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Article

Evaluation of the Intracellular Signaling Activities of κ -Opioid Receptor Agonists, Nalfurafine Analogs; Focusing on the Selectivity of G-Protein- and β -Arrestin-Mediated Pathways

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Abstract: Opioid receptors (ORs) are classified into three types (μ , δ , and κ), and opioid analgesics are mainly mediated by μ OR activation; however, their use is sometimes restricted by unfavorable effects. The selective κ OR agonist nalfurafine was initially developed as an analgesic, but its indication was changed because of the narrow safety margin. The activation of ORs mainly induces two intracellular signaling pathways: a G-protein-mediated pathway and a β -arrestin-mediated pathway. Recently, the expectations for κ OR analgesics that selectively activate these pathways have increased; however, the structural properties required for the selectivity of nalfurafine are still unknown. Therefore, we evaluated the partial structures of nalfurafine that are necessary for the selectivity of these two pathways. We assayed the properties of nalfurafine and six nalfurafine analogs (SYKs) using cells stably expressing κ ORs. The SYKs activated κ ORs in a concentration-dependent manner with higher EC₅₀ values than nalfurafine. Upon bias factor assessment, only SYK-309 (possessing the 3S-hydroxy group) showed higher selectivity of G-protein-mediated signaling activities than nalfurafine, suggesting the direction of the 3S-hydroxy group may affect the β -arrestin-mediated pathway. In conclusion, nalfurafine analogs having a 3S-hydroxy group, such as SYK-309, could be considered G-protein-biased κ OR agonists.

Keywords: analgesic; κ -opioid receptor; G-protein-biased agonists; nalfurafine; bias factor

1. Introduction

Opioid analgesics such as morphine are widely used to improve various forms of pain, including chronic pain, perioperative pain, and cancer pain [1–4]. ORs belong to the G-protein-coupled receptor (GPCR) family [5] and are classified into three subtypes (μ ORs, δ ORs, and κ ORs), where each type of OR is associated with analgesic effects. Current opioid analgesics mainly bind to μ OR to exert their analgesic effects [6]. The typical

OR agonists, such as morphine, activate μ ORs and show strong antinociceptive effects. Therefore, μ OR agonists have been used as effective analgesics to alleviate acute and severe chronic pain. However, their use is sometimes restricted by unfavorable side effects, such as opioid dependence, tolerance, constipation, itch, or respiratory depression [7–9], while increasing opioid misuse and opioid-associated mortality have also been observed [10]. In fact, approximately 20% of patients with chronic pain using opioids were abusing them [11]. Thus, the investigation of novel opioids as an adjuvant to μ OR-targeting analgesics, which have sufficient analgesic effects and lower side effects, is necessary.

Besides selective μ OR agonists, several selective δ OR or κ OR compounds have been investigated [12,13]. In particular, κ ORs are ubiquitously and widely expressed throughout the central nervous system and activated by opioid peptides, such as dynorphins [14–17]. The κ OR/dynorphin system is linked to reducing pruritus as a potential therapeutic action [18]. In practice, it has been proven that κ OR agonists were efficacious in treating intractable itch or pruritus [18–21]. Moreover, like other ORs, activating the κ OR promotes antinociception, and κ OR agonists produce an analgesic effect without respiratory depression that is often seen with μ OR agonists [22]. Therefore, κ OR agonists have been investigated as alternatives to μ OR agonists for pain treatment [1]; however, their therapeutic potential is limited by negative side effects such as sedation, motor incoordination, dysphoria, and psychotomimesis [23–26].

After the ligand conjugates to ORs, including κ ORs, the intracellular signaling from the ORs is transmitted through two major pathways: one is a G-protein-mediated pathway induced by decreasing the intracellular cAMP levels and is required for analgesia, and the other is a β -arrestin-mediated pathway that affects the recruitment of β -arrestin and is associated with unfavorable side effects [27–33]. Moreover, there is evidence which calls into question the concept of developing G-protein-biased μ OR agonists as a strategy for developing safer opioid analgesic drugs [34]. Thus, there is no definite conclusion that the G-protein-mediated pathway is “good”, and the β -arrestin-mediated pathway is “bad”, which is still under discussion. However, to develop safer and more effective opioid analgesics, investigating G-protein-biased agonists or those with a pharmacological profile that prioritize activation of the G-protein-mediated pathway over the β -arrestin-mediated pathway may be desirable [35,36]. From these viewpoints, some molecules were investigated and indicated as G-protein-biased agonists [37,38]. Among them, oliceridine was evaluated by intravenous administration in clinical studies and was approved as the first G-protein-biased μ OR agonist that could be prescribed [39]. Subsequently, the expectations in researching the selective activation of G-protein- or β -arrestin-mediated pathways via κ ORs have also increased. In fact, the analgesic therapeutic outcomes of G-protein-mediated signaling through κ ORs have been investigated in some studies [40–42]. These results suggest that G-protein-mediated signaling after binding to the κ OR could be involved in analgesic effects. In contrast, β -arrestin-mediated signaling could be related to unfavorable effects [40]. Thus, κ OR agonists with a pharmacological profile of selectively activating the G-protein-mediated pathway over the β -arrestin-mediated pathway may be promising targets for more effective analgesics with fewer unfavorable effects [35,36,43].

Nalfurafine was discovered in 1998 by Dr. Hiroshi Nagase in Japan and was found to promote antinociception without aversion [44], acting as a selective κ OR agonist [45]. Nalfurafine also reduced pruritus [46] and was launched as nalfurafine hydrochloride (Remitch[®]), an antipruritic drug in Japan [47,48]. Nalfurafine is the first and, currently, only available selective κ OR agonist for the treatment of intractable pruritus suffered by patients on hemodialysis [49,50]. In addition, κ OR agonists, including nalfurafine, may not be addictive because they do not induce euphoria, nor do they promote increases in dopamine release as abused drugs do [51–53]. In fact, nalfurafine, initially, was developed as an analgesic; however, its indication was changed from an analgesic to an antipruritic drug because the analgesic and sedative effects were not well separated [47,54]. Even now, no published clinical evidence indicates the efficacy of nalfurafine for the treatment of

pain, and nalfurafine is no longer approved as an analgesic. Therefore, in our previous study, we investigated the affinity of nalfurafine and its analogs for κ ORs using binding assays and revealed the structure–activity relationship (SAR) of nalfurafine for κ ORs [55]. The differences in affinity of each nalfurafine analog for κ ORs were observed; however, how the structural properties of nalfurafine and its analogs affect the selectivity of the G-protein- and β -arrestin-mediated pathways remains unknown. Accordingly, to mitigate the negative effects of κ OR agonists, especially nalfurafine analogs, and effectively utilize them for the treatment of pain, further investigation into the relationship between the structural features of nalfurafine and G-protein-/ β -arrestin-mediated signaling activities is required.

Therefore, in the present study, we aimed to evaluate the partial structures of nalfurafine and six nalfurafine analogs (SYK-160, -186, -245, -308, -309, and -406; Figure 1) that are necessary for the selectivity of G-protein- and β -arrestin-mediated pathways. We used the CellKey™, GloSensor® cAMP, and PathHunter® β -arrestin recruitment assays in cells stably expressing κ ORs. We estimated the G-protein-biased factor of each nalfurafine analog in comparison with nalfurafine to contribute to the development of more useful opioid analgesics.

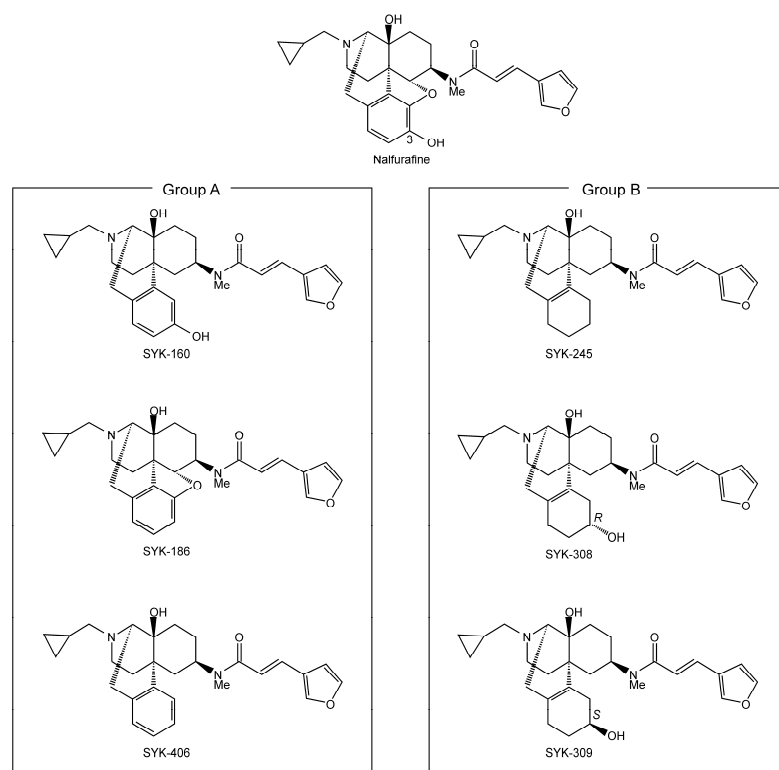


Figure 1. Molecular structures of nalfurafine and six nalfurafine analogs. Nalfurafine analogs were divided into two groups according to their structural characteristics. Group A (SYK-160, -186, and -406) includes nalfurafine analogs with a maintained benzene ring. Group B (SYK-245, -308, and -309) includes nalfurafine analogs with a cyclohexene ring converted from the benzene ring.

