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Comparison of Pharmacological Properties between the Kappa Opioid Receptor Agonist Nalfurafine and 42B, Its 3-Dehydroxy Analogue: Disconnect between *in Vitro* Agonist Bias and *in Vivo* Pharmacological Effects

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Abstract

Nalfurafine, a moderately selective kappa opioid receptor (KOR) agonist, is used in Japan for treatment of itch without causing dysphoria or psychotomimesis. Here we characterized pharmacology of compound 42B, a 3-dehydroxy analog of nalfurafine and compared with that of nalfurafine. Nalfurafine and 42B acted as full KOR agonists and partial μ opioid receptor (MOR) agonists, but 42B showed much lower potency for both receptors and lower KOR/MOR selectivity, different from previous reports. Molecular modeling revealed that water-mediated hydrogen-bond formation between 3-OH of nalfurafine, and KOR accounted for its higher KOR potency than 42B. The higher potency of both at KOR over MOR may be due to hydrogen-bond formation between non-conserved $Y^{7.35}$ of KOR and their carbonyl groups. Both showed modest G protein signaling biases. In mice, like nalfurafine, 42B produced antinociceptive and anti-scratch effects and did not cause conditioned place aversion (CPA) in the effective dose ranges. Unlike nalfurafine, 42B caused motor incoordination and hypolocomotion. As both agonists showed G protein biases, yet produced different effects on locomotor activity and motor incoordination, the

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findings and those in the literature suggest caution in correlating *in vitro* biochemical data with *in vivo* behavior effects. The factors contributing to the disconnect, including pharmacodynamic and pharmacokinetic issues, are discussed. In addition, our results suggest that among the KOR-induced adverse behaviors, CPA can be separated from motor incoordination and hypolocomotion.

Keywords

kappa opioid receptor; nalfurafine; antipruritic effect; conditioned place aversion; sedation; motor incoordination

Introduction

The κ opioid receptor (KOR), one of the three opioid receptors, belongs to the rhodopsin subfamily of G protein-coupled receptors. KOR agonists produce analgesic and antipruritic effects[1–4]; however, clinical development of these compounds has been limited by the side effects such as psychotomimetic effect, dysphoria, and sedation[2, 4, 5], except for nalfurafine. Nalfurafine, a moderately selective KOR agonist, has been used in Japan for treatment of itch in patients undergoing hemodialysis and those with chronic liver diseases[6–10]. Clinical reports have shown that different from other KOR agonists that have been tested in humans, at therapeutic doses, nalfurafine did not produce dysphoria or psychotomimetic effects[7–9]. Recently, we have demonstrated that in mice nalfurafine exerted analgesic and anti-scratch effects without adverse effects such as sedation, motor incoordination, or conditioned place aversion (CPA)[11], thus recapitulating the observations in humans. Because of its favorable side effect profile, nalfurafine was also investigated as an adjuvant to μ opioid receptor (MOR)-targeting analgesics[12] and as a therapeutic agent for drug abuse and alcohol treatment[13–16]. Nalfurafine enhanced analgesic effects of morphine, but reduced morphine-induced conditioned place preference (CPP) and hyperlocomotion in mice[12]. Preclinical research showed that nalfurafine augmented antinociceptive effect of oxycodone, but attenuated oxycodone-induced reinforcing effects and respiratory depression[13, 14]. In addition, nalfurafine reduced alcohol intake and relapse-like alcohol drinking and repeated administration did not induce tolerance[15, 16].

Although nalfurafine is a full agonist for the KOR and a partial agonist for the MOR in [^{35}S]GTPγS binding assays[17], its analgesic and anti-scratching effects were shown to be mediated by the KOR[18–22]. Its effects on reducing drug abuse was also mediated by KOR since oxycodone self-administration and alcohol drinking were blocked by the selective KOR antagonist nor-binaltorphimine (norBNI)[13–16]. Nalfurafine did not cause CPA in MOR knockout mice, indicating that its lack of CPA in wildtype mice is not due to its combined actions on the MOR and KOR[11]. Thus, selective KOR agonists with similar chemical structures as nalfurafine may be promising therapeutic agents with fewer side effects.

42B, a nalfurafine analog which lacks the 3-hydroxy moiety of the phenolic group (Figure 1 a,b), was reported to show higher selectivity for KOR over MOR than nalfurafine, albeit with lower affinities for both receptors in radioligand binding assays[23, 24]. However, no *in vivo* characterization was performed on 42B. We hypothesized that 42B may show favorable pharmacological properties *in vivo* due to its similar chemical structure to nalfurafine and

higher selectivity for KOR than nalfurafine. Although the 3-OH group of morphinan opioids was described long ago as one of the most important pharmacophore groups, such a conclusion mainly came from the small molecule structure-activity relationship with very limited information from structural biology due to technical limitation previously. Recently, as all the three major opioid receptors have been crystallized in both active and inactive states, different conclusions have been reported on the role of 3-OH group for its influence on binding affinity and selectivity for the KOR activated structure[24]. It was suggested that modifications of the phenol group may be a possible path towards producing selective KOR ligands with desirable pharmacological characteristics[24]. Thus, a revisit of many take-for-granted concepts and opinions in this field is warranted. Therefore, in this study, we examined *in vitro* and *in vivo* pharmacological properties of the 3-dehydroxy analog of nalfurafine, 42B, and compared with those of nalfurafine.

Results and Discussion

Maximal responses and potencies of nalfurafine and 42B in stimulating [^{35}S]GTPγS binding via MOR, δ opioid receptor (DOR), KOR and nociceptin / orphanin FQ receptor (NOR).

[^{35}S]GTPγS binding was applied as a functional measure of activation of opioid receptors and NOR. Nalfurafine and its 3-dehydroxy analog 42B stimulated the KOR to enhance [^{35}S]GTPγS binding to membranes, both having the maximal effects 91% of that of the full agonist (−)U50,488 (Figure 2 a–d; Table 1), indicating that both are full agonists at the KOR in this assay. The potency of nalfurafine (EC_{50} = 0.097 ± 0.018 nM) at the KOR was ~250-fold higher than that of 42B (EC_{50} = 25.56 ± 1.50 nM).

For the MOR, both nalfurafine and 42B acted as partial agonists, with the maximal effect being 74% and 49%, respectively, of that of the full agonist DAMGO. Nalfurafine also showed higher potency at the MOR than 42B, with the EC_{50} values of 3.11 ± 0.63 nM and 214.9 ± 50.4 nM, respectively. Thus, nalfurafine not only showed higher potency in activating both the KOR and the MOR, but also had higher selectivity for the KOR over the MOR (32-fold) than 42B (8.4-fold).

In addition, nalfurafine and 42B demonstrated full agonism at the DOR and NOR with relatively low potency.

Our results that nalfurafine showed greater selectivity for the KOR over the MOR than 42B in [^{35}S]GTPγS binding assays are different from the previous radioligand binding results that nalfurafine showed lower ratio of KOR/MOR affinity than 42B[23, 24]. Nagase and Fujii[23] reported that the ratios of KOR/MOR affinity were 2.4 for nalfurafine and 60 for 42B (Supplementary Materials, Table S1). Che, et al.[24] showed that the KOR/MOR affinity ratios of nalfurafine and 42B (compound 18) were 13.1 and 355, respectively (Table S1). Assays and tissues used may account for the discrepancy. We performed [^{35}S]GTPγS binding, a functional measure of receptor activation, whereas both Nagase and Fujii[23] and Che et al.[24] did inhibition of radioligand binding. As shown in Table S1, Che et al.[24] did inhibition of [^{3}H]diprenorphine binding to the KOR and MOR stably expressed in Sf9 cells. Nagase and Fujii[23] determined the affinities by inhibition of [^{3}H]U69,593 binding to the KOR in guinea

pig cerebellum membranes and of [^{3}H]DAMGO binding to the MOR in mouse brain membranes.

Effects of nalfurafine and 42B on KOR-mediated G protein and β-arrestin signaling

For determination of the effects of nalfurafine and 42B on G protein- and β-arrestin-mediated signaling following KOR activation, GloSensor cAMP assay and Tango assay were performed by the Psychoactive Drug Screening Program (PDSP) of the National Institute of Mental Health (NIMH) for G protein activation and β-arrestin2 recruitment, respectively. The prototypic KOR agonist U50,488H was used as the reference agonist. Nalfurafine and 42B activated G proteins and recruited β-arrestin in a dose-dependent fashion with high maximal responses and varying potencies in the two assays (Figure 3, Table 2). For inhibition of cAMP accumulation, both nalfurafine and 42B had nearly maximal effects as U50,488H, but nalfurafine (EC_{50} = 0.17 ± 0.04 nM) and 42B (EC_{50} = 0.82 ± 0.18 nM) were ~9-fold and ~2-fold more potent than U50,488H (EC_{50} = 1.61 ± 0.19 nM), respectively. For β-arrestin recruitment, the maximal effect of nalfurafine (E_{max} = 108.0 ± 2.6%) was higher than that of U50,488H (E_{max} = 99.93 ± 0.07%), and the maximal effect of 42B (E_{max} = 116.8 ± 1.0%) was higher than those of nalfurafine and U50,488H. While the potency of nalfurafine (EC_{50} = 0.74 ± 0.07 nM) was not significantly different from that of U50,488 (EC_{50} = 1.43 ± 0.13 nM), 42B (EC_{50} = 3.22 ± 0.42 nM) showed ~2-fold and ~4-fold lower potency for β-arrestin recruitment than U50,488H and nalfurafine, respectively.

