Acta Pharmacologica Sinica 2017: 1–13
 1671-4083/17
www.nature.com/aps

Original Article

The opioid receptor triple agonist DPI-125 produces analgesia with less respiratory depression and reduced abuse liability

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Abstract

Opioid analgesics remain the first choice for the treatment of moderate to severe pain, but they are also notorious for their respiratory depression and addictive effects. This study focused on the pharmacology of a novel opioid receptor mixed agonist DPI-125 and attempted to elucidate the relationship between the δ-, μ- and κ-receptor potency ratio and respiratory depression and abuse liability. Five diarylmethylpiperazine compounds (DPI-125, DPI-3290, DPI-130, KUST202 and KUST13T02) were selected for this study. PKA fluorescence redistribution assays in CHO cells individually expressing δ-, μ- or κ-receptors were used to measure the agonist potency. The respiratory safety profiles were estimated in rats by the ratio of ED_{50} (pCO2 increase)/ED_{50} (antinociception). The abuse liability of DPI-125 was evaluated with a self-administration model in rhesus monkeys. The observed agonist potencies of DPI-125 for δ-, μ- and κ-opioid receptors were 4.29±0.36, 11.10±3.04, and 16.57±4.14 nmol/L, respectively. The other four compounds were also mixed agonists with varying potencies. DPI-125 exhibited a high respiratory safety profile (14.4), clearly related to its high δ-receptor potency. The ratio of the EC_{50} potencies for the μ- and δ-receptors was found to be positively correlated with the respiratory safety ratio. DPI-125 has similar potencies for μ- and κ-receptors, which is likely the reason for its reduced abuse potential. Our results demonstrate that the opioid receptor mixed agonist DPI-125 is safer and less addictive than traditional μ-agonist analgesics. These findings suggest that the development of δ>μ~κ opioid receptor mixed agonists is feasible, and such compounds could represent a promising class of potent analgesics with wider therapeutic windows.

Keywords: opioid receptor; mixed agonist; DPI-125; analgesia; respiratory depression; addiction

Acta Pharmacologica Sinica advance online publication, 15 May 2017; doi: 10.1038/aps.2017.14

Introduction

Opioid receptors (ORs) include μ, δ, κ and opioid receptor-like 1 (ORL-1) subtypes. This receptor family plays a key role in the sensing of pain, thus drawing tremendous attention to their functional and structural details[1–7]. Small-molecule μ-OR agonists remain the first choice for the clinical treatment of moderate to severe pain. Unfortunately, μ-agonists are notorious for their life-threatening and/or debilitating adverse effects, including respiratory depression, physical dependence, euphoric addiction, vomiting, constipation and urinary retention[8]. All of the above side effects are known to be primarily mediated through μ-ORs. Although substantial advances have been achieved in our understanding of the biology of ORs, safer analgesics are still lacking, resulting in patients being under-dosed or deprived of treatment altogether.

The discoveries of the systemically active δ-antagonist naltrindole (NTI)[9] and the δ-agonists BW373U86[10] and SNC80[11] have advanced our understanding of the functions of δ-ORs *in vivo* over the past two decades[12]. Initial studies with small-molecule δ-ligands indicated that δ-ORs mediate milder analgesic effects than morphine[13, 14] and cause convulsive symptoms in rodents[15]. The δ-agonists BW373U86, SNC80 and AZD2327 were shown to lack addictive effects in rodents and monkeys[16–18]. Since then, investigators have continued to discover potential therapeutic uses for δ-agonists in depression[19], Parkinson's disease[20], cardioprotection[21], neuropro-

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Received 2016-10-14 Accepted 2017-01-23

tection[22] and overactive bladder[23]. Some of these indications are currently under clinical investigation.

