdrawal assay. Wild type C57BL/6 mice were injected with the indicated doses of U50,488 (A) or nalfurafine (B) i.p, and tail flick withdrawal latency was recorded before and 30 min post-injection. For U50, 488, a significant interaction ($p < 0.001$), effect of drug treatment ($p < 0.0001$), and effect of dose ($p < 0.001$) was observed based on two-way ANOVA with repeated measures ($n = 16$). For nalfurafine, a significant interaction ($p < 0.001$) and significant effect of drug treatment ($p < 0.001$) was observed based on two-way ANOVA with repeated measures ($n = 16$). Significance according to Bonferroni post-hoc test, as compared to pretest is indicated by ** ($p < 0.01$) or *** ($p < 0.001$). KOR-dependence of increased tail flick withdrawal latency by U50,488 (C) and nalfurafine (D) was determined by measuring tail flick withdrawal latency following pretreatment with norBNI. Latencies are reported the change from baseline (post-pre). A significant effect of antagonist was observed for U50,488 ($p < 0.001$, $n = 16$) and nalfurafine ($p < 0.05$, $n = 16$) based on two-way ANOVA with repeated measures followed by Student's *t*-test.

Fig. 4.

Nalfurafine results in acute but not prolonged tolerance. Wild type C57BL/6 mice were pretreated with saline or nalfurafine (1 mg/kg). Tail flick withdrawal latency was measured before and 30 min after injection of U50,488 (15 mg/kg, i.p.) at 3 h (A) and 24 h (B) following pretreatment with saline or nalfurafine. At 3 h following pretreatment, a significant interaction ($p < 0.05$) and significant effect of time ($p < 0.05$) were observed based on two-way ANOVA with repeated measures ($n = 13$ for saline, $n = 12$ for nalfurafine). At 24 h following pretreatment, a significant effect of time ($p < 0.05$, inset) was observed, but no interaction or effect of pretreatment. Significance according to Bonferroni post-hoc test, as compared to pretest, is indicated by ** ($p < 0.01$).

Fig. 5.

Nalfurafine has KOR-mediated anti-pruritic effects. (A) The number of scratches in 30 min following injection with saline or 30 µg/kg 5'-GNTI (s.c.) were counted in wild type (n=10) or KOR^{-/-} (n=8) C57BL/6 mice. A significant effect of 5'-GNTI ($p < 0.0001$) was observed based on two-way ANOVA with repeated measures. Significance according to Bonferroni post-hoc test, as compared to saline injection, is indicated by **** ($p < 0.0001$). (B) Wild type, KOR^{-/-}, and MOR^{-/-} C57BL/6 mice were injected with saline or 50 µg/kg nalfurafine, s.c. 20 min prior to injection of 30 µg/kg 5'-GNTI (s.c.) and the number of scratches over 30 min following 5'-GNTI injection were counted in 5 min bins. A significant effect of nalfurafine pretreatment ($p < 0.0001$) and time ($p < 0.0001$) was observed in wild type mice based on two-way ANOVA with repeated measures (n = 9). In KOR^{-/-} mice, a significant effect of time ($p < 0.0001$) was observed based on two-way ANOVA with repeated measures (n = 8), but no significant effect of nalfurafine pretreatment was seen. A significant effect of nalfurafine pretreatment ($p < 0.0001$), time ($p < 0.0001$), and an interaction between time and nalfurafine pretreatment ($p < 0.001$) was observed in MOR^{-/-} mice based on two-way ANOVA with repeated measures (n = 8). Significance according to Bonferroni post-hoc, as compared to the saline pretreatment of the same genotype, is indicated by * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$) or **** ($p < 0.0001$).

Table 1

Pharmacological data for hKOR and rKOR expressing HEK293.

Nalfurafine	hKOR Mean (error; n)	rKOR Mean (error; n)	rKOR/hKOR ratio
pp38 relative efficacy	1.0 (0.30; 4)	1.0 (0.35; 4)	1.0
pERK relative efficacy	0.99 (0.11; 5)	1.1 (0.08; 5)	1.1
pp38/pERK ratio	1.0	0.91	0.70
pp38 EC50	110 nM (100–6000 nM; 4)	5.2 nM (4.4–160 nM; 4)	0.081*
pERK EC50	1.4 nM (1.1–3.8 nM; 5)	0.50 nM (0.38 nM–1.6 nM; 5)	0.36
pp38/pERK ratio	79*	10*	0.10

Summary of pharmacological properties of nalfurafine. EC50 and relative efficacy values for ERK1/2 and p38 phosphorylation following nalfurafine were calculated based on three parameter dose response curves. Numbers depict the average values from 4 to 5 replicates, with SEM(efficacy) or 95% confidence interval (EC50) and number of replicates in parenthesis. Significance of differences, based *t*-test with Holm's Sidak correction for multiple comparisons, are indicated (**p* < 0.05).

Table 2

Bias factor for ERK1/2 signaling vs p38 phosphorylation.

	rKOR		hKOR	
	log(RA) Mean (SEM)	Bias factor	log(RA) Mean (SEM)	Bias factor
U50,488	0 (0.61)	1.0	0 (0.78)	1.00
Nalfurafine	1.15 (0.74)	7.2	3.2 (1.02)	300
Pentazocine	0.14 (0.79)	1.4	−0.20 (0.78)	0.62

Summary of signaling bias of U50,488, nalfurafine, and pentazocine for ERK1/2 phosphorylation (G protein) relative to p38 phosphorylation. Signaling bias was calculated based on $\log(RA)$, as described in Materials and methods. The $\log(RA)$, standard error of $\log(RA)$, and bias factor are reported.