The intrinsic relative activity (RA_i) of the agonists for G protein activation (RA_{i-G}) and β-arrestin recruitment (RA_{i-b}) were calculated from the agonist concentration-response curves (Figure 3) and summarized in Table 3, using the method of Ehlert and colleagues[25–27]. This parameter represents the product of affinity ($1/K_{obs}$) and efficacy (ε) of a test agonist, divided by the corresponding product for a reference agonist (ε'/K_{obs}') ($RA_i = \varepsilon K_{obs}'/\varepsilon' K_{obs}$). In our experiments, we designated U50,488H as the reference agonist. RA_i was estimated using global nonlinear regression analysis with a modified form of the operational model[26, 27]. It has been shown that RA_i is a relative estimate of active state affinity, which is why RA_i is useful in quantifying biased signaling. The bias factor represents the ratio of the RA_i value for G protein signaling divided by that for arrestin signaling, and these values are also summarized in Table 3. In the present study, the agonists exhibit Hill slopes <1 in the GloSensor assay (0.64 – 0.74) and Hill slopes >1 in the Tango assay (1.1, 1.4), which were taken into consideration in our calculations.

A bias factor > 1 indicates a preference for G protein signaling, whereas a bias factor < 1 indicate a preference for β-arrestin recruitment. As shown in Table 3, the bias factors of nalfurafine and 42B were 4.49 and 2.85, respectively, suggesting that both nalfurafine and 42B have small, significant biases for G protein signaling.

Thus, these results indicate that both nalfurafine and 42B are biased agonists for G protein signaling. Earlier research on whether nalfurafine is a biased agonist yielded different results, however, including G protein-biased[12, 28], un-biased[11] and β-arrestin-biased[29]. We previously demonstrated that *in vitro* nalfurafine was not biased using [^{35}S]GTPγS binding and β-arrestin1 and β-arrestin2 recruitment as the end points[11]. Thus, there are discrepancies between our own two studies. In these earlier studies, for [^{35}S]GTPγS binding we used

mouse neuro2A cells stably transfected with the mouse KOR and for β-arrestin recruitment assay we used β-galactosidase fragment complementation assay and HEK293 cells stably transfected with the mouse KOR conjugated with β-galactosidase donor fragment and β-arrestin-1 or β-arrestin-2 fused with β-galactosidase acceptor fragment[11]. In the present study, experiments were performed by the PDSP program, in which HEK293-T cells stably expressing the human KOR were transfected with the GloSensor cAMP DNA construct for GloSensor cAMP assay and HEK293 cells stably expressing a tTA-dependent luciferase reporter and a β-arrestin2-TEV fusion gene were transfected with the human KOR for Tango assays. In both studies, U50,488H was used as the reference balanced agonist. Thus, the assay, cell line, species origin of the receptor, receptor expression level and kinetics of agonist-receptor interaction may contribute to the differences, as discussed previously[30–33]. The recent finding of Gillis, et al.[34], which revealed that responses that have amplification factors and have higher receptor reserves tend to enhance potency and efficacy of an agonist, thus confounding agonist bias calculation. The inhibition of adenylyl cyclase response has a high signal amplification[34], whereas [^{35}S]GTPγS binding and β-arrestin recruitment do not, thus making nalfurafine to be G protein-biased in the present study, not in our previous one[11]. In addition, while both Schattauer et al.[28] and Kaski et al.[12] reported nalfurafine to be a G protein-biased agonist using various assays for G protein and β-arrestin signaling in human or rat KOR-expressing HEK293 or HEK293T cells with U50,488 as the reference balanced agonist, Dunn, et al.[29] showed that nalfurafine was a β-arrestin-biased agonist using [^{35}S]GTPγS binding and β-arrestin2 recruitment assays in human KOR-expressing U2OS cells with U69,593 as the reference balanced agonist. Again, the inconsistency may be attributed to different assays, cell lines and the reference agonist used and the kinetics of agonist action can be a factor if not addressed by the experimental protocol. In addition to amplification factors of signaling end points, kinetics of agonist action on the receptor also is a factor, thus end points of assays also play important roles in determining the bias factors.

Molecular docking studies

Nalfurafine and 42B have identical chemical structure skeletons, except for 3-hydroxy group on the phenyl moiety of nalfurafine (Figure 1). Nalfurafine and 42B were docked into the agonist-bound crystal structures of the MOR (PDB ID: 5C1M)[35] and KOR (PDB ID: 6B73)[24] to explore how nalfurafine and 42B may interact with the receptors so that they produce different potency profiles for the MOR and KOR. The docking poses with the highest CHEM-PLP scores were selected as the optimal binding poses, named as nalfurafine_MOR^{active}, nalfurafine_KOR^{active}, 42B_MOR^{active}, and 42B_KOR^{active} complexes (Figure 4 a–d), respectively.

From the docking study results, nalfurafine and 42B in the active MOR and KOR adopted similar binding modes as BU72 and MP1104 did in the crystal structures of the active MOR (PDB ID: 5C1M)[35] and KOR (PDB ID: 6B73)[24], respectively (Supporting Information, Figure S1 a–b). Especially, the cyclopropylmethyl moieties of nalfurafine and 42B were accommodated by the hydrophobic pockets formed by residues W$^{6.48}$, G$^{7.42}$, and Y$^{7.43}$ of the MOR and KOR. The furan moieties of nalfurafine and 42B seemed to interact with the transmembrane helices 2 and 3 (TMs 2 and 3). Notably, these interactions were thought to

stabilize the active states of the MOR and KOR (Figure S1 a–b)[24, 35]. Therefore, that may help explain why nalfurafine and 42B exhibited agonist activity to the MOR and KOR.

Further zooming in, there were at least 22 residues within 5 Å of nalfurafine and 42B in the nalfurafine_MOR^{active}, nalfurafine_KOR^{active}, 42B_MOR^{active}, and 42B_KOR^{active} complexes. As shown in Figure 4, conserved residues M$^{3.36}$, W$^{6.48}$, I$^{6.51}$, H 6.52, and I$^{7.39}$ located at the orthosteric binding sites may form direct hydrophobic interactions with the epoxymorphinan scaffold of both ligands. In addition, the highly conserved D$^{3.32}$ may form ionic interactions with the 17-quaternary nitrogen atom of nalfurafine and 42B, and Y$^{3.33}$ may form hydrogen bond interactions with the dihydrofuran oxygen atom of both ligands (Table S2). Notably, among the 22 residues, two residues (K$^{6.58}$ and W$^{7.35}$ in the MOR, and E$^{6.58}$ and Y$^{7.35}$ in the KOR) were non-conserved ones. In the nalfurafine_KOR^{active} and 42B_KOR^{active} complexes, Y$^{7.35}$ may form hydrogen bonds with the carbonyl groups of nalfurafine and 42B (Table S2), which may help stabilize the binding of the furan moieties of nalfurafine and 42B. However, this hydrogen bond was not observed with W$^{7.35}$ in the nalfurafine_MOR^{active} and 42B_MOR^{active} complexes. As discussed above, the furan moieties of nalfurafine and 42B interacting with the TMs 2 and 3 was involved in the activation of the MOR and KOR. The hydrogen bonds formed between Y$^{7.35}$ in the KOR and the carbonyl groups of nalfurafine and 42B may provide a possible explanation for the observations that nalfurafine and 42B exhibited higher binding affinities with the KOR than the MOR while both acted as KOR full agonists, but MOR partial agonists.

In addition, as described in the crystal structures of the active MOR (PDB ID: 5C1M)[35] and KOR (PDB ID: 6B73)[24], water-mediated hydrogen bonding interactions were formed between the hydroxy groups of the phenolic moiety of the ligands, BU72 and MP1104, and conserved residues Y$^{3.33}$, K$^{5.39}$, and H$^{6.52}$, respectively (Figure S1). These interactions may retain the position of the side chain of W$^{6.48}$ and further stabilize the active state of the MOR and KOR. Moreover, in the nalfurafine_KOR^{active} complex, the same water-mediated hydrogen bonding interactions were observed between the 3-hydroxy group of the phenolic moiety of nalfurafine and residues Y$^{3.33}$, K$^{5.39}$, and H$^{6.52}$ (Table S2), while there were no water-mediated hydrogen bonding interactions in the 42B_KOR^{active} complex due to the lack of the 3-hydroxy group in 42B. Therefore, the lack of the water-mediated hydrogen bonding interaction in the 42B_KOR^{active} complexes, compared with that of the nalfurafine_KOR^{active} complex, may be the cause of lower potency of 42B at the KOR, compared to nalfurafine.

42B produced antinociceptive and anti-scratching effects in a dose-dependent manner, similar to nalfurafine.

Activation of the KOR produces antinociceptive and anti-scratching effects[1, 3, 11]. We examined activities of 42B in inhibiting pain-like behaviors in the formalin test[11, 36] and in attenuating compound 48/80-induced scratching[3, 11, 17].

42B (1, 3, 5 mg/kg) reduced licking time in phase II of the formalin test in a dose-dependent manner in mice, indicating antinociceptive effects (Figure 5a). The A_{50} value was determined to be 2.08 mg/kg. Our previous results demonstrated that nalfurafine dose-

dependently reduced pain behavior in the late phase of the formalin test (Figure 5b), with an A_{50} value of 5.8 μg/kg[11].

42B (1, 3, 5 mg/kg) significantly inhibited compound 48/80-induced scratching in a dose-dependent manner in mice, indicating anti-scratch effects. The A_{50} value was determined to be 2.82 mg/kg. Similarly, we previously showed that nalfurafine (2.5, 5, 10, 20, 30 μg/kg) displayed dose-dependent anti-scratch effect in mice with an A_{50} value of 8.0 μg/kg[11] (Figure 5c,d).

Thus, nalfurafine was 400x and about 340x more potent than 42B, respectively, in the formalin test and in the anti-scratch test, consistent with the differences in their *in vitro* potency (260X) for the KOR in [^{35}S]GTPγS binding. However, in inhibition of forskolin-stimulated adenylyl cyclase (Glosensor assay), nalfurafine was only ~5X more potent than 42B.