2. Results

2.1. The Effects of Nalfurafine and Nalfurafine Analogs on the Functions of κ ORs Using the CellKey™ System

We evaluated the effects of nalfurafine and six nalfurafine analogs (SYK-160, -186, -245, -308, -309, and -406) on κ OR activities using the CellKey™ system in HEK293 cells stably expressing Halotag®- κ OR/pGS22F. The CellKey™ system detects the activities of GPCRs, including κ ORs, as changes in cellular impedance [56]. The E_{max} and EC_{50} values

were calculated, and we compared them between nalfurafine and its analogs. Nalfurafine and each nalfurafine analog activated κ ORs in a concentration-dependent manner; however, none of the six analogs exhibited E_{\max} (%) values higher than those of nalfurafine (Figure 2). In contrast, the $\log EC_{50}$ (M) values of SYK-186 (removed the 3-hydroxy group from nalfurafine in Group A), -245 (removed the 3-hydroxy group and 4,5-ether bridge from nalfurafine, and converted the benzene ring to a cyclohexene ring in Group B), -308 (removed the 4,5-ether bridge from nalfurafine, converted the benzene ring to a cyclohexene ring, and added a 3*R*-hydroxy group in Group B), and -406 (removed the 3-hydroxy group and 4,5-ether bridge from nalfurafine in Group A) were significantly increased compared to those of nalfurafine (Table 1).

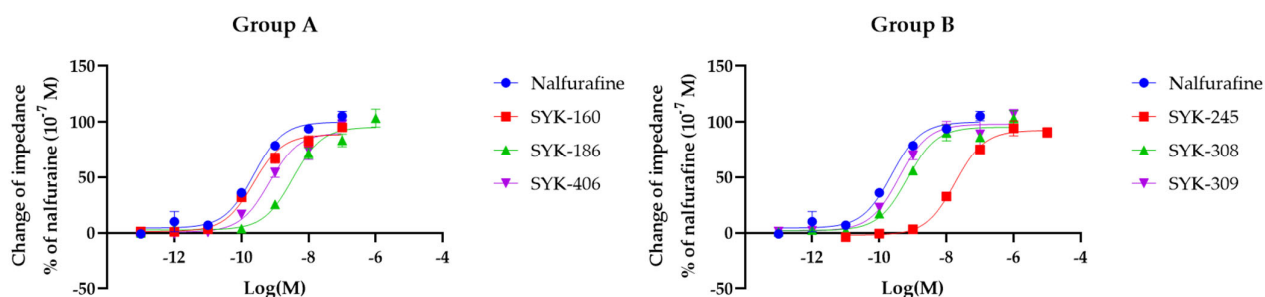


Figure 2. Effect of nalfurafine and six nalfurafine analogs on κ ORs observed using the CellKey™ system. The cells expressing κ ORs were treated with nalfurafine (positive control) and six nalfurafine analogs (Group A: SYK-160, -186, -406; Group B: SYK-245, -308, -309) at concentrations of 10^{-13} – 10^{-5} M, and changes in impedance (ΔZ_{iec}) were measured using the CellKey™ system. Concentration-response curves were prepared by calculating ΔZ_{iec} relative to the data obtained for the positive control: 10^{-7} M nalfurafine. All data points are presented as means \pm standard error of the mean (SEM) ($n = 3$ – 6).

Table 1. E_{\max} and $\log EC_{50}$ values for nalfurafine and six nalfurafine analogs in the CellKey, GloSensor cAMP, and PathHunter assays for κ ORs.

	CellKey Assay		GloSensor cAMP Assay		PathHunter Assay		
	Compounds	Log EC_{50} (M)	E_{\max} (%)	Log EC_{50} (M)	E_{\max} (%)	Log EC_{50} (M)	E_{\max} (%)
	Nalfurafine	-9.64 ± 0.10	100.00 ± 3.09	-10.09 ± 0.06	100.00 ± 1.74	-9.27 ± 0.19	100.00 ± 7.43
Group A	SYK-160	-9.64 ± 0.90	88.74 ± 2.55	-9.47 ± 0.10 ***	96.51 ± 1.97	-8.83 ± 0.12	107.27 ± 4.64
	SYK-186	-8.47 ± 0.10 ***	95.42 ± 3.18	-8.67 ± 0.09 ***	101.14 ± 2.74	-7.56 ± 0.11 ***	81.76 ± 3.83
	SYK-406	-9.17 ± 0.12 **	90.33 ± 3.94	-9.52 ± 0.12 ***	104.26 ± 3.44	-8.64 ± 0.09 **	87.47 ± 2.77
Group B	SYK-245	-7.74 ± 0.07 ***	92.51 ± 2.36	-7.42 ± 0.11 ***	103.21 ± 3.60	-6.91 ± 0.09 ***	88.78 ± 3.35
	SYK-308	-9.16 ± 0.09 **	95.41 ± 2.57	-9.20 ± 0.10 ***	98.16 ± 3.21	-8.20 ± 0.14 ***	93.29 ± 5.51
	SYK-309	-9.41 ± 0.12	98.06 ± 3.54	-9.13 ± 0.07 ***	102.03 ± 2.16	-7.81 ± 0.08 ***	85.92 ± 3.08

Nalfurafine was used as the positive control. E_{\max} (%) and $\log EC_{50}$ (M) values (means \pm SEM) were calculated according to the results shown in Figures 2–4. Statistical comparisons were made using GraphPad Prism 9 software and are expressed as means \pm SEM. Differences between the means were analyzed with one-way analysis of variance (ANOVA) or *t*-tests. One-way ANOVA was followed by Bonferroni post hoc analysis. Significant levels are ** $p < 0.01$ and *** $p < 0.001$ compared with nalfurafine. The number of samples of EC_{50} and E_{\max} are indicated as follows: $n = 3$ – 6 (the CellKey™ assay), $n = 3$ – 9 (the GloSensor® cAMP assay), and $n = 5$ – 8 (the PathHunter® recruitment assay).

2.2. The Effects of Nalfurafine Analogs on the Intracellular cAMP Levels Evaluated Using the GloSensor® cAMP Assay

We evaluated the actions of test compounds on κ OR-induced G-protein signaling by measuring the intracellular cAMP levels using HEK293 cells stably expressing Halotag®- κ OR/pGS22F. The E_{\max} and EC_{50} values were calculated using nalfurafine as a positive

control, and the six nalfurafine analogs caused a concentration-dependent decrease in cAMP levels (Figure 3). In detail, there were no nalfurafine analogs that showed E_{max} (%) values higher than nalfurafine, and the $\log EC_{50}$ (M) values of all nalfurafine analogs were significantly increased compared to those of nalfurafine (Table 1). These results suggested that the six nalfurafine analogs used in this study showed lower G-protein-mediated signaling activities than nalfurafine.

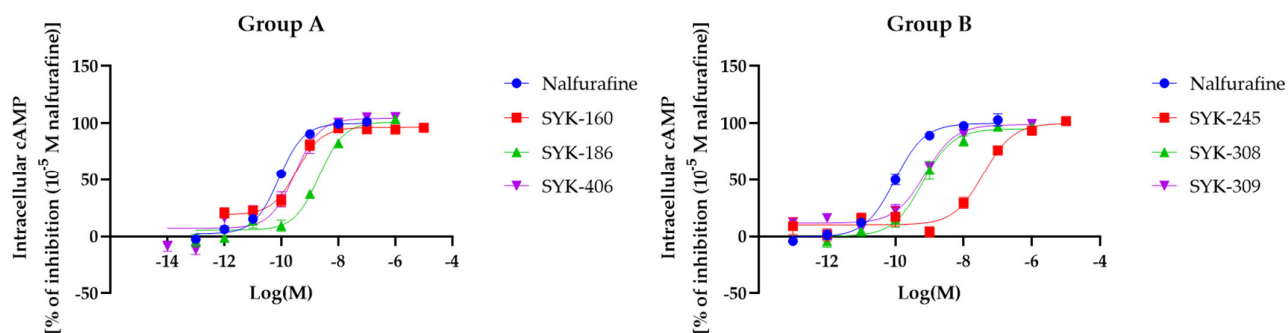


Figure 3. Changes in intracellular cAMP levels induced by nalfurafine (positive control) and six nalfurafine analogs (Group A: SYK-160, -186, -406; Group B: SYK-245, -308, -309). Cells expressing κ ORs were treated with the listed compounds (10^{-14} – 10^{-5} M), and intracellular cAMP levels were measured with the GloSensor[®] cAMP assay. Concentration-response curves were prepared by calculating cAMP levels relative to the data obtained with 10^{-7} M nalfurafine. Data are presented as means \pm SEM ($n = 3$ –9).