Given that endogenous opioids, which have mixed receptor effects, do not exhibit the deleterious effects of μ-OR agonists, there have been numerous efforts since the 1990s to demonstrate the therapeutic implications of targeting δ- and μ-ORs simultaneously. One of these efforts attempted to activate both δ- and μ-ORs to reduce the side effects of opioid analgesics. O'Neill *et al*[24] reported antagonistic modulation between the δ-agonist BW373U86 and the μ-agonist fentanyl. When they are administered separately, BW373U86 causes convulsive effects and fentanyl causes muscle rigidity in mice, but their co-administration significantly reduced these side effects. Su *et al*[25] further reported that δ-OR ligands reversed the life-threatening respiratory depression induced by alfentanil without interfering with its analgesic activity. On the other hand, κ-agonists are known to induce dysphoria, aversion and other effects[26] that are often opposite to the euphoric effects of μ-agonists[27, 28]. κ-Agonists have been shown to counteract reward behavior in conditioned place preference tests[29], inhibit the dopamine release in the nucleus accumbens induced by heroin self-administration[30], and decrease the rate of fentanyl self-administration[31].

These results sparked intense efforts in the drug discovery community to develop analgesic mixed agonists. Mixed μ-agonist/κ-agonist compounds have been reported to have less abuse liability[32, 33]. Our contribution in these efforts was the discovery of an analgesic diarylmethylpiperazine mixed δ-agonist/μ-agonist, DPI-3290[34–36] (Figure 1). DPI-3290 demonstrated reduced respiratory depression compared with morphine, suggesting that a mixed δ-agonist/μ-agonist could be a safer clinical choice than currently available options.

Following this work, we carried out in-depth structure-activity relationship (SAR) studies on DPI-3290 analogs and discovered another diarylmethylpiperazine compound, DPI-125 (Figure 1), with a similarly high respiratory safety profile. Our results indicated that the κ-OR activity of these compounds also played an important role in their *in vivo* effect profiles. These compounds are in fact δ-, μ- and κ-OR mixed agonists, a class combining both δ-agonist/μ-agonist and μ-agonist/κ-agonist effects.

Herein, we report our findings on the pharmacology of diarylmethylpiperazine δ-, μ- and κ-OR mixed agonists. Five compounds, DPI-125[36, 37], DPI-3290, DPI-130[36, 38], KUST202[38] and KUST13T02, were selected for this study (Figure 1). These compounds demonstrate various levels of mixed δ-, μ-, κ-OR activities. Compounds with higher δ-OR potency than μ-OR potency were safer in terms of their propensity to cause respiratory depression, while compounds with relatively high and balanced μ- and κ-OR activity levels exhibited lower addiction liability. Our results demonstrate that DPI-125 is safer than classical μ-agonists in terms of both respiratory depression and addictive effects and suggest that δ-, μ-, κ-OR mixed agonists may represent a promising avenue to effective analgesia with reduced respiratory depression and abuse potential.

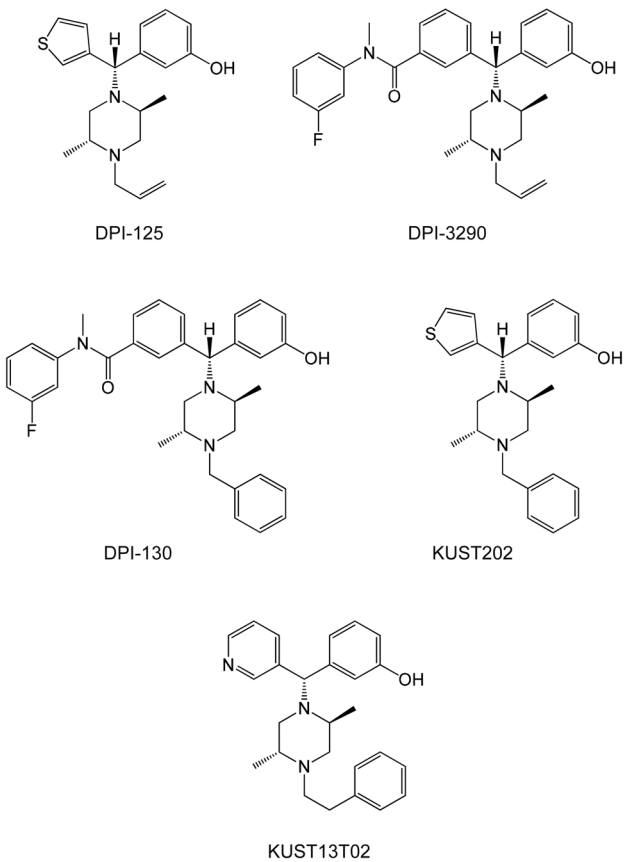

Figure 1. Diarylmethylpiperazine opioid receptor mixed agonists used in this study.