We then examined if 42B produced anti-scratching effects via KOR activation by pretreatment of mice with the selective KOR antagonist norBNI (20 mg/kg, i.p.) 20 h prior to saline or 42B injection. While norBNI alone had no effects on compound 48/80-induced scratching, administration of norBNI 20 h before 42B (3 mg/kg) eliminated the anti-scratch activity of 42B, indicating that 42B produces its anti-scratching effects through activation of the KOR (Figure 5e).

Effects of 42B on adverse behaviors induced by KOR activation

Motor incoordination and sedation—KOR activation results in impaired performance in the rotarod test and inhibition of novelty-induced hyperlocomotion[11, 37], measures of motor incoordination and sedation in rodents. We tested if 42B would produce these effects in mice.

42B (1, 3, 5 mg/kg) dose-dependently caused performance impairment in the rotarod test (Figure 6a), indicating motor incoordination. Even at 1 mg/kg, which is lower than its A_{50} values in the antinociceptive and anti-scratch tests, 42B induced significant effect on rotarod test at 10 min. Interestingly, the effect of 42B was short-lived. The peak effect was at 10 min among the time points tested, and returned to the control level at 40 min. We previously showed nalfurafine at 20 μg/kg nalfurafine had small, yet significant, effects at 30 min and 40 min following drug injection[11]. The prototypic KOR agonist U50,488H was used as a positive control. The effect induced by 20 μg/kg is like that of U50,488H at 2 mg/kg, but much attenuated than 5 mg/kg of U50,488H (Figure 6b). U50,488H (5 mg/kg) significantly reduced the time mice stayed on the rod at 10, 20, 30, and 40 min after drug administration and the effect remained robust at 40 min. The doses of 20 μg/kg of nalfurafine and 5 mg/kg of U50,488 were 2.5 × their A_{50} values in the anti-scratching test[11].

42B (1, 3, 5 mg/kg) caused significant and dose-dependent reduction in novelty-induced hyperlocomotion in mice (Figure 6c). U50,488 at 5 mg/kg showed significant inhibition, whereas nalfurafine at 20 μg/kg did not (Figure 6d)[11].

Thus, unlike nalfurafine, 42B caused motor incoordination and sedation in the dose range producing antinociceptive and anti-scratching effects.

CPA—KOR agonists have been shown to cause dysphoria and psychotomimetic effects in humans[2, 4, 5] and CPA in rodents[11, 38, 39]. We examined if 42B caused CPA. 42B did not cause CPA in mice at the doses (1, 3, 5 mg/kg) producing antinociceptive and anti-scratching effects (Figure 6e). We reported previously that nalfurafine did not produce CPA even at 20 μg/kg, while U50,488 (5 mg/kg) induced significant CPA, compared to the saline group (Figure 6f)[11]. The doses of nalfurafine and U50,488H are 2.5-fold of the A_{50} in the anti-scratch test[11]. Thus, like nalfurafine, 42B did not produce CPA test in mice.

42B showed only 8.4-fold functional selectivity for the KOR over the MOR *in vitro*. Lack of CPA by 42B may be due to a combination of its actions on both the MOR and the KOR, causing conditioned place preference (CPP) and CPA, respectively. We tested this possibility by blocking the MOR with naloxone at a dose (1 mg/kg) which is selective for the MOR. Neither naloxone (1 mg/kg) alone nor a combination of naloxone (1 mg/kg) and 42B (3 mg/kg) caused CPA or CPP, compared to the saline group, suggesting that MOR did not contribute to lack of CPA by 42B (Figure 6g). As a positive control in this experiment, U50,488 (5 mg/kg) induced significant CPA.

Our results that neither nalfurafine up to 20 μg/kg nor 42B up to 5 mg/kg produced CPA demonstrated that there is dose separation between antinociception/anti-scratch and CPA for both nalfurafine[11, 18, 40] and 42B.

42B acts on the KOR, but not the MOR, to produce its behavioral effects

42B showed relatively low KOR/MOR selectivity. The following evidence strongly suggests that 42B likely acts on the KOR to produce its *in vivo* effects. The anti-scratch effect of 42B was blocked by norBNI pretreatment (20 mg/kg, i.p.) for 20 h or longer (see Figure 5e). Such a norBNI treatment paradigm was shown to selectively block the KOR, but not MOR or DOR[41–43], indicating that 42B at 3 mg/kg (and likely all the doses we used) acts on the KOR. In addition, KOR agonists and MOR agonists produce different effects in some behavior end points in mice. KOR agonists, but not MOR agonists, produce anti-scratch effect[17, 21, 44]. Moreover, in mice KOR agonists produce hypolocomotion, whereas MOR agonists cause hyperlocomotion[11, 37, 45]. Here we demonstrated that 42B induced anti-scratch effect and hypolocomotion, indicating KOR-mediated effects. In addition, blockade of MOR by naloxone (1 mg/kg, s.c.) did not affect its lack of CPA by 42B (see Figure 6g). We did not use the selective MOR antagonist CTAP or CTOP because these compounds required intracerebroventricular injection. Naloxone has a higher affinity for the MOR than for the KOR. In vivo, naloxone is 20–36 times more potent in antagonizing morphine- than U50,488- or spiradoline-induced antinociception in the mouse tail flick test[1, 46]. A low dose (0.5 – 1 mg/kg) of naloxone has been often used to determine the role of MOR in physiological or pharmacological responses (for example,[47–49]).

Nalfurafine and 42B showed similar G protein biases, but had different effects on locomotor activity and motor coordination

It is believed that G protein- and β-arrestin-mediated signaling pathways were linked to different KOR-mediated behaviors. Deletion of β-arrestin2 in mice impaired KOR-mediated motor incoordination in the rotarod test, but did not affect antinociceptive effects in the hot plate test, CPA and hypolocomotion produced by the KOR agonists U69,593 or salvinorin A[37]. Neither did it affect inhibitory effect of U50,488H on chloroquine-induced scratching[50]. These results indicate that β-arrestin2 is involved in motor incoordination in the rotarod test, but has no role in KOR-mediated analgesic effect (in the hot plate test), anti-scratch effect, CPA and hypolocomotion, and suggest that these effects may be mediated by G protein signaling. However, the possibility cannot be excluded that β-arrestin1 may compensate for the absence of β-arrestin2 in these mice. In contrast, KOR activation produces GRK3-dependent p38 MAPK activation[51] and inhibition of this pathway blocked KOR-mediated CPA[52–54], suggesting that CPA is mediated by GRK3- and arrestin-dependent pathway.

In this study, we demonstrated that both nalfurafine and 42B had biases towards G protein signaling *in vitro* and did not cause CPA, which is different from the findings of White et al. that G protein-biased RB64 caused CPA. However, 42B, unlike nalfurafine, produced motor incoordination and hypolocomotion, which is opposite to the previous finding that β-arrestin2 is involved in motor incoordination. Furthermore, Dunn, et al.[29] found that impaired rotarod performance is correlated with β-arrestin bias by examining nine KOR agonists of different biases in the rotarod test[29]. These inconsistent observations underscore an important consideration, that is, one should be cautious in correlating *in vitro* biochemical data with *in vivo* behavior results. As discussed above, different *in vitro* assay conditions yielded varying bias results for the same compound.

In addition, prior studies of distinct G protein-biased KOR agonists showed varying pharmacological properties, further indicating that bias factors *in vitro* may not predict pharmacological properties of agonists *in vivo*. Bohn and colleagues reported that the selective KOR agonists triazole 1.1 and Isoquinolinone 2.1 showed G protein biases, using U69,593 or U50,488H as the reference balanced agonist[55, 56]. These two G protein-biased KOR agonists produced antinociception in the warm water tail withdrawal test and inhibited chloroquine-promoted scratching similar to U50,488H, but they did not decrease locomotor activity in the open field test[50, 55, 56]. Roth and colleagues reported that RB-64, a salvinorin A derivative, is a G protein-biased KOR agonist, with salvinorin A as the reference balanced agonist[37]. RB-64 produced antinociceptive effect in the hot plate test, caused CPA, but no motor incoordination in the rotarod test and no hypolocomotion[37]. In addition, triazole 1.1 and RB-64 did not cause an increase in baseline threshold in the intracranial self-stimulation test as U50,488H did[37, 56], suggesting lack of anhedonia; however, U50,488H, as a positive control in the ICSS test, decreased the maximal spinning rate, confounding data interpretation. Taken together, among the five KOR agonists reported to have G protein-biases (triazole 1.1, Isoquinolinone 2.1, RB-64, nalfurafine and 42B), all produced antinociception, four had anti-scratch effects (RB-64 not examined) and four did not affect locomotor activity (42B did). In the rotarod test, RB-64 had no effect, nalfurafine had a

slight effect, but 42B showed profound effect (triazole 1.1 and isoquinone 2.1 not tested). While RB-64 caused CPA, nalfurafine and 42B did not.