2.3. Effects of Nalfurafine Analogs on β -Arrestin Recruitment Using the PathHunter[®] Recruitment Assay

To evaluate the actions of nalfurafine and six nalfurafine analogs on κ OR-induced β -arrestin signaling, the PathHunter[®] β -arrestin recruitment assay was performed using U2OS cells stably expressing κ ORs (DiscoverX, Fremont, CA, USA). Nalfurafine and each nalfurafine analog induced β -arrestin recruitment to κ ORs in a concentration-dependent manner (Figure 4). We calculated the E_{max} and EC_{50} values of these compounds, and no nalfurafine analogs showed E_{max} (%) values higher than nalfurafine. However, the $\log EC_{50}$ (M) values of all nalfurafine analogs were increased, except SYK-160, which has a non-significant increase compared to those of nalfurafine.

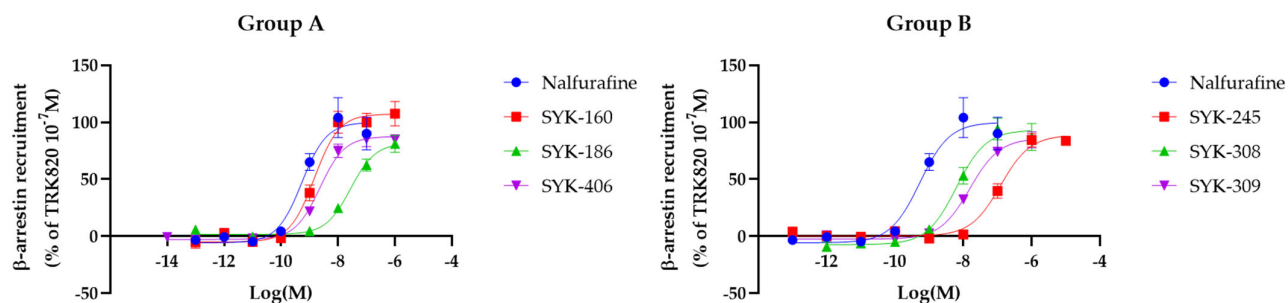


Figure 4. Levels of β -arrestin recruitment through κ ORs induced by nalfurafine and six nalfurafine analogs. We performed a PathHunter[®] β -arrestin assay in cells expressing κ ORs by treatment with nalfurafine (positive control) and the six nalfurafine analogs (Group A: SYK-160, -186, -406; Group B: SYK-245, -308, -309) at concentrations of 10^{-14} – 10^{-5} M. Concentration-response curves were prepared by calculating intracellular β -arrestin levels relative to the data obtained for nalfurafine (positive control: 10^{-7} M). All data points are presented as means \pm SEM ($n = 5$ –8).

2.4. The Selectivity of G-Protein- and β -Arrestin-Mediated Pathways (G-Protein-Biased Factors)

The selectivity of G-protein- and β -arrestin-mediated pathways is indicated as a G-protein-biased factor representing the ratio of the value for G-protein signaling divided by that of β -arrestin signaling [57]. A biased factor > 1 indicates a preference for G-protein-mediated signaling activities, whereas a biased factor < 1 indicates a preference for the recruitment of β -arrestin as β -arrestin-mediated signaling activities compared to the control compound [56]. Subsequently, we estimated the G-protein-biased factor of each nalfurafine analog compared to nalfurafine to identify G-protein-biased analogs. The present study calculated G-protein-biased factors using data from the GloSensor[®] cAMP assay (G-protein-mediated signaling) and PathHunter[®] recruitment assay (β -arrestin-mediated signaling). As shown in Table 2, the G-protein-biased ratio of SYK-309 was significantly higher than that of nalfurafine (mean \pm SEM: 4.46 ± 1.87 , $p = 0.0055$, Table 2). These results indicated that, compared to nalfurafine, SYK-309 was the only G-protein-biased κ OR agonist among the six nalfurafine analogs.

Table 2. G-protein-biased factors of nalfurafine analogs for G-protein and β -arrestin coupling.

Compounds	G-Protein-Biased Ratio (Mean \pm SEM)	<i>p</i> -Value
Nalfurafine	1	n.s.
SYK-160	0.36 ± 0.05	n.s.
SYK-186	1.47 ± 0.30	n.s.
SYK-406	0.89 ± 0.18	n.s.
SYK-245	0.35 ± 0.67	n.s.
SYK-308	1.83 ± 0.80	n.s.
SYK-309	$4.46 \pm 1.87^{**}$	0.0055

The parameters were calculated from the same agonist concentration-response curves used to estimate EC_{50} and E_{max} values in Figures 2 and 3, and in Table 1, using the method described by Ehlert and colleagues [58–60]. The prototype of the selective κ OR agonist, nalfurafine, was designated as a standard reference ligand. The bias factor of G-protein signaling for a given ligand is defined as the ratio of the intrinsic activity (RA_{i-G}) divided by RA_{i-b} . The G-protein-biased ratios (means \pm SEM) were calculated according to the results shown in Table 1, $** p < 0.01$, compared to bias factor of 1 by *t*-test.

3. Discussion

Here, we evaluated the effects of nalfurafine and six nalfurafine analogs (SYK-160, -186, -245, -308, -309, and -406) on κ OR-activated intracellular signaling using the CellKey[™], GloSensor[®] cAMP, and PathHunter[®] β -arrestin recruitment assays. Our results revealed that all tested compounds activated κ OR-mediated intracellular signaling in a concentration-dependent manner as full κ OR agonists. Furthermore, most of the EC_{50} values of these test compounds were higher than nalfurafine. In addition, similar results were obtained in our CellKey[™] assay and GloSensor[®] cAMP assay and their correlation seemed to be high. These results suggest that in an impedance assay using the CellKey[™] system, the results of impedance changes were reflected mostly with changes in cAMP levels, but not changes in β -arrestin activity. Our previous studies also showed a similar pattern to the present results [61,62]. Our previous study examined the binding affinity of six nalfurafine analogs (the same ones used in the present study) for κ ORs; nitrogen, with an *N*-cyclopropylmethyl substituent, and 6-amide side chains were indispensable for nalfurafine to bind to κ ORs, and the phenol ring (3-hydroxy group) was also important for increasing the κ OR binding affinity. Compared to our present study, the binding ability K_i (nM) value of each nalfurafine analog for κ ORs tended to correlate with the EC_{50} values of CellKey[™], cAMP, and β -arrestin recruitment assays [55]. Moreover, the results of one analog, SYK-309, indicated that there was a significant difference in the ratio of G-protein-mediated signaling to β -arrestin-mediated signaling in our present study.

The κ OR agonists have been proposed as antinociceptive drugs in humans [24,46,63,64]. These agonists can independently activate multiple signaling mechanisms, making it difficult to screen them using one assay [65]. For this reason, we used three assays in our present study. Schattauer S.S. et al. showed that nalfurafine was a G-protein-biased κ OR agonist compared to other κ OR agonists such as (-)-U50488H [41]. Nalfurafine exerted anti-scratch and analgesic effects without adverse events such as sedation, motor incoordination, or conditioned place aversion in mice [66]. Many investigations indicated that antinociception induced by κ OR agonists is caused by the G-protein-mediated pathway [67], whereas unfavorable effects are caused by the β -arrestin-mediated pathway [68–70], suggesting that G-protein-biased κ OR agonists could lead to the development of safer and more effective opioid analgesics. Indeed, nalfurafine was initially developed as an analgesic; however, the analgesic and sedative effects were not well separated at analgesic doses [47,54]. Taking the previous studies into consideration, we focused on nalfurafine and investigated the relationships between κ OR signaling selectivity and the structural features of nalfurafine using six nalfurafine-based analogs.