Materials and methods

Animals

Male albino Wistar Hannover rats (Harlan, Madison, WI, USA) weighing 200 to 300 g were maintained on a 12-h light/dark cycle (lights on between 7:00 AM and 7:00 PM) and allowed access to food and water *ad libitum*. A self-administration model in alfentanil-trained and alfentanil-maintained adult male rhesus monkeys[39–41] was used to evaluate the reinforcing effects of DPI-125 in Prof James H Woods' laboratory at the University of Michigan; these experiments were organized through the Drug Evaluation Committee of the National Institute on Drug Abuse (NIDA) College on the Problems of Drug Dependence (CPDD) (see supplementary information). All experimental procedures were conducted in accordance with guidelines for the use of experimental animals and were approved by the local ethics committee and the Institutional Review Committee on Animal Care and Use.

Cell lines

Recombinant CHO-K1 cells stably expressing human μ-OR and the catalytic domain of human Protein Kinase A (PKAcat) fused to the N-terminus of enhanced green fluorescent protein (eGFP), known as CHO-PKAcat-eGFP/μ-OR cells, were purchased from ThermoFisher (Waltham, MA, USA). PKA

G_s- or $G_{i/o}$-coupled GPCR redistribution cell lines, consisting of CHO-K1 cells stably expressing PKAcat fused to the N-terminus of eGFP (CHO-PKAcat-eGFP) (ThermoFisher, Waltham, MA, USA) and stably transfected with human κ-OR (named CHO-PKAcat-eGFP/κ-OR) or δ-OR (named CHO-PKAcat-eGFP/δ-OR), have previously been established at the Beijing Institute of Pharmacology and Toxicology. Standard methods were used to generate plasmid DNA. Briefly, full-length cDNAs for the wild-type human δ- and κ-opioid receptors were subcloned individually into pcDNA3.1+ plasmids (Invitrogen, Carlsbad, CA, USA) at the *Bam*HI (5′) and *EcoR*I (3′) sites (δ-OR) or at the *Kpn* I (5′) and *Xho* I (3′) sites (κ-OR). Cells were transfected using the reagent Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) as described in the reagent supplier's user manual. These three cell lines were used for PKA redistribution assays; they were cultured at 37 °C in F12 medium supplemented with 10% fetal bovine serum and 200 μg/mL geneticin (G418) in a humidified atmosphere of 95% air and 5% CO_2.

Materials

[^{3}H]DPDPE, [^{3}H]DAMGO, and [^{3}H]U69593 were purchased from PerkinElmer Life Sciences (Boston, MA, USA). Morphine, fentanyl, naloxone, U69593, U50488, DAMGO, SNC80 and other chemical reagents were purchased from Sigma-Aldrich (St Louis, MO, USA). All diarylmethylpiperazine mixed OR agonists except KUST13T02 were synthesized according to previously reported methods.

For *in vitro* experiments, samples of each test compound were dissolved in DMSO to a concentration of 1 mmol/L and then diluted to the required concentrations with F12 medium or buffer solution. The highest concentration of the solutions was 10 μmol/L, corresponding to DMSO concentrations of 1% or less. For *in vivo* experiments, solutions of the test compounds were prepared by dissolving samples in a minimum amount of ethanol, converting the compounds to hydrochlorides by adding a molar equivalent of concentrated hydrochloric acid, completely drying the solutions under nitrogen flow, and dissolving the solid residues in sterilized 5% dextrose solution. All compound solutions were prepared freshly before use.