There are many other factors involved in *in vivo* pharmacological effects of drugs besides *in vitro* pharmacological properties. Pharmacokinetic characteristics of a drug are critical in determining its in vivo actions. Our bias estimates are made in vitro to circumvent pharmacokinetic issues. The log RAi estimate is a relative estimate of active state affinity, which is why the parameter is used to estimate agonist-receptor bias for different signaling pathways. Even at the pharmacodynamic level, the in vitro bias estimate does not directly translate to in vivo behavior of agonists for three reasons, 1) it is a relative estimate and the reference agonist may not be strictly neutral for different pathways, 2) the sensitivities of the signaling pathways measured in vivo may be different from those measured in vitro (sensitivity refers to how much redundancy and amplification occurs in the pathway), 3) the signaling pathway measured in vitro may be different from that measured in vivo. In addition, pharmacokinetic issues may alter the potencies of agonists in vivo, such as absorption, distribution, metabolism into active or inactive metabolites and excretion following drug administration. In the case of drugs acting on the CNS, the degree of drugs crossing blood-brain barrier is also an important consideration. 42B appears to get into the brain because it caused hypolocomotion and motor incoordination. Nalfurafine was shown to get into the brain previously. Systemic nalfurafine suppressed intracisternal or intrathecal morphine-induced scratching[21, 57] and affected several signaling pathways in brain regions[11]. The anti-scratch activity of systemic nalfurafine was blocked by intra-cerebroventricularly injected norBNI[21]. It is conceivable that there are differences between the two drugs in some pharmacokinetic parameters, such as membrane partitioning, tissue binding, time course of the free drug concentrations in brain and volume of distribution. Pharmacokinetic properties of 42B have not been characterized, which is outside of the scope of our current study. Nalfurafine was shown to be a P-glycoprotein substrate[58], decreasing its influx into the brain. 3-OH group is likely to play a role in P-glycoprotein efflux, thus 42B may have higher influx into the brain and likely have higher brain concentration. In the rotarod test, 42B produced profound inhibitory effect with the peak effect at 10 min, whereas nalfurafine caused its slight lowering effect with a peak at 30 min, suggesting that 42B is distributed into the brain more readily than nalfurafine. Nalfurafine was shown to be metabolized by N17-decyclopropylmethylation and 3-OH glucuronidation[59]. 42B, lacking the 3-OH group, is more lipophilic and also will not undergo glucuronidation and may impact on its half-life in the blood. Thus, pharmacokinetic properties of the drug, particularly the time course of the free drug concentrations in brain, play critical roles in its pharmacological effects in vivo. Most studies on in vivo effects of biased agonists defined in vitro did not examine detailed pharmacokinetic properties of the compounds.

The behavioral effects are likely driven by the brain concentration of the drug and therefore temporally sensitive. As mentioned above, the effects of 42B and nalfurafine in the rotarod test reached peaks at 10 min and 30 min after injection, respectively, which likely approximated the times of the maximal concentrations of the drugs. The endpoints we examined were measured cumulatively for the following time periods post drug injection: from 20 to 40 min for the formalin test, from 20 to 50 min for the anti-scratch test, from 0 to

30 min for the locomotor activity, from 0 to 40 min for the rotarod test and from 0 to 30 min for the conditioning phase of the CPA test (similar results were obtained when conditioning was done from 10 to 40 min). Thus, we may underestimate the potency/efficacy or 42B in the formalin test and the anti-scratch test, but we have captured the peak effects of both nalfurafine and 42B in all the other tests.

Our observations that 42B produced hypolocomotion and motor incoordination, but not CPA, suggest that the signaling mechanism underlying CPA is different from those of hypolocomotion and motor incoordination. These results are in line with our previous observation that rapamycin, a mTOR inhibitor, abrogated U50,488H-promoted CPA, but not hypolocomotion and motor incoordination, in mice[11]. Our findings demonstrated more complexity in KOR-mediated behaviors which can be grouped based on mechanisms involved: analgesia / anti-scratch effect; hypolocomotion / motor incoordination; CPA. The separation of motor incoordination from CPA is consistent with the findings of White, et al. [37].

Conclusion

Our *in vitro* data that 42B had lower KOR/MOR selectivity than nalfurafine are different from published data. The observations that 42B showed lower affinity for the KOR and MOR and lower KOR/MOR selectivity were explained by molecular modeling results on their interactions with active states of the MOR and KOR, highlighting the importance of 3-OH group of nalfurafine. Furthermore, both nalfurafine and 42B showed biases towards G protein signaling. Like nalfurafine, 42B produced analgesic and anti-scratching effects, and did not cause CPA in mice. In addition, antipruritic effects of 42B was KOR-mediated and MOR did not contribute to lack of CPA by 42B, like nalfurafine. In contrast, unlike nalfurafine, 42B impaired motor coordination and induced sedation in mice. Therefore, 3-hydroxy group of nalfurafine is important for its unique and favorable pharmacological profile. Among the KOR-induced adverse behaviors, CPA was separated from motor incoordination and hypolocomotion. As both agonists showed G protein biases yet produced different effects on locomotor activity and motor incoordination, the findings and those in the literature suggest caution in correlating in vitro biochemical data with in vivo behavior effects.

Materials and Methods

Drugs and Materials

(−)U50,488 (U111), DAMGO (E-7384), DPDPE (E3888) and Nociceptin / OFQ (487960) were purchased from Sigma-Aldrich (St. Louis, MO). Nalfurafine was obtained from the National Institute on Drug Abuse (Bethesda, MD). 42B was synthesized by the laboratory of Dr. Yan Zhang of Virginia Commonwealth University (Richmond, VA) (See Supporting Information for details in chemical synthesis). [^{35}S]GTPγS (1250 Ci/mmol) was purchased from PerkinElmer Life Sciences (Boston, MA). The following materials were purchase from Sigma-Aldrich (St. Louis, MO): formalin, compound 48/80, GDP and GTPγS. Other commonly used chemicals were obtained from Sigma-Aldrich or ThermoFisher Scientific.

Cell Lines

CHO cells that stably express the rat MOR[60], the mouse DOR[61], the human KOR[62] or the human NOR were cultured in Minimum Essential medium (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (Gibco), 200 μg/ml geneticin, 100 U/ml penicillin and 100 U/ml streptomycin (Sigma-Aldrich, St. Louis, MO) at 37 °C in a humidified atmosphere containing 5% CO_2. CHO cells stably expressing the mouse DOR were kindly supplied by Dr. Ping-Yi Law, formerly Department of Pharmacology, University of Minnesota School of Medicine, Minneapolis, MN. CHO cells stably transfected with the human NOR were a gift from Dr. Lawrence Toll, formerly SRI International, Menlo Park, CA and currently Florida Atlantic University[63]. Others were established as we described previously[64, 65].

Cell Membrane Preparation and [^{35}S]GTPγS Binding

Membranes were prepared according to a modified procedure of Huang, et al.[66]. Cells were washed twice and harvested in Versene solution (EDTA 0.54 mM, NaCl 140 mM, KCl 2.7 mM, Na_2HPO4 8.1 mM, KH_2PO4 1.46 mM, and glucose 1 mM) and centrifuged at 130 × g for 5 min. The cell pellet was suspended in buffer A [50 mM Tris (pH 7.4), 1 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride] and kept on ice for 30 min, then centrifuged at 50,000 × g for 30 min. The pellet was re-suspended in buffer A and centrifuged again. Then, the membrane pellet was re-suspended in buffer A, homogenized and then aliquoted at about 1.5 mg/ml, frozen in dry ice/ethanol, and stored at −80℃. All the procedures were performed at 4°C.

Determination of [^{35}S]GTPγS binding to G proteins was carried out using a modified procedure of Huang, et al.[66]. Immediately before the [^{35}S]GTPγS binding assay, membranes were thawed on ice, sonicated and diluted with buffer B [50 mM Tris (pH 7.4), 100 mM (KOR) or 50 mM (MOR, DOR or NOR) NaCl, 5 mM $MgCl_2$, and 1 mM EDTA]. Membranes (10 μg) were incubated with [^{35}S]GTPγS (80 pM, 190,000–220,000 dpm) and 15 μM GDP with or without a ligand (10^{-13} to 5×10^{-5} M) in a total volume of 1 ml buffer B for 60 min at 30℃. Nonspecific binding was defined by incubation in the presence of 10 μM GTPγS. Bound and free [^{35}S]GTPγS were separated by filtration with GF/B filters under reduced pressure. Radioactivity on filters was determined by liquid scintillation counting. EC_{50} values and E_{max} of drugs were determined by curve fitting to the equation for a sigmoidal curve using GraphPad Prism version 8.2.1 (GraphPad Software Inc., La Jolla, CA).

Molecular docking studies

As nalfurafine and 42B (Figure 1a,b) are MOR partial agonists and KOR full agonists[17], the crystal structures of active MOR (PDB ID: 5C1M)[35] and KOR (PDB ID: 6B73)[24] were downloaded from the Protein Data Bank at http://www.rcsb.org to be applied as target protein templates. Before conducting the molecular docking studies, the N terminus, the seven transmembrane helices, the C terminus, and the three crystal water molecules involved in the water-mediated interaction between residues $Y^{3.33}$, $K^{5.39}$, and $H^{6.52}$ and the hydroxyl group of the original ligands, BU72 and MP1104, were retained[24, 35, 67–70], and other molecules were removed from both crystal structures. The missing residues in extracellular

loop 2 and intracellular loop 3 of 6B73 were then constructed and implemented through Sybyl 8.0[71]. The missing hydrogen atoms were added to both receptors. Meanwhile, nalfurafine and 42B were sketched, assigned with Gasteiger-Hückel charges, and further energy minimized to a gradient of 0.05 with 10,000 iterations in Sybyl 8.0[71].

The molecular docking studies were conducted by using GOLD 5.6. Similar to the studies of the epoxymorphinan analogs developed in our group[2435, 72–77], $D^{3.32}$ in the MOR and KOR may form ionic interactions with the protonated nitrogen atom at the 17-amino group of nalfurafine and 42B. $Y^{3.33}$ in the MOR and KOR may form hydrogen bonding interactions with the dihydrofuran oxygen atom of both ligands. Thus, the binding sites of the MOR and KOR were defined by the atoms within 10 Å of the γ-carbon atom of $D^{3.32}$ in the MOR and KOR, respectively. The distances between the protonated nitrogen atom in the 17-amino group of nalfurafine and 42B and the oxygen atom at the side chain of $D^{3.32}$ in the MOR and KOR were restricted to 4.0 Å. The distances between the two ligands' dihydrofuran oxygen atom and the phenolic oxygen atom of $Y^{3.33}$ were restricted to 3.5 Å. In addition, the *Allow early termination option* in GOLD 56 was activated during our docking studies, the number of docking solutions were set to 50. Except for above parameters, other parameters were applied their standard default settings. In the process of the molecular docking, flexible ligand was docked into a rigid protein. After the molecular docking, the docking poses with the highest CHEM-PLP score were chosen as the optimal binding poses.