Among the nalfurafine analogs in Group A (maintained benzene ring), none had E_{\max} values that significantly exceeded those of nalfurafine in both G-protein- and β -arrestin-mediated signaling. In contrast, for G-protein-mediated signaling, SYK-160 (removed the 4,5-ether bridge), SYK-186 (removed the 3-hydroxy group), and SYK-406 (removed both the 4,5-ether bridge and 3-hydroxy group) caused significant increases in EC_{50} values. However, for β -arrestin-mediated signaling, the EC_{50} value of SYK-160 was not significantly changed, whereas those of SYK-186 and SYK-406 were significantly increased. Compared to nalfurafine, the G-protein-biased ratio of SYK-160 was decreased. Still, there was no significant change in the ratios of the Group A compounds (Table 2). Therefore, these data suggest that the 4,5-ether bridge and 3-hydroxy group on the benzene ring were important to activate both G-protein- and β -arrestin-mediated signaling.

Among Group B (converted benzene ring to cyclohexene ring), SYK-245 (removed both the 4,5-ether bridge and 3-hydroxy group) remarkably lost potency in both G-protein- and β -arrestin-mediated signaling compared to nalfurafine; the EC_{50} value increased significantly by ~100 times in the GloSensor[®] cAMP assay, whereas that in the PathHunter[®] recruitment assay increased significantly by ~1000 times (Table 1). Furthermore, the G-protein-biased ratio of SYK-245 tended to decrease; however, the change was not significant compared to nalfurafine (Table 2). It is notable that both SYK-308 and -309 significantly increased G-protein- and β -arrestin-mediated signaling compared to SYK-245, as seen in the log EC_{50} value in GloSensor[®] cAMP assay: SYK-245 vs. SYK-308 (-7.42 ± 0.11 vs. -9.20 ± 0.10 , $p = 0.0001$), SYK-245 vs. SYK-309 (-7.42 ± 0.11 vs. -9.13 ± 0.07 , $p = 0.0001$); and in the PathHunter[®] recruitment assay: SYK-245 vs. SYK-308 (-6.91 ± 0.09 vs. -8.20 ± 0.14 , $p = 0.0001$), SYK-245 vs. SYK-309 (-6.91 ± 0.09 vs. -7.81 ± 0.08 , $p = 0.0001$) (Table 1). These data suggest that the 3-hydroxy group on the cyclohexene ring is important for κ OR activities.

Moreover, our present study showed that SYK-309, but not SYK-308, significantly increased the G-protein-biased ratio compared to nalfurafine (Table 2). As a cyclohexene ring is not an aromatic ring, the carbon bound to the 3-hydroxy group is a stereogenic center. Therefore, SYK-308 and SYK-309 possess a 3R and 3S configuration, respectively. Between SYK-308 and -309, the direction of the 3-hydroxy group was the only structural difference; however, there were significant differences in the EC_{50} values of β -arrestin-mediated signaling (PathHunter[®] recruitment assay, $p = 0.031$), but not in G-protein-mediated signaling (GloSensor[®] cAMP assay, $p = 0.576$). Therefore, our present study, for the first time, suggests that the difference in the direction of a hydroxy group at the 3-position of the cyclohexene ring may cause a change in the selectivity of β -arrestin-mediated signaling, and the direction of the 3S-hydroxy group could be one of the important key factors for G-protein-biased κ OR signaling.

Molecular docking studies of nalfurafine and SYK-186 (removal of the 3-hydroxy group) into κ ORs have shown that the 3-hydroxy group of the phenolic moiety of nalfurafine interacted with residues Y3.33, K5.39, and H6.52 in κ ORs via water-mediated hydrogen bonds. In contrast, SYK-186 did not interact with these residues in κ ORs [57]. However, it is unknown which of the residues in κ ORs interacts with SYK-309. Therefore, considering the structural features of nalfurafine and the expectation for the development of novel κ OR agonists, further investigation with a 3-D docking model using SYK-309 could elucidate the direction of the hydroxy group at the 3-position of nalfurafine analogs that induces G-protein-biased signaling.

There are some limitations in the present study. Since this was an *in vitro* study, we cannot clinically evaluate the analgesic effects or side effects of the nalfurafine analogs compared to nalfurafine. As a result, we cannot conclude whether SYK-309 can maintain analgesic effects and reduce any unfavorable side effects mediated by the β -arrestin-mediated pathway. In addition, whether G-protein-biased κ OR agonists have safer and more beneficial profiles than opioid analgesics is still under discussion. Therefore, further studies in different models are necessary to develop more efficacious opioids without any negative impacts on disorders such as chronic pain and pruritus.

Several studies, particularly by Laura Bohn, claim that arrestin is primarily responsible for the adverse effects of ORs; however, numerous recent studies contradict this assertion [34,71]. Therefore, it is necessary to analyze not only analgesic effects, but also sedative effects of SYK-309, as well as its property as a G-protein-biased agonist against κ ORs. Moreover, we measured the κ OR activities (the G-protein-mediated and β -arrestin-mediated signaling) for only the κ OR agonists, the nalfurafine and nalfurafine analogs, in this study. Therefore, it is also necessary that the κ OR activities in other morphinan or benzomorphan derivatives which have the A ring modified like the SYK-309 are measured and have their profiles compared with nalfurafine and nalfurafine analogs in the future.

Opioid analgesics, especially μ OR agonists, are used to treat pain; however, their usage is sometimes complicated by detrimental side effects [7]. Therefore, developing novel opioids with fewer adverse events is strongly desirable. Recent research indicated that functionally selective κ OR agonists elicited neither addictive nor adverse effects [72], and, subsequently, several groups have screened for G-protein-biased κ OR agonists [73–76]. This study showed that the direction of the 3-hydroxy group in nalfurafine is crucial in inducing G-protein-biased signaling. However, to clinically introduce κ OR agonists as painkillers, further investigation of the structural properties of nalfurafine—which selectively activate G-protein-mediated pathways via κ ORs—is necessary.

4. Materials and Methods

4.1. Chemicals

We used the following reagents: nalfurafine—(2E)-N-[(5R,6R)-17-(cyclopropylmethyl)-4,5-epoxy-3,14-dihydromorphinan-6-yl]-3-(furan-3-yl)-N-methylprop-2-enamide, and six nalfurafine analogs that were divided into two groups according to structural characteristics (Figure 1). Group A: SYK-160 (removed the 4,5-ether bridge from nalfurafine), SYK-186 (removed the 3-hydroxy group from nalfurafine), and SYK-406 (removed the 4,5-ether bridge and 3-hydroxy group from nalfurafine). Group B: SYK-245 (removed the 4,5-ether bridge and 3-hydroxy group from nalfurafine, and converted the benzene ring to a cyclohexene ring), SYK-308 (removed the 4,5-ether bridge from nalfurafine and converted the benzene ring to a cyclohexene ring, with a 3R-hydroxy group), and SYK-309 (removed the 4,5-ether bridge from nalfurafine and converted the benzene ring to a cyclohexene ring, with a 3S-hydroxy group). All chemicals were diluted with dimethyl sulfoxide. All compounds employed were synthesized as described previously [77,78].

4.2. Cell Lines

We amplified Halotag[®]-fused κ ORs (Halotag[®]- κ OR, from Kazusa DNA Research Institute, Chiba, Japan) with the pGlosensor[™]-22F plasmid (pGS22F) from Promega (Madison, WI, USA), following the manufacturer's instructions. Human embryonic kidney 293 (HEK293) cells were obtained from the American Type Culture Collection (ATCC[®], Manassas, VA, USA), and stably expressing Halotag[®]- κ ORs were generated by transfection of the constructed plasmids using the Lipofectamine reagent (Life Technologies, Carlsbad, CA, USA), which were selected based on OR activity measured by the CellKey[™] assay or the cAMP assay with Glosensor[®].

4.3. Cell Culture

We cultured HEK293 cells that stably expressed Halotag[®]- κ OR/pGS22F in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum albumin, penicillin (100 U/mL), streptomycin (100 μ g/mL), 700 μ g/mL genistein (Glico, Palo Alto, CA, USA), and 100 μ g/mL hygromycin in a humidified atmosphere containing 95% air and 5% CO₂ at 37 °C.

4.4. Functional Analysis of ORs Using the CellKey[™] System

We examined the effects of nalfurafine and nalfurafine analogs on κ ORs by the CellKey[™] assay system, as described previously [56,79]. We seeded cells at a density of 5.0×10^4 in CellKey[™] poly-D-Lysine (Sigma-Aldrich)-coated 96-well microplates with an embedded electrode at the bottom of each well, for 24 h of incubation. After washing with the CellKey[™] buffer composed of Hanks' balanced salt solution (1.3 mM CaCl₂·2H₂O, 0.81 mM MgSO₄, 5.4 mM KCl, 0.44 mM KH₂PO₄, 4.2 mM NaHCO₃, 136.9 mM NaCl, 0.34 mM Na₂HPO₄, and 5.6 mM d-glucose) containing 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and 0.1% bovine serum albumin (BSA), we incubated the cells for 30 min at 28 °C and treated them with the vehicle or one of the reagents. The change in impedance of an induced extracellular current (dZiec) in each well was measured for 25 min, following a 5 min baseline measurement. The magnitude of change in the dZiec value was defined as Δ Ziec. The value for nalfurafine analogs was calculated as a percentage using the highest value for nalfurafine (positive control).

4.5. Intracellular cAMP Levels Measured with the GloSensor[®] cAMP Assay

We performed the GloSensor[®] cAMP assay as described previously [30,79,80]. In brief, cAMP accumulation was analyzed using cells stably expressing Halotag[®]- κ OR/pGS22F. We seeded the cells at 4.0×10^4 cells/well in 96-well clear-bottom white plates (Corning, Corning, NY, USA) and then incubated them for 24 h. After washing the cells with the CellKey[™] buffer without BSA, the cells were equilibrated with the diluted GloSensor[®] reagent at room temperature for 2 h, and the baseline fluorescence intensity was measured for 15 min. After the baseline measurement, cells were treated with the test compounds for 10 min, after which forskolin (3.0×10^{-6} M) was added. The fluorescence intensity was measured every 2.5 min for 30 min using Synergy[™] H1 (Bio Tek Instruments Inc., Winooski, VT, USA); time-fluorescence curves and the area under the curve (AUC) values of time-fluorescence intensities were calculated. The responses of test compounds were expressed as the AUC of each test compound subtracted from that of the negative control sample (forskolin alone). Data were transformed from each well as the percentage (%) of intracellular cAMP inhibition and calculated by dividing the corrected AUC by those of the standard sample. The standard sample was nalfurafine (10^{-7} M) for Halotag[®]- κ OR/pGS22F.

4.6. β -Arrestin Recruitment Assay with PathHunter[®]

This was performed as described previously [81]. In brief, U2OS OPRM1, CHO-K1 OPRD1, or U2OS OPRK1 cells were seeded at a density of 1.0×10^4 cells/well in 96-well

clear-bottom white plates and incubated for 48 h. The cells were stimulated for 180 min at 37 °C under 5% CO₂, and the PathHunter® working detection solution was added. The luminescence intensity was measured using the FlexStation 3 (Bio Tek Instruments Inc., Winooski, VT, USA) for 1 h at 25 ± 3 °C. Data are expressed as the maximum signal intensity of each test compound as a percentage of the maximum signal intensity of the positive control.

4.7. The Estimated Intrinsic Reactive Activity (RA_i) and Biased Factors

According to the method developed and refined by Ehlert and colleagues [58–60], the G-protein-biased factor was estimated. In brief, each agonist's intrinsic reactive activity (RA_i) was estimated by global nonlinear regression analysis [59,82,83]. The RA_i of each nalfurafine analog was estimated from the concentration-response curves used to estimate EC₅₀ and E_{max} values (Table 1). The G-protein-biased factor was defined as the ratio of the RA_{i,g} value divided by RA_{i-B} (Table 2).

4.8. Statistical Analysis and Approval for the Study

Data analyses and concentration-response curve fitting were performed using GraphPad Prism 9 (GraphPad Software, San Diego, CA, USA). Data are presented as means with the standard error of the mean (SEM) for at least three independent experiments. Statistical analysis was performed using a one-way ANOVA, followed by the Bonferroni multiple comparison tests or *t*-tests. A value of *p* < 0.05 was considered statistically significant. All analyses and experiments were approved and performed in accordance with the Guide for Genetic Modification Safety Committee, National Cancer Center, Japan.

5. Conclusion

The present study revealed that the direction of the 3-hydroxy group of nalfurafine may be the partial structure inducing G-protein-biased signaling via the weakening of β-arrestin-mediated signaling. Therefore, nalfurafine analogs having a 3''S''-hydroxy group, such as SYK-309, could be considered κOR agonists with a pharmacological profile that selectively activates the G-protein-mediated pathway. This evaluation of the structure–activity relationship is expected to help the development of novel selective κOR agonists.

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References

1. Kivell, B.; Priszano, T.E. Kappa Opioids and the Modulation of Pain. *Psychopharmacology* **2010**, *210*, 109–119. <https://doi.org/10.1007/s00213-010-1819-6>.
2. Berterame, S.; Erthal, J.; Thomas, J.; Fellner, S.; Vosse, B.; Clare, P.; Hao, W.; Johnson, D.T.; Mohar, A.; Pavadia, J.; et al. Use of and Barriers to Access to Opioid Analgesics: A Worldwide, Regional, and National Study. *Lancet* **2016**, *387*, 1644–1656. [https://doi.org/10.1016/S0140-6736\(16\)00161-6](https://doi.org/10.1016/S0140-6736(16)00161-6).
3. Caudill-Slosberg, M.A.; Schwartz, L.M.; Woloshin, S. Office Visits and Analgesic Prescriptions for Musculoskeletal Pain in US: 1980 vs. 2000. *Pain* **2004**, *109*, 514–519. <https://doi.org/10.1016/j.pain.2004.03.006>.
4. Olsen, Y.; Daumit, G.L.; Ford, D.E. Opioid Prescriptions by U.S. Primary Care Physicians From 1992 to 2001. *J. Pain* **2006**, *7*, 225–235. <https://doi.org/10.1016/j.jpain.2005.11.006>.
5. Machelska, H.; Celik, M.Ö. Advances in Achieving Opioid Analgesia Without Side Effects. *Front. Pharmacol.* **2018**, *9*, 1388. <https://doi.org/10.3389/fphar.2018.01388>.
6. Trescot, A.M.; Datta, S.; Lee, M.; Hansen, H. Opioid Pharmacology. *Pain Phys.* **2008**, *11*, S133–S153.
7. Chou, R.; Fanciullo, G.J.; Fine, P.G.; Adler, J.A.; Ballantyne, J.C.; Davies, P.; Donovan, M.I.; Fishbain, D.A.; Foley, K.M.; Fudin, J.; et al. Clinical Guidelines for the Use of Chronic Opioid Therapy in Chronic Noncancer Pain. *J. Pain* **2009**, *10*, 113–130. <https://doi.org/10.1016/j.jpain.2008.10.008>.
8. Benyamin, R.; Trescot, A.M.; Datta, S.; Buenaventura, R.; Adlaka, R.; Sehgal, N.; Glaser, S.E.; Vallejo, R. Opioid Complications and Side Effects. *Pain Phys.* **2008**, *11*, S105–S120.
9. Khademi, H.; Kamangar, F.; Brennan, P.; Malekzadeh, R. Opioid Therapy and Its Side Effects: A Review. *Arch. Iran. Med.* **2016**, *19*, 870–876. <https://doi.org/10.0161912/AIM.0010>.
10. Rummans, T.A.; Burton, M.C.; Dawson, N.L. How Good Intentions Contributed to Bad Outcomes: The Opioid Crisis. *Mayo Clin. Proc.* **2018**, *93*, 344–350. <https://doi.org/10.1016/j.mayocp.2017.12.020>.
11. Webster, L.R.; Webster, R.M. Predicting Aberrant Behaviors in Opioid-Treated Patients: Preliminary Validation of the Opioid Risk Tool. *Pain Med.* **2005**, *6*, 432–442. <https://doi.org/10.1111/j.1526-4637.2005.00072.x>.
12. Vanderah, T.W. Delta and Kappa Opioid Receptors as Suitable Drug Targets for Pain. *Clin. J. Pain* **2010**, *26*, S10–S15. <https://doi.org/10.1097/AJP.0b013e3181c49e3a>.
13. Nagase, H.; Saitoh, A. Research and Development of Kappa Opioid Receptor Agonists and Delta Opioid Receptor Agonists. *Pharmacol. Ther.* **2020**, *205*, 107427. <https://doi.org/10.1016/j.pharmthera.2019.107427>.
14. Zhou, L.; Stahl, E.L.; Lovell, K.M.; Frankowski, K.J.; Priszano, T.E.; Aubé, J.; Bohn, L.M. Characterization of Kappa Opioid Receptor Mediated, Dynorphin-Stimulated [35S]GTPγS Binding in Mouse Striatum for the Evaluation of Selective KOR Ligands in an Endogenous Setting. *Neuropharmacology* **2015**, *99*, 131–141. <https://doi.org/10.1016/j.neuropharm.2015.07.001>.
15. Chavkin, C.; James, I.F.; Goldstein, A. Dynorphin Is a Specific Endogenous Ligand of the Kappa Opioid Receptor. *Science* **1982**, *215*, 413–415. <https://doi.org/10.1126/science.6120570>.
16. Mansour, A.; Khachaturian, H.; Lewis, M.E.; Akil, H.; Watson, S.J. Anatomy of CNS Opioid Receptors. *Trends Neurosci.* **1988**, *11*, 308–314. [https://doi.org/10.1016/0166-2236\(88\)90093-8](https://doi.org/10.1016/0166-2236(88)90093-8).
17. Dhawan, B.N.; Cesselin, F.; Raghubir, R.; Reisine, T.; Bradley, P.B.; Portoghese, P.S.; Hamon, M. International Union of Pharmacology. XII. Classification of Opioid Receptors. *Pharmacol. Rev.* **1996**, *48*, 567–592.
18. Kardon, A.P.; Polgár, E.; Hachisuka, J.; Snyder, L.M.; Cameron, D.; Savage, S.; Cai, X.; Karnup, S.; Fan, C.R.; Hemenway, G.M.; et al. Dynorphin Acts as a Neuromodulator to Inhibit Itch in the Dorsal Horn of the Spinal Cord. *Neuron* **2014**, *82*, 573–586. <https://doi.org/10.1016/j.neuron.2014.02.046>.
19. Cowan, A.; Kehner, G.B.; Inan, S. Targeting Itch With Ligands Selective for Kappa Opioid Receptors. *Handb. Exp. Pharmacol.* **2015**, *226*, 291–314. https://doi.org/10.1007/978-3-662-44605-8_16.
20. Inan, S.; Cowan, A. Kappa Opioid Agonists Suppress Chloroquine-Induced Scratching in Mice. *Eur. J. Pharmacol.* **2004**, *502*, 233–237. <https://doi.org/10.1016/j.ejphar.2004.09.010>.
21. Morgenweck, J.; Frankowski, K.J.; Priszano, T.E.; Aubé, J.; Bohn, L.M. Investigation of the Role of β arrestin2 in Kappa Opioid Receptor Modulation in a Mouse Model of Pruritus. *Neuropharmacology* **2015**, *99*, 600–609. <https://doi.org/10.1016/j.neuropharm.2015.08.027>.
22. Martin, W.R. Pharmacology of Opioids. *Pharmacol. Rev.* **1983**, *35*, 283–323.
23. Land, B.B.; Bruchas, M.R.; Schattauer, S.; Giardino, W.J.; Aita, M.; Messinger, D.; Hnasko, T.S.; Palmiter, R.D.; Chavkin, C. Activation of the Kappa Opioid Receptor in the Dorsal Raphe Nucleus Mediates the Aversive Effects of Stress and Reinstates Drug Seeking. *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 19168–19173. <https://doi.org/10.1073/pnas.0910705106>.
24. Pfeiffer, A.; Brantl, V.; Herz, A.; Emrich, H.M. Psychotomimesis Mediated by Kappa Opiate Receptors. *Science* **1986**, *233*, 774–776. <https://doi.org/10.1126/science.3016896>.
25. Dykstra, L.A.; Gmerek, D.E.; Winger, G.; Woods, J.H. Kappa Opioids in Rhesus Monkeys. I. Diuresis, Sedation, Analgesia and Discriminative Stimulus Effects. *J. Pharmacol. Exp. Ther.* **1987**, *242*, 413–420.
26. Roth, B.L.; Baner, K.; Westkaemper, R.; Siebert, D.; Rice, K.C.; Steinberg, S.; Ernsberger, P.; Rothman, R.B. Salvinorin A: A Potent Naturally Occurring Nonnitrogenous Kappa Opioid Selective Agonist. *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 11934–11939. <https://doi.org/10.1073/pnas.182234399>.
27. Raehal, K.M.; Schmid, C.L.; Groer, C.E.; Bohn, L.M. Functional Selectivity at the μ -Opioid Receptor: Implications for Understanding Opioid Analgesia and Tolerance. *Pharmacol. Rev.* **2011**, *63*, 1001–1019. <https://doi.org/10.1124/pr.111.004598>.