Animals

Adult male CD-1 mice (30–35 g) were purchased from Charles River Laboratories (Wilmington, MA). All the mice were housed in a temperature- and humidity-controlled room on a light-dark cycle (22°C, 50–60 % humidity, 12:12 h light / dark cycle). All experiments were conducted when lights were on. The animals received food and water ad libitum, except during the experimental sessions. All procedures were approved by Temple University School of Medicine Institutional Animal Care and Use Committee.

Behavior tests

Formalin test was performed as we described[11], which was based on the procedures of Murray, et al.[78]. Briefly, after acclimation, mice were pretreated with saline (10 ml/kg, s.c.) or 42B (1, 3, 5 mg/kg, s.c.) five minutes before 5 % formalin (20 μl, s.c.). The time each animal licked / groomed the formalin-injected paw was recorded from 15 to 35 min post-injection (phase II reaction).

Compound 48/80 scratching test was performed as we described[11], which was based on the procedures of Wang, et al.[17]. Briefly, after acclimation, mice were injected with saline (10 ml/kg, s.c.) or 42B (1, 3, 5 mg/kg, s.c.) 20 min before compound 48/80 (50 μg, s.c), then the bouts of scratching were counted for 30 min. To determine whether the antipruritic effects of 42B is mediated by KOR, a selective KOR antagonist norBNI (20 mg/kg, i.p.) was given 20 h before saline (10 ml/kg, s.c.) or 42B (3 mg/kg, s.c.), then compound 48/80 scratching test was performed in mice.

Rotarod test was performed as we described[11], which was adapted from the procedures of White, et al.[37]. Briefly, after one-day training, the mice with baseline > 240 s were injected

with 42B (1, 3, 5 mg/kg, s.c.) or saline (10 ml/kg, s.c.) before the test, then the time each mouse stayed on the rotarod was recorded at 10, 20, 30, and 40 min after injection for a total of 5 minutes, respectively.

Measurement of locomotor activities was performed as we described[11], which was according to our previous procedures[79]. Mice were treated with saline (10 ml/kg, s.c.) or 42B (1, 3, 5 mg/kg, s.c.) and put into locomotor chambers immediately. Activities were continuously monitored over a 60-min period.

CPA was performed as we described[11], which was adapted from our procedures of CPP[79]. Due to the short-term effects of 42B shown in rotarod test, mice were pretreated with saline (10 ml/kg, s.c.) or 42B (1, 3, 5 mg/kg, s.c.) immediately before each conditioning session, respectively, in which mouse was confined to one chamber for 30 min. The time each mouse spent in the drug-paired chamber during pre-test and post-test was recorded, respectively. To exclude 42B's action on MOR in lack of CPA, mice were administered with the selective MOR antagonist naloxone (1 mg/kg, s.c.) or saline (10 ml/kg, s.c.) 25 min prior to saline (10 ml/kg, s.c.) or 42B (1, 3, 5 mg/kg, s.c.), then confined to one chamber for 30 min during each conditioning session.

GloSensor cAMP and Tango assays

GloSensor cAMP and Tango assays, which are used to measure G protein activation and β-arrestin recruitment, respectively, were performed by PDSP of the NIMh, directed by Bryan Roth of University of North Carolina (Web site: https://pdsp.unc.edu/ims/investigator/web/). Briefly, HEK293-T cells stably expressing the human KOR were transfected with the GloSensor cAMP DNA construct overnight and grown in Poly-L-Lys-coated 384-well plates in DMEM + 1% FBS at a density of 15–20K cells in a volume of 40 μl per well for GloSensor cAMP assay. After 6 h or overnight recovery, cells were removed from culture medium and received 20 μl/well buffer C (20 mM HEPES, 1x HBSS, pH 7.40), 10 μl different concentrations of nalfurafine, 42B or U50,488H was added and incubated at room temperature for 15 min, then 10 μl of 4 mM luciferin supplemented with isoproternol at a final concentration of 200 nM were added and the luminescence counter was used to record chemiluminescence in relative luminescence units after 15 minutes. For Tango assay, HEK293 cells stably expressing a tTA-dependent luciferase reporter and a β-arrestin2-TEV fusion gene were transfected with the human KOR overnight[80, 81] and plated in DMEM supplemented + 1% FBS in Poly-L-Lys-coated 384-well plates at a density of 15,000 cells in a volume of 40 μl per well. After 6 h or overnight recovery, each well was treated with different concentrations of nalfurafine, 42B or U50,488H dissolved in 10 μl buffer C overnight. Then, medium and drug solutions were removed and 20 μl per well of BrightGlo reagent (diluted 20-fold with buffer C, Promega, Madison, WI) was added. Cells were incubated for 20 min at room temperature in the dark before being counted on a luminescence counter. Both GloSensor cAMP and Tango assay were performed triplicate three times. EC_{50}, E_{max} and RA_i of each agonist were calculated from the same concentration-response curves with the method developed and refined by Ehlert and colleagues[25–27]. The difference between the log RA_i values of an agonist for the GloSensor cAMP (log RA_{i-G}) and Tango (log RA_{i-b}) assays was calculated as a measure of the log

agonist-bias [log (RA_{i-G}/RA_{i-b})]. For each agonist, RA_{i-G} was divided by RA_{i-b} to estimate the bias factor to G protein.

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 8.2.1 (GraphPad Software Inc., La Jolla, CA). All data are expressed as the mean ± standard error of the mean. One- or two-way analysis of variance or t-tests were used. Individual group comparisons were performed with Dunnett's, Tukey's or Sidak's *post-hoc* test. $P < 0.05$ was considered to indicate a statistically significant difference.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

CPA	conditioned place aversion
CPP	conditioned place preference
DOR	δ opioid receptor
KOR	κ opioid receptor
MOR	μ opioid receptor
NOR	nociceptin / orphanin FQ receptor
norBNI	norbinaltorphimine
RA_i	intrinsic reactive activity

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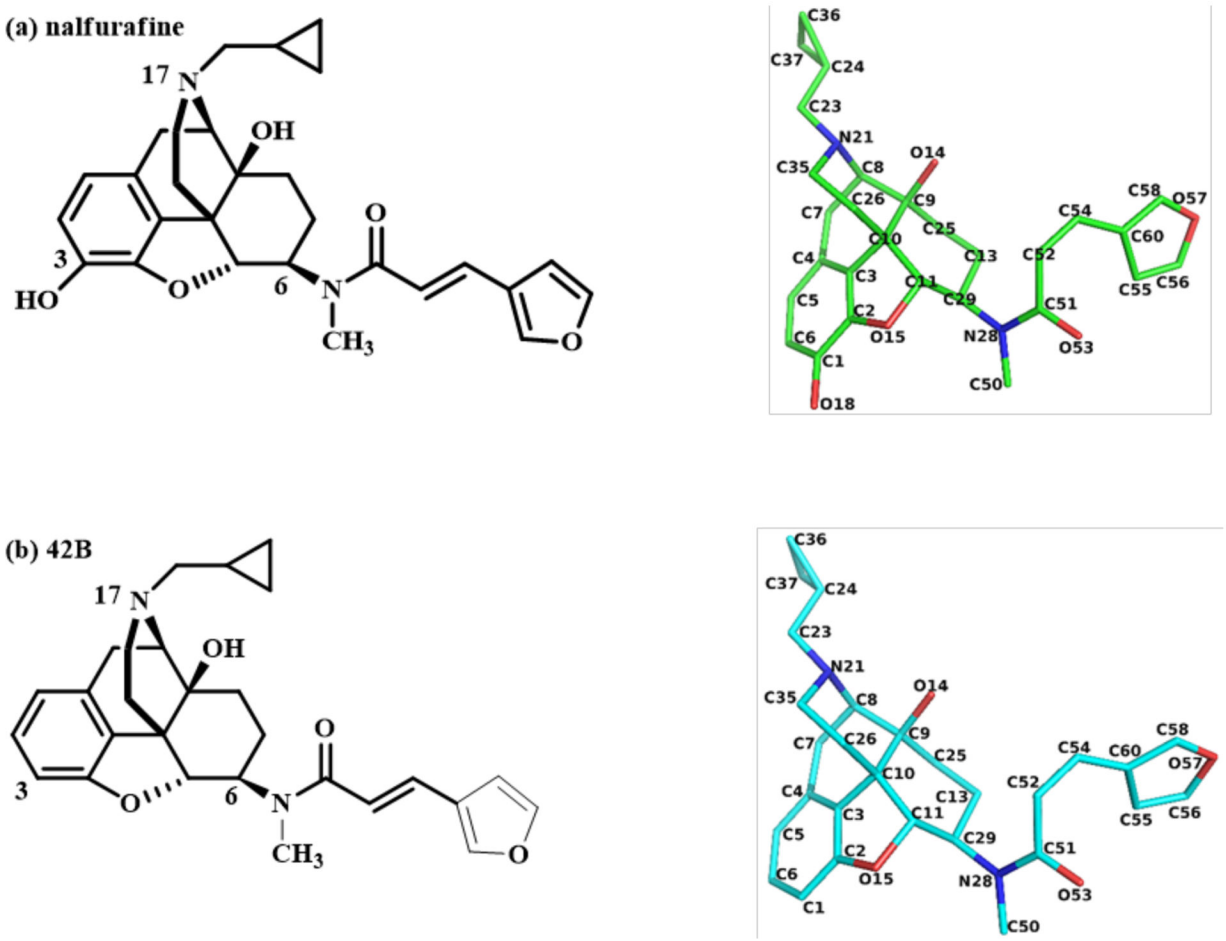

Figure 1.
The chemical structures of nalfurafine (a) and 42B (b) with atom notations.

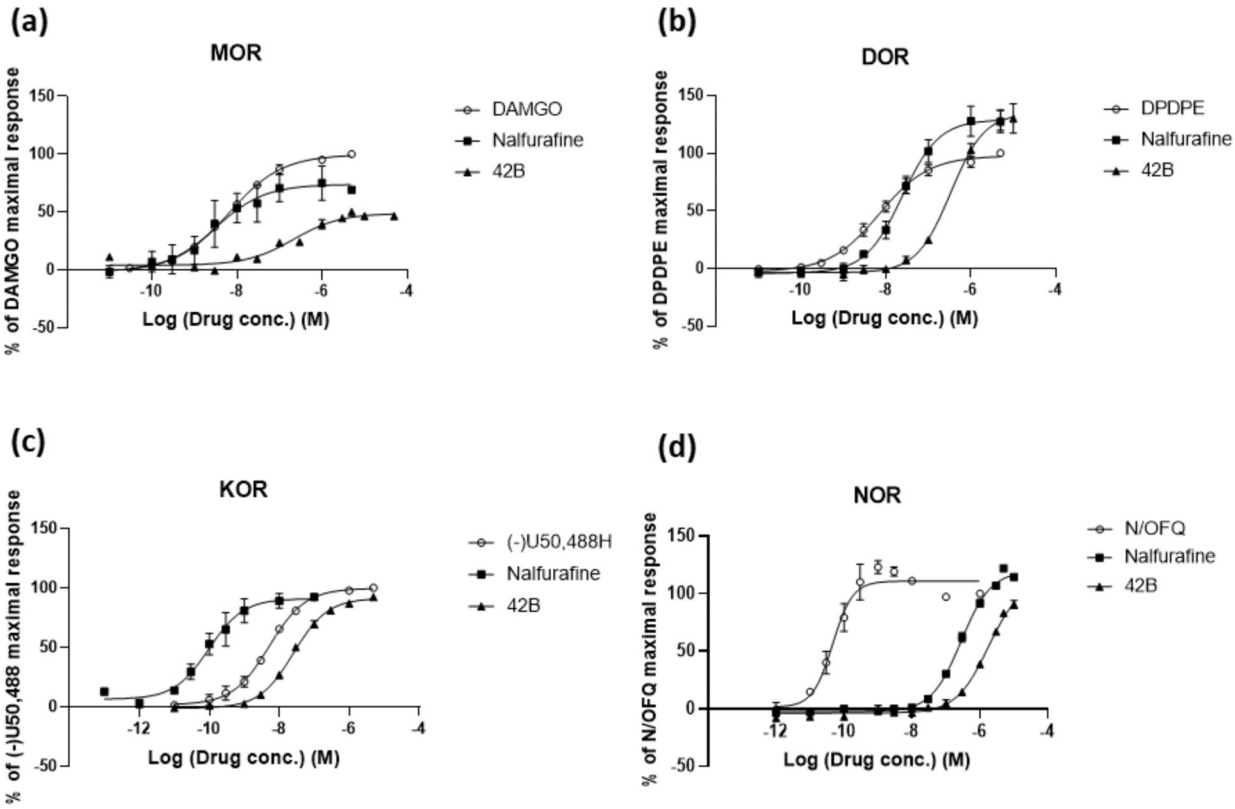

Figure 2. Stimulation of [^{35}S]GTPγS binding to membranes of CHO cells stably transfected with the MOR, DOR, KOR or NOR by nalfurafine, 42B, and a reference full agonist for each receptor.

[^{35}S]GTPγS binding to membranes was performed with various concentrations of each compound in 50 mM Tris buffer (pH 7.4) in the presence of 50 mM (for the MOR, DOR or NOR) or 100 mM NaCl (for the KOR), 5 mM $MgCl_2$, 15 μM GDP, 1 mM EDTA with ~10 μg membrane proteins and ~80 pM [^{35}S]GTPγS at 30°C for 60 min. Nonspecific binding, determined using 10 μM cold GTPγS, was ~500 dpm. Data were normalized and expressed as percentage of the maximal [^{35}S]GTPγS binding of the respective reference full agonist. Basal [^{35}S]GTPγS binding in the absence of added compounds was ~2000 dpm and maximal binding (with basal binding subtracted) was ~2500 dpm for the MOR and DOR, ~5000 dpm for the KOR, ~1500 dpm for the NOR. Each value represents the mean ± SEM of at least three independent experiments performed in duplicate. EC_{50} values and maximal responses are shown in Table 1. Each value is mean ± SEM (n=3–4).

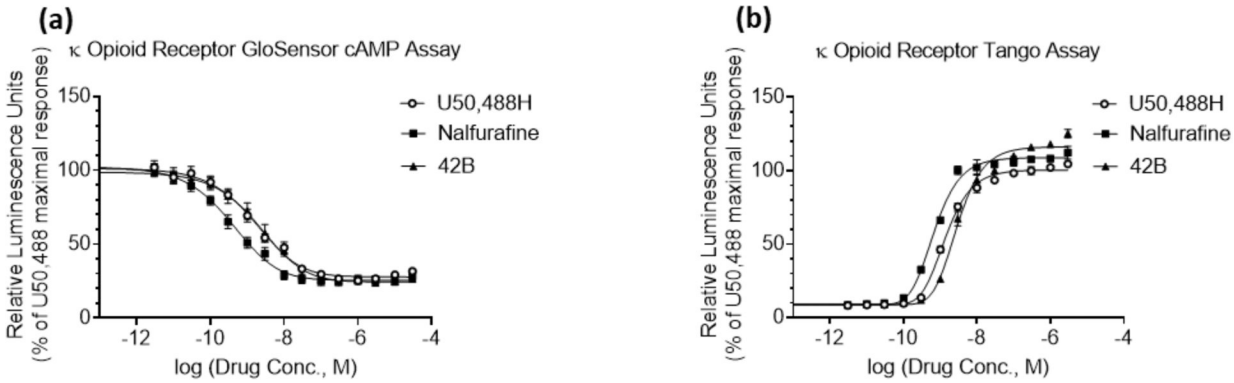

Figure 3. KOR agonists-induced G protein activation (GloSensor cAMP assay) and β-arrestin recruitment (Tango assay).

GloSensor cAMP and Tango assays were performed by PDSP of the NIMH, which is directed by Dr. Bryan Roth of University of North Carolina (Web site: https://pdsp.unc.edu/ims/investigator/web/). EC_{50} values and maximal responses are shown in Table 2. Each value is mean ± SEM (n=3).

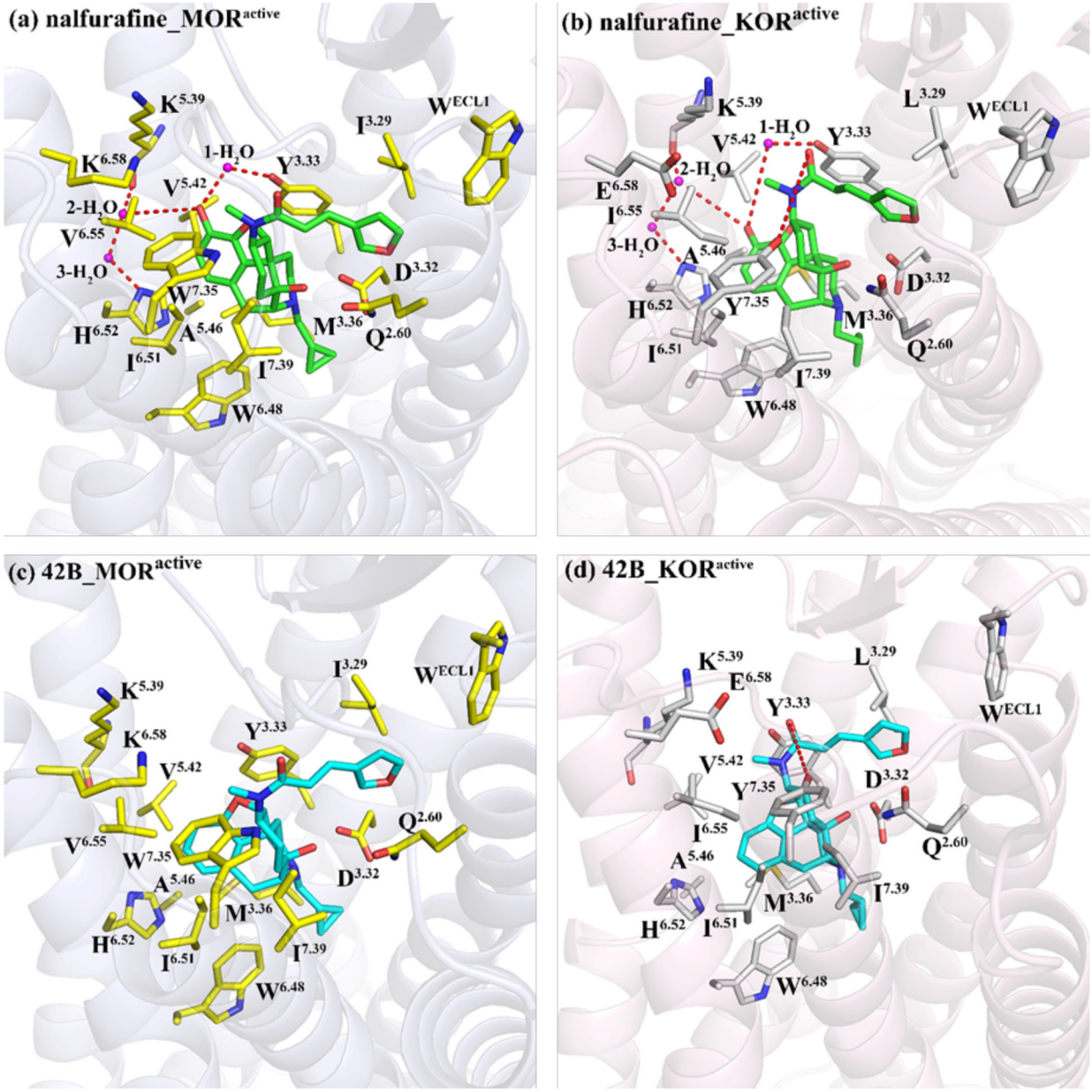

Figure 4. Binding poses of nalfurafine and 42B with the active MOR and KOR from molecular docking studies.