28. Schmid, C.L.; Kennedy, N.M.; Ross, N.C.; Lovell, K.M.; Yue, Z.; Morgenweck, J.; Cameron, M.D.; Bannister, T.D.; Bohn, L.M. Bias Factor and Therapeutic Window Correlate to Predict Safer Opioid Analgesics. *Cell* **2017**, *171*, 1165–1175. <https://doi.org/10.1016/j.cell.2017.10.035>.
29. Rovira, X.; Pin, J.P.; Giraldo, J. The Asymmetric/Symmetric Activation of GPCR Dimers as a Possible Mechanistic Rationale for Multiple Signalling Pathways. *Trends Pharmacol. Sci.* **2010**, *31*, 15–21. <https://doi.org/10.1016/j.tips.2009.10.008>.
30. Townsend, E.A.; Naylor, J.E.; Negus, S.S.; Edwards, S.R.; Qureshi, H.N.; McLendon, H.W.; McCurdy, C.R.; Kapanda, C.N.; do Carmo, J.M.; da Silva, F.S.; et al. Effects of nalfurafine on the reinforcing, thermal antinociceptive, and respiratory-depressant effects of oxycodone: modeling an abuse-deterrent opioid analgesic in rats. *Psychopharmacology* **2017**, *234*, 2597–2605. <https://doi.org/10.1007/s00213-017-4652-3>.
31. Okude, J.; Ueda, T.; Kofuku, Y.; Sato, M.; Nobuyama, N.; Kondo, K.; Shiraishi, Y.; Mizumura, T.; Onishi, K.; Natsume, M.; et al. Identification of a Conformational Equilibrium That Determines the Efficacy and Functional Selectivity of the μ -Opioid Receptor. *Angew. Chem. Int. Ed. Engl.* **2015**, *54*, 15771–15776. <https://doi.org/10.1002/anie.201508794>.
32. Raffa, R.B.; Martinez, R.P.; Connelly, C.D. G-Protein Antisense Oligodeoxyribonucleotides and Mu-Opioid Supraspinal Antinociception. *Eur. J. Pharmacol.* **1994**, *258*, R5–R7. [https://doi.org/10.1016/0014-2999\(94\)90073-6](https://doi.org/10.1016/0014-2999(94)90073-6).
33. Bohn, L.M.; Lefkowitz, R.J.; Gainetdinov, R.R.; Poppel, K.; Caron, M.G.; Lin, F.T. Enhanced Morphine Analgesia in Mice Lacking Beta-Arrestin 2. *Science* **1999**, *286*, 2495–2498. <https://doi.org/10.1126/science.286.5449.2495>.
34. Kliewer, A.; Gillis, A.; Hill, R.; Schmiedel, F.; Bailey, C.; Kelly, E.; Henderson, G.; Christie, M.J.; Schulz, S. Morphine-induced respiratory depression is independent of beta-arrestin2 signalling. *Br. J. Pharmacol.* **2020**, *177*, 2923–2931. <https://doi.org/10.1111/bph.15004>.
35. Chen, X.T.; Pitis, P.; Liu, G.; Yuan, C.; Gotchev, D.; Cowan, C.L.; Rominger, D.H.; Koblisch, M.; Dewire, S.M.; Crombie, A.L.; et al. Structure-Activity Relationships and Discovery of a G Protein Biased μ Opioid Receptor Ligand, [(3-Methoxythiophen-2-yl) Methyl][(2-[(9R)-9-(Pyridin-2-yl)-6-Oxaspiro-[4.5] Decan- 9-yl] Ethyl)] Amine (TRV130), for the Treatment of Acute Severe Pain. *J. Med. Chem.* **2013**, *56*, 8019–8031. <https://doi.org/10.1021/jm4010829>.
36. Faouzi, A.; Varga, B.R.; Majumdar, S. Biased Opioid Ligands. *Molecules* **2020**, *25*, 4527. <https://doi.org/10.3390/molecules25184257>.
37. Bermudez, M.; Nguyen, T.N.; Omieczynski, C.; Wolber, G. Strategies for the Discovery of Biased GPCR Ligands. *Drug Discov. Today* **2019**, *24*, 1031–1037. <https://doi.org/10.1016/j.drudis.2019.02.010>.
38. Kudla, L.; Przewlocki, R. Influence of G Protein-Biased Agonists of μ -Opioid Receptor on Addiction-Related Behaviors. *Pharmacol. Rep.* **2021**, *73*, 1033–1051. <https://doi.org/10.1007/s43440-021-00251-1>.
39. Miyano, K.; Manabe, S.; Komatsu, A.; Fujii, Y.; Mizobuchi, Y.; Uezono, E.; Ohshima, K.; Nonaka, M.; Kuroda, Y.; Narita, M.; et al. The G Protein Signal-Biased Compound TRV130; Structures, Its Site of Action and Clinical Studies. *Curr. Top. Med. Chem.* **2020**, *20*, 2822–2829. <https://doi.org/10.2174/1568026620999201027224229>.
40. Spetea, M.; Eans, S.O.; Ganno, M.L.; Lantero, A.; Mairegger, M.; Toll, L.; Schmidhammer, H.; McLaughlin, J.P. Selective Kappa Receptor Partial Agonist HS666 Produces Potent Antinociception Without Inducing Aversion After i.c.v. Administration in Mice. *Br. J. Pharmacol.* **2017**, *174*, 2444–2456. <https://doi.org/10.1111/bph.13854>.
41. Chavkin, C. The Therapeutic Potential of Kappa-Opioids for Treatment of Pain and Addiction. *Neuropsychopharmacology* **2011**, *36*, 369–370. <https://doi.org/10.1038/npp.2010.137>.
42. Schattauer, S.S.; Kuhar, J.R.; Song, A.; Chavkin, C. Nalfurafine Is a G-Protein Biased Agonist Having Significantly Greater Bias at the Human Than Rodent Form of the Kappa Opioid Receptor. *Cell Signal.* **2017**, *32*, 59–65. <https://doi.org/10.1016/j.cellsig.2017.01.016>.
43. Brust, T.F.; Morgenweck, J.; Kim, S.A.; Rose, J.H.; Locke, J.L.; Schmid, C.L.; Zhou, L.; Stahl, E.L.; Cameron, M.D.; Scarry, S.M.; et al. Biased Agonists of the Kappa Opioid Receptor Suppress Pain and Itch Without Causing Sedation or Dysphoria. *Sci. Signal.* **2016**, *9*, ra117. <https://doi.org/10.1126/scisignal.aai8441>.
44. Nagase, H.; Hayakawa, J.; Kawamura, K.; Kawai, K.; Takezawa, Y.; Matsuura, H.; Tajima, C.; Endo, T. Discovery of a Structurally Novel Opioid Kappa-Agonist Derived From 4,5-Epoxymorphinan. *Chem. Pharm. Bull.* **1998**, *46*, 366–369. <https://doi.org/10.1248/cpb.46.366>.
45. Seki, T.; Awamura, S.; Kimura, C.; Ide, S.; Sakano, K.; Minami, M.; Nagase, H.; Satoh, M. Pharmacological Properties of TRK-820 on Cloned μ -, Δ - and Kappa-Opioid Receptors and Nociceptin Receptor. *Eur. J. Pharmacol.* **1999**, *376*, 159–167. [https://doi.org/10.1016/s0014-2999\(99\)00369-6](https://doi.org/10.1016/s0014-2999(99)00369-6).
46. Togashi, Y.; Umeuchi, H.; Okano, K.; Ando, N.; Yoshizawa, Y.; Honda, T.; Kawamura, K.; Endoh, T.; Utsumi, J.; Kamei, J.; et al. Antipruritic Activity of the Kappa-Opioid Receptor Agonist, TRK-820. *Eur. J. Pharmacol.* **2002**, *435*, 259–264. [https://doi.org/10.1016/s0014-2999\(01\)01588-6](https://doi.org/10.1016/s0014-2999(01)01588-6).
47. Nagase, H.; Fujii, H. Opioids in Preclinical and Clinical Trials. *Top. Curr. Chem.* **2011**, *299*, 29–62. https://doi.org/10.1007/128_2010_74.
48. Inui, S. Nalfurafine Hydrochloride to Treat Pruritus: A Review. *Clin. Cosmet. Investig. Dermatol.* **2015**, *8*, 249–255. <https://doi.org/10.2147/CCID.S55942>.
49. Kumagai, H.; Ebata, T.; Takamori, K.; Muramatsu, T.; Nakamoto, H.; Suzuki, H. Effect of a Novel Kappa-Receptor Agonist, Nalfurafine Hydrochloride, on Severe Itch in 337 Haemodialysis Patients: A Phase III, Randomized, Double-Blind, Placebo-Controlled Study. *Nephrol. Dial. Transplant.* **2010**, *25*, 1251–1257. <https://doi.org/10.1093/ndt/gfp588>.

50. Nakao, K.; Mochizuki, H. Nalfurafine Hydrochloride: A New Drug for the Treatment of Uremic Pruritus in Hemodialysis Patients. *Drugs Today* **2009**, *45*, 323–329. <https://doi.org/10.1358/dot.2009.45.5.1362067>.
51. Bruijnzeel, A.W. Kappa-Opioid Receptor Signaling and Brain Reward Function. *Brain Res. Rev.* **2009**, *62*, 127–146. <https://doi.org/10.1016/j.brainresrev.2009.09.008>.
52. Chefer, V.I.; Czyzyk, T.; Bolan, E.A.; Moron, J.; Pintar, J.E.; Shippenberg, T.S. Endogenous Kappa-Opioid Receptor Systems Regulate Mesoaccumbal Dopamine Dynamics and Vulnerability to Cocaine. *J. Neurosci.* **2005**, *25*, 5029–5037. <https://doi.org/10.1523/JNEUROSCI.0854-05.2005>.
53. Shippenberg, T.S.; Chefer, V.I.; Zapata, A.; Heidbreder, C.A. Modulation of the Behavioral and Neurochemical Effects of Psychostimulants by Kappa-Opioid Receptor Systems. *Ann. N. Y. Acad. Sci.* **2001**, *937*, 50–73. <https://doi.org/10.1111/j.1749-6632.2001.tb03558.x>.
54. Fujii, H.; Hirayama, S.; Nagase, H. Opioid kappa receptor selective agonist TRK820 (nal-furafine hydrochloride). In *InTech*; Luca, G., Ed.; Pharmacology: Rijeka, Croatia, 2012; pp. 81–98.
55. Nagase, H.; Fujii, H. Essential Structure of the Kappa Opioid Receptor Agonist Nalfurafine for Binding to the Kappa Receptor. *Curr. Pharm. Des.* **2013**, *19*, 7400–7414. <https://doi.org/10.2174/138161281942140105165011>.
56. Miyano, K.; Sudo, Y.; Yokoyama, A.; Hisaoka-Nakashima, K.; Morioka, N.; Takebayashi, M.; Nakata, Y.; Higami, Y.; Uezono, Y. History of the G Protein-Coupled Receptor (GPCR) Assays From Traditional to a State-of-the-Art Biosensor Assay. *J. Pharmacol. Sci.* **2014**, *126*, 302–309. <https://doi.org/10.1254/jphs.14R13CP>.
57. Cao, D.; Huang, P.; Chiu, Y.T.; Chen, C.; Wang, H.; Li, M.; Zheng, Y.; Ehlert, F.J.; Zhang, Y.; Liu-Chen, L.Y. 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. *ACS Chem. Neurosci.* **2020**, *11*, 3036–3050. <https://doi.org/10.1021/acscemneuro.0c00407>.
58. DiMattio, K.M.; Ehlert, F.J.; Liu-Chen, L.Y. Intrinsic Relative Activities of Kappa Opioid Agonists in Activating G α Proteins and Internalizing Receptor: Differences Between Human and Mouse Receptors. *Eur. J. Pharmacol.* **2015**, *761*, 235–244. <https://doi.org/10.1016/j.ejphar.2015.05.054>.
59. Griffin, M.T.; Figueroa, K.W.; Liller, S.; Ehlert, F.J. Estimation of Agonist Activity at G Protein-Coupled Receptors: Analysis of M2 Muscarinic Receptor Signaling Through Gi/o, Gs, and G15. *J. Pharmacol. Exp. Ther.* **2007**, *321*, 1193–1207. <https://doi.org/10.1124/jpet.107.120857>.
60. Tran, J.A.; Chang, A.; Matsui, M.; Ehlert, F.J. Estimation of Relative Microscopic Affinity Constants of Agonists for the Active State of the Receptor in Functional Studies on M2 and M3 Muscarinic Receptors. *Mol. Pharmacol.* **2009**, *75*, 381–396. <https://doi.org/10.1124/mol.108.051276>.
61. Komatsu, A.; Miyano, K.; Nakayama, D.; Mizobuchi, Y.; Uezono, E.; Ohshima, K.; Karasawa, Y.; Kuroda, Y.; Nonaka, M.; Yamaguchi, K.; et al. Novel Opioid Analgesics for the Development of Transdermal Opioid Patches That Possess Morphine-Like Pharmacological Profiles Rather Than Fentanyl: Possible Opioid Switching Alternatives Among Patch Formula. *Anesth. Analg.* **2022**, *134*, 1082–1093. <https://doi.org/10.1213/ANE.0000000000005954>.
62. Manabe, S.; Miyano, K.; Fujii, Y.; Ohshima, K.; Yoshida, Y.; Nonaka, M.; Uzu, M.; Matsuoka, Y.; Sato, T.; Uezono, Y.; et al. Possible biased analgesic of hydromorphone through the G protein-over beta-arrestin-mediated pathway: cAMP, CellKey, and receptor internalization analyses. *J. Pharmacol. Sci.* **2019**, *140*, 171–177. <https://doi.org/10.1016/j.jphs.2019.06.005>.
63. Land, B.B.; Bruchas, M.R.; Lemos, J.C.; Xu, M.; Melief, E.J.; Chavkin, C. The Dysphoric Component of Stress Is Encoded by Activation of the Dynorphin Kappa-Opioid System. *J. Neurosci.* **2008**, *28*, 407–414. <https://doi.org/10.1523/JNEUROSCI.4458-07.2008>.
64. Van't Veer, A.; Carlezon, W.A., Jr. Role of Kappa-Opioid Receptors in Stress and Anxiety-Related Behavior. *Psychopharmacology* **2013**, *229*, 435–452. <https://doi.org/10.1007/s00213-013-3195-5>.
65. Mores, K.L.; Cummins, B.R.; Cassell, R.J.; van Rijn, R.M. A Review of the Therapeutic Potential of Recently Developed G Protein-Biased Kappa Agonists. *Front. Pharmacol.* **2019**, *10*, 407. <https://doi.org/10.3389/fphar.2019.00407>.
66. Liu, J.J.; Chiu, Y.T.; DiMattio, K.M.; Chen, C.; Huang, P.; Gentile, T.A.; Muschamp, J.W.; Cowan, A.; Mann, M.; Liu-Chen, L.Y. Phosphoproteomic Approach for Agonist-Specific Signaling in Mouse Brains: mTOR Pathway Is Involved in Kappa Opioid Aversion. *Neuropsychopharmacology* **2019**, *44*, 939–949. <https://doi.org/10.1038/s41386-018-0155-0>.
67. McLaughlin, J.P.; Myers, L.C.; Zarek, P.E.; Caron, M.G.; Lefkowitz, R.J.; Czyzyk, T.A.; Pintar, J.E.; Chavkin, C. Prolonged Kappa Opioid Receptor Phosphorylation Mediated by G-Protein Receptor Kinase Underlies Sustained Analgesic Tolerance. *J. Biol. Chem.* **2004**, *279*, 1810–1818. <https://doi.org/10.1074/jbc.M305796200>.
68. Bruchas, M.R.; Land, B.B.; Aita, M.; Xu, M.; Barot, S.K.; Li, S.; Chavkin, C. Stress-Induced p38 Mitogen-Activated Protein Kinase Activation Mediates Kappa-Opioid-Dependent Dysphoria. *J. Neurosci.* **2007**, *27*, 11614–11623. <https://doi.org/10.1523/JNEUROSCI.3769-07.2007>.
69. Bruchas, M.R.; Macey, T.A.; Lowe, J.D.; Chavkin, C. Kappa Opioid Receptor Activation of p38 MAPK Is GRK3- and Arrestin-Dependent in Neurons and Astrocytes. *J. Biol. Chem.* **2006**, *281*, 18081–18089. <https://doi.org/10.1074/jbc.M513640200>.
70. Redila, V.A.; Chavkin, C. Stress-Induced Reinstatement of Cocaine Seeking Is Mediated by the Kappa Opioid System. *Psychopharmacology* **2008**, *200*, 59–70. <https://doi.org/10.1007/s00213-008-1122-y>.
71. Grundmann, M.; Merten, N.; Malfacini, D.; Inoue, A.; Preis, P.; Simon, K.; Ruttiger, N.; Ziegler, N.; Benkel, T.; Schmitt, N.K.; et al. Lack of beta-arrestin signaling in the absence of active G proteins. *Nat. Commun.* **2018**, *9*, 341. <https://doi.org/10.1038/s41467-017-02661-3>.