(a) nalfurafine_MOR^{active}, (b) nalfurafine_KOR^{active}, (c) 42B_MOR^{active}, (d) 42B_KOR^{active}. The MOR and KOR shown as cartoon models in light-blue and light-pink, respectively. Nalfurafine, 42B, and key amino acid residues shown as stick models. Carbon atoms: nalfurafine in green; 42B in cyan; key amino acid residues of the MOR in yellow, KOR in gray. The red dashed line represented possible hydrogen bonds. The water molecules shown as sphere models in magenta.

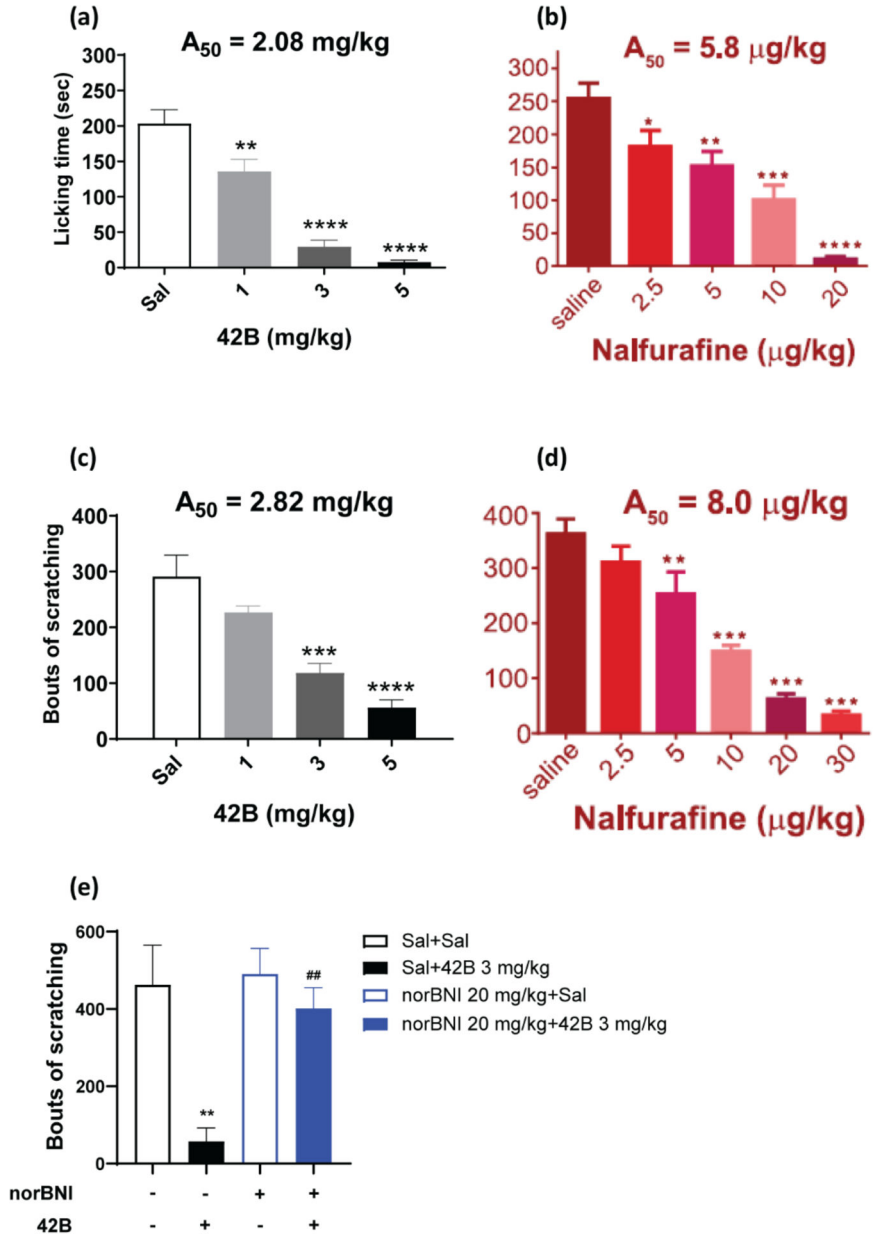

Figure 5. 42B produced antinociceptive and anti-scratching effects in mice, like nalfurafine.

(a) and (b) 42B inhibited formalin-induced pain behaviors in mice like nalfurafine. Saline or one of several doses of 42B or nalfurafine was injected (s.c.) 5 min before formalin and the amount of time the animal spent licking the injected paw was counted for 20 min starting at 15 min after formalin injection. A_{50} doses were determined as described[11]. Data were analyzed using one-way ANOVA followed by Dunnett's *post-hoc* test. Results of one-way ANOVA are: 42B, $F(3,24) = 41.63$, $p < 0.001$; nalfurafine, $F(4, 41) = 18.65$, $p < 0.0001$. Significance levels are $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$, compared to saline control, by Dunnett's *post-hoc* test (mean ± SEM, n = 6–10 animals/group). Data on nalfurafine were from Liu, et al.[11] and are shown for comparison.

(c) and (d) 42B induced inhibition of scratching behavior induced by compound 48/80, like nalfurafine. Saline or one of the different doses of 42B or nalfurafine was injected (s.c.) 20 min before compound 48/80 and the bouts of scratching were counted for 30 min. A_{50} doses were determined as described previously[11]. Data were analyzed using one-way ANOVA followed by Dunnett's *post-hoc* test. Results of one-way ANOVA were: 42B, $F(3,28) = 11.68$, $p < 0.001$; nalfurafine, $F(5,44) = 31.04$, $p < 0.0001$. Significance levels are $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$, compared to saline control by Dunnett's *post-hoc* test (mean ± SEM, n = 6–12 animals/group). Data on nalfurafine were from Liu, et al.[11] and are shown for comparison.

(e) The selective KOR antagonist norBNI blocked anti-scratching effects of 42B in compound 48/80 scratching test. Saline or norBNI was injected (20 mg/kg, i.p.) 20 h before saline or 42B, 20 min later compound 48/80 was injected and the bouts of scratching were counted for 30 min. Data were analyzed with two-way ANOVA followed by Sidak's *post-hoc* test. Results of two-way ANOVA showed a significant main effects of 42B [$F(1,21) = 13.26$, $p < 0.01$], a significant main effects of norBNI [$F(1,21) = 7.47$, $p < 0.05$] and a significant interaction [$F(1,21) = 5.39$, $p < 0.05$]. Significance levels are $^{**}p < 0.01$, compared to saline+saline group; $^{\#\#}p < 0.01$, compared to saline+42B group, by Sidak's *post-hoc* test (mean ± SEM, n = 5–8 animals/group).

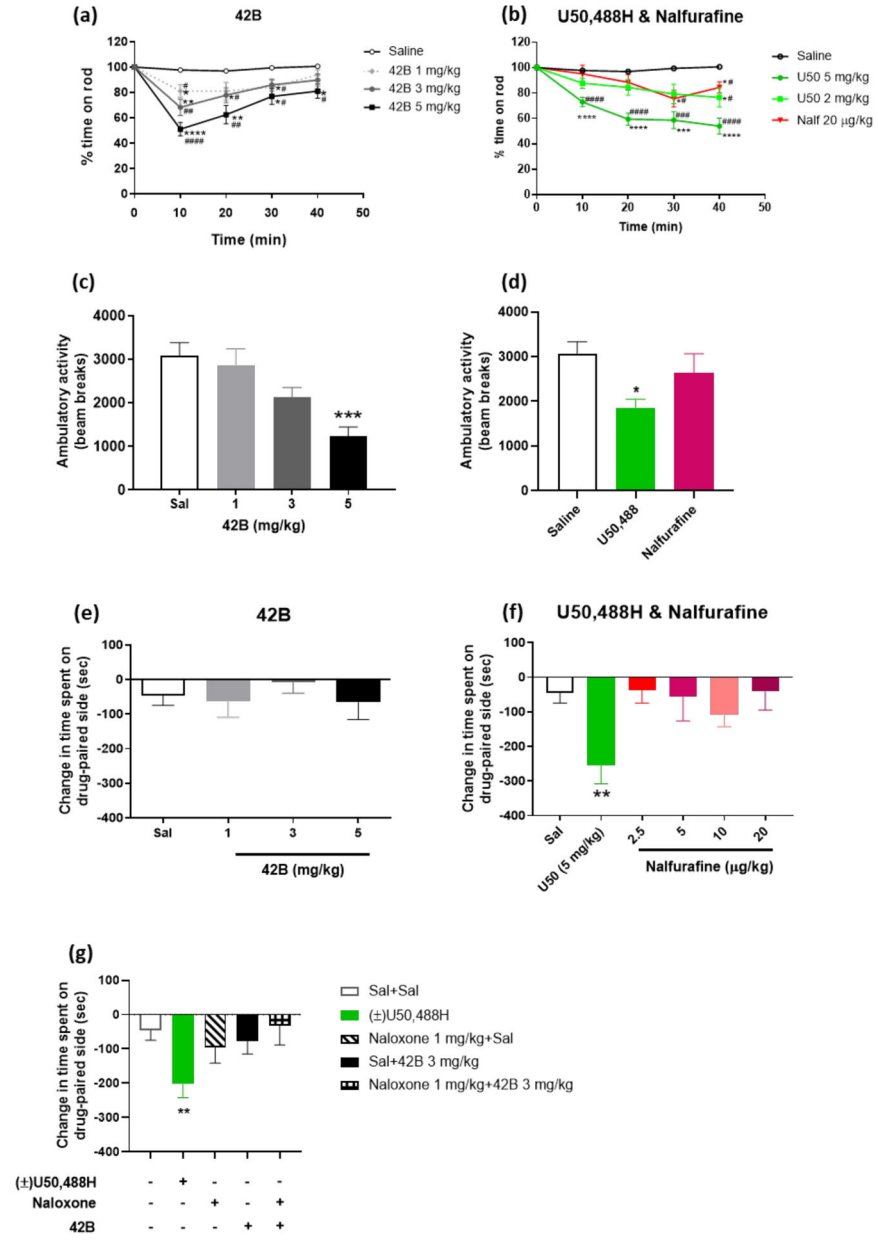

Figure 6. Comparison of 42B and nalfurafine in KOR agonist-induced adverse behaviors: motor incoordination, sedation, and conditioned place aversion (CPA) in mice.