72. Urban, J.D.; Clarke, W.P.; von Zastrow, M.; Nichols, D.E.; Kobilka, B.; Weinstein, H.; Javitch, J.A.; Roth, B.L.; Christopoulos, A.; Sexton, P.M.; et al. Functional Selectivity and Classical Concepts of Quantitative Pharmacology. *J. Pharmacol. Exp. Ther.* **2007**, *320*, 1–13. <https://doi.org/10.1124/jpet.106.104463>.
73. Rives, M.L.; Rossillo, M.; Liu-Chen, L.Y.; Javitch, J.A. 6'-Guanidinonaltrindole (6'-GNTI) Is a G Protein-Biased Kappa-Opioid Receptor Agonist That Inhibits Arrestin Recruitment. *J. Biol. Chem.* **2012**, *287*, 27050–27054. <https://doi.org/10.1074/jbc.C112.387332>.
74. Schmid, C.L.; Streicher, J.M.; Groer, C.E.; Munro, T.A.; Zhou, L.; Bohn, L.M. Functional Selectivity of 6'-Guanidinonaltrindole (6'-GNTI) at Kappa-Opioid Receptors in Striatal Neurons. *J. Biol. Chem.* **2013**, *288*, 22387–22398. <https://doi.org/10.1074/jbc.M113.476234>.
75. Zhou, L.; Lovell, K.M.; Frankowski, K.J.; Slauson, S.R.; Phillips, A.M.; Streicher, J.M.; Stahl, E.; Schmid, C.L.; Hodder, P.; Maddoux, F.; et al. Development of Functionally Selective, Small Molecule Agonists at Kappa Opioid Receptors. *J. Biol. Chem.* **2013**, *288*, 36703–36716. <https://doi.org/10.1074/jbc.M113.504381>.
76. White, K.L.; Scopton, A.P.; Rives, M.L.; Bikbulatov, R.V.; Polepally, P.R.; Brown, P.J.; Kenakin, T.; Javitch, J.A.; Zjawiony, J.K.; Roth, B.L. Identification of Novel Functionally Selective Kappa-Opioid Receptor Scaffolds. *Mol. Pharmacol.* **2014**, *85*, 83–90. <https://doi.org/10.1124/mol.113.089649>.
77. Nagase, H.; Imaide, S.; Hirayama, S.; Nemoto, T.; Fujii, H. Essential Structure of Opioid Kappa Receptor Agonist Nalfurafine for Binding to the Kappa Receptor 2: Synthesis of Decahydro(Iminoethano)Phenanthrene Derivatives and Their Pharmacologies. *Bioorg. Med. Chem. Lett.* **2012**, *22*, 5071–5074. <https://doi.org/10.1016/j.bmcl.2012.05.122>.
78. Fujii, H.; Imaide, S.; Hirayama, S.; Nemoto, T.; Gouda, H.; Hirono, S.; Nagase, H. Essential Structure of Opioid Kappa Receptor Agonist Nalfurafine for Binding to the Kappa Receptor 3: Synthesis of Decahydro(Iminoethano)Phenanthrene Derivatives With an Oxygen Functionality at the 3-Position and Their Pharmacologies. *Bioorg. Med. Chem. Lett.* **2012**, *22*, 7711–7714. <https://doi.org/10.1016/j.bmcl.2012.09.101>.
79. Meguro, Y.; Miyano, K.; Hirayama, S.; Yoshida, Y.; Ishibashi, N.; Ogino, T.; Fujii, Y.; Manabe, S.; Eto, M.; Nonaka, M.; et al. Neuropeptide Oxytocin Enhances μ Opioid Receptor Signaling as a Positive Allosteric Modulator. *J. Pharmacol. Sci.* **2018**, *137*, 67–75. <https://doi.org/10.1016/j.jphs.2018.04.002>.
80. Tobo, M.; Tomura, H.; Mogi, C.; Wang, J.Q.; Liu, J.P.; Komachi, M.; Damirin, A.; Kimura, T.; Murata, N.; Kurose, H.; et al. Previously Postulated 'ligand-independent' Signaling of GPR4 Is Mediated Through Proton-Sensing Mechanisms. *Cell Signal.* **2007**, *19*, 1745–1753. <https://doi.org/10.1016/j.cellsig.2007.03.009>.
81. Yin, H.; Chu, A.; Li, W.; Wang, B.; Shelton, F.; Otero, F.; Nguyen, D.G.; Caldwell, J.S.; Chen, Y.A. Lipid G protein-coupled receptor ligand identification using beta-arrestin PathHunter assay. *J. Biol. Chem.* **2009**, *284*, 12328–12338. <https://doi.org/10.1074/jbc.M806516200>.
82. Ehlert, F.J. On the Analysis of Ligand-Directed Signaling at G Protein-Coupled Receptors. *Naunyn Schmiedeberg's Arch. Pharmacol.* **2008**, *377*, 549–577. <https://doi.org/10.1007/s00210-008-0260-4>.
83. Ehlert, F.J.; Suga, H.; Griffin, M.T. Quantifying Agonist Activity at G Protein-Coupled Receptors. *J. Vis. Exp.* **2011**, *58*, e3179. <https://doi.org/10.3791/3179>.