(a) and (b) unlike nalfurafine, 42B caused motor incoordination in the rotarod test in mice. After training the previous day, mice were injected s.c. with saline, U50,488H (2, 5 mg/kg), 42B (1, 3, 5 mg/kg) or nalfurafine (20 μg/kg) and tested on the rotarods 10, 20, 30, and 40 min after injection. The time each stayed on the rods was recorded and normalized against the baseline. Data were analyzed with two-way ANOVA followed by Dunnett's *post-hoc* test (mean ± SEM, n= 9–12/group). 42B: Results of two-way ANOVA showed a significant main effect of treatment [$F(3,36) = 10.48$, $p < 0.0001$], a significant main effect of time [$F(4,144) = 32.33$, $p < 0.0001$] and a significant interaction [$F(12,199) = 4.65$, $p < 0.0001$]. Nalfurafine: Results of two-way ANOVA showed a significant main effect of treatment [$F(3,36) = 15.43$, $p < 0.0001$], a significant main effect of time [$F(4,144) = 24.14$, $p < 0.0001$]

and a significant interaction [$F\ (12,199) = 5.46$, $p < 0.0001$]. *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$, compared with 0 min of each group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, ####$p < 0.0001$, compared with the saline group at the same time, by Dunnett's *post-hoc* test. Data on nalfurafine and 5 mg/kg U50,488H were from Liu, et al.[11] and are shown for comparison.

(c) and (d) Unlike nalfurafine, 42B caused inhibition of novelty-induced locomotor activity in mice. Mice were treated s.c. with saline, 42B (1, 3, 5 mg/kg), U50,488H (5 mg/kg), or nalfurafine (20 μg/kg) (2.5x A_{50} values in the anti-scratching test) and locomotor activities were monitored. Cumulative data between 0–30 min post-injection are shown here. Each value represents mean ± SEM (n = 8–14). (c) Ambulatory activity of 42B: ***$p < 0.001$, compared with the saline group by one-way ANOVA [$F\ (3,34) = 7.59$, $p < 0.001$] followed by Dunnett's *post-hoc* test. (d) Ambulatory activity of U50,488H and nalfurafine: *$p < 0.05$, compared with the saline group by one-way ANOVA [$F\ (2,27) = 6.254$, $p < 0.01$] followed by Dunnett's *post-hoc* test. Data on nalfurafine and 5 mg/kg U50,488H were from Liu, et al.[11] and are shown for comparison.

(e) and (f) 42B did not cause CPA in mice, like nalfurafine. On Day 0, mice were subject to pre-test. On Days 1–6, Mice were injected with saline or one of the various doses of U50,488H, 42B or nalfurafine before each 30-min conditioning session (1 saline session and 1 drug session/day) for 6 days. On Day 7 (post-test), the length of time the animal spent on the drug-paired side was measured. The graph shows the time the animal spent during the post-test subtracting the amount of time spent during the pre-test. Data were analyzed with one-way ANOVA followed by Dunnett's *post-hoc* test (for the 42B and nalfurafine experiments) and unpaired t-test (for U50,488H) (mean ± SEM, n = 9–10/group). 42B, $F(3,36) = 0.45$, $p > 0.05$; nalfurafine, $F(4,47) = 0.38$, $p > 0.05$. Significance levels are **$p < 0.01$, compared to saline control by unpaired t-test. Data on nalfurafine and U50,488H were from Liu, et al.[11] and are shown for comparison.

(g) Naloxone at a dose (1 mg/kg) selective for the MOR did not alter lack of CPA of 42B. On Day 0, mice were subject to pre-test. On Days 1–6, saline or naloxone was injected 25 min before saline or 42B, then 30-min conditioning session began immediately (1 saline session and 1 drug session/day) for 6 days. U50,488H alone was used as the CPA positive control. On Day 7 (post-test), the length of time the animal spent on the drug-paired side was measured. The graph shows the time the animal spent during the post-test subtracting the amount of time spent during the pre-test. Data were analyzed with two-way ANOVA followed by Sidak's *post-hoc* test (for the 42B-naloxone experiments) and unpaired t-test (for U50,488H) (mean ± SEM, n = 10/group). Results of two-way ANOVA showed no significant main effects of 42B [$F(1,36) = 0.13$, $p > 0.05$], naloxone [$F(1,36) = 0.004$, $p > 0.05$] or interaction [$F\ (1,36) = 1.16$, $p > 0.05$]. Significance levels are **$p < 0.01$, compared to saline + saline by unpaired t-test.

Table 1.

EC_{50} values and maximal effects of nalfurafine and 42B in stimulating [^{35}S]GTPγS binding to membranes of CHO cells stably expressing the MOR, DOR, KOR or NOR. DAMGO, DPDPE, (−)U50,488H, and N/OFQ were used as the reference full agonists for the MOR, DOR, KOR and NOR, respectively. Data were derived from the curves in Figure 2. Mean ± SEM (n=3–4)

	MOR		DOR		KOR		NOR	
	EC50	Maximal Effect	EC50	Maximal Effect	EC50	Maximal Effect	EC50	Maximal Effect
	nM	%	nM	%	nM	%	nM	%
DAMGO	6.30 ± 0.43	99.42 ± 1.07						
DPDPE			7.06 ± 0.76	97.67 ± 1.92				
(−)U50,488H					5.12 ± 0.37	99.65 ± 1.24		
Nociceptin / OFQ							0.046 ± 0.007	110.8 ± 2.8
Nalfurafine	3.11 ± 0.63	73.88 ± 2.93	24.22 ± 2.56	129.1 ± 3.2	0.097 ± 0.018	90.90 ± 3.25	279.7 ± 17.7	119.7 ± 2.2
42B	214.9 ± 50.4	48.87 ± 2.45	334.5 ± 35.1	134.7 ± 3.4	25.56 ± 1.50	91.34 ± 1.15	1760 ± 284	110.9 ± 7.4

Table 2.

Summary of EC_{50} and E_{max} values of KOR agonists for G protein activation as measured by GloSensor cAMP assay and G protein independent β-arrestin recruitment as measured by Tango assay. The prototypic selective KOR agonist U50,488H was used as the balanced agonist. E_{max} values in the GloSensor assay denote the maximal degree of inhibition. Data were derived from the curves in Figure 3. Mean ± SEM (n = 3). EC_{50} and E_{max} data of GloSensor cAMP and Tango assays were analyzed with one-way ANOVA followed by Tukey's *post-hoc* test, respectively. Results of one-way ANOVA are: E_{max} of GloSensor cAMP assay: $F(2,6) = 2.77$, $p > 0.05$; EC_{50} of GloSensor cAMP assay: $F(2,6) = 22.91$, $p < 0.01$; E_{max} of Tango assay: $F(2,6) = 28.32$, $p < 0.001$;

	GloSensor cAMP assay			Tango assay		
	EC_{50}(nM)	**E_{max}(%)**	**N**	**EC_{50}(nM)**	**E_{max}**	**N**
U50,488H	1.61±0.19	72.95±0.86	3	1.43±0.13	99.93±0.07	3
Nalfurafine	0.17±0.04 **	69.82±1.53	3	0.74±0.07	108.0±2.6 *	3
42B	0.82±0.18 *	74.40±1.68	3	3.22±0.42 **,##	116.8±1.0 ***,#	3

EC_{50} of Tango assay: $F(2,6) = 24.80$, $p < 0.01$. Significance levels are

* $p < 0.05$,

** $p < 0.01$,

*** $p < 0.001$, compared with U50,488H in the same assay;

\# $p < 0.05$,

\#\# $p < 0.01$, compared with nalfurafine in the same assay, by Tukey's *post-hoc* test.

Table 3.

log RA_{i-G}, log RA_{i-b}, log (RA_{i-G}/RA_{i-b}), bias factors to G protein signaling and probability values of KOR agonists. The parameters were calculated from the same agonist concentration-response curves that were used to calculate EC_{50} and E_{max} values (Figure 3, Table 2) using the method described by Ehlert and colleagues[25–27]. The prototypic selective KOR agonist U50,488H was designated as the "balanced" or standard reference ligand. The bias factor to G protein signaling for a given ligand is defined as the ratio of RA_{i-G} divided by RA_{i-b}. Mean ± SEM (n = 3).

	Log RA_{i-G}	**Log RA_{i-b}**	$\text{Log}\frac{RA_{i-g}}{RA_{i-b}}$	**G Biased factor (95% CI)**	(***p* value**)*
U50,488H	0	0	0	1	
Nalfurafine	0.94±0.14	0.28±0.02	0.65±0.40	4.49 (1.79–11.1)	0.010
42B	0.17±0.06	−0.28±0.03	0.46±0.20	2.85 (1.82–4.48)	0.003

G, G proteins; b, β-arrestin; CI: confidence interval.

*compared to bias factor of 1 by *t* test.