Synthesis of KUST13T02

In an oven-dried flask, nicotinaldehyde (**1**, Figure 2, 1.07 g, 10.0 mmol), (2*R*,5*S*)-2,5-dimethyl-1-phenethylpiperazine (**2**, 2.18 g, 10.0 mmol), benzotriazole (BtH, 1.19 g, 10.0 mmol) and toluene (120 mL) were combined. The mixture was stirred under reflux, and the water generated in the reaction was collected by a Dean-Stark trap. After 3 h, the solvent was removed under reduced pressure. The residue was re-dissolved in anhydrous THF under nitrogen and added to a freshly prepared solution of {3-[(*tert*-butyldimethylsilyl)oxy] phenyl}magnesium bromide (**3**, 20.0 mmol) in THF at room temperature. After stirring at room temperature for 5 h, the reaction was quenched by adding a saturated aqueous solution of ammonium chloride. THF was removed under reduced pressure. Ethyl acetate was added, and the mixture was filtered. The organic layer was washed with brine, dried over anhydrous magnesium sulfate, and concentrated. The crude product was purified by flash column chromatography (silica gel, ethyl acetate) to obtain a TBS-protected intermediate (**4**), which was then dissolved in THF and treated with tetrabutylammonium fluoride (TBAF, 1 mol/L in THF). The mixture was concentrated and the residue was partitioned between ethyl acetate and water. The organic layer was separated, dried over anhydrous magnesium sulfate, and concentrated. The crude product was purified by flash column chromatography (silica gel, ethyl acetate) to obtain KUST13T02 as a white powder (18% yield for two steps). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.55 (s, 1H), 8.35–8.31 (m, 1H), 7.76 (d, *J*=8.0 Hz, 1H), 7.18–7.01 (m, 8H), 6.68–6.64 (m, 1H), 6.55–6.50 (m, 1H), 5.09 (bs, 1H), 2.89–2.74 (m, 2H), 2.73–2.58 (m, 3H), 2.56–2.47 (m, 2H), 2.42 (d, *J*=10.5 Hz, 1H), 2.26 (dd, *J*=10.5, 9.5 Hz, 1H), 1.87 (dd, *J*=10.5, 9.5 Hz, 1H), 1.05 (d, *J*=6.0 Hz, 3H), 0.89 (d, *J*=6.0 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 157.58, 149.07, 146.76, 140.12, 138.83, 136.81, 129.43, 128.73, 128.52, 126.16, 123.45, 121.02, 117.14, 115.30, 77.45, 63.56, 55.38, 55.24, 53.21, 51.76, 31.60, 28.54, 16.02. HRMS (ESI) calcd. for $C_{26}H_{32}N_3O^+$ $[M+H]^+$ 402.2545; found 402.2541.

Membrane preparation for radio-ligand binding

Membrane preparation was performed as previously described[10]. The brains of male albino Wistar Hannover rats were rinsed with ice-cold 50 mmol/L Tris-HCl buffer (pH 7.4, 25 °C) containing the following protease inhibitors: 50 μg/mL soybean trypsin inhibitor, 0.1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 1 mmol/L EDTA, 10 μg/mL leupeptin, 200 μg/mL bacitracin, and 0.5 μg/mL aprotinin. Brains were minced with scissors and homogenized in 10 volumes/g wet weight of ice-cold 50 mmol/L Tris-HCl buffer containing pro-

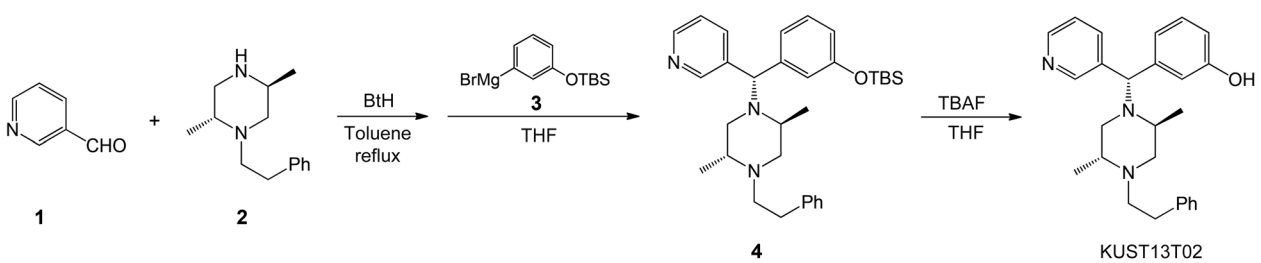

Figure 2. Synthesis of diarylmethylpiperazine opioid receptor agonist KUST13T02.

tease inhibitors, and then centrifuged at 6000×*g* for 15 min at 4 °C. The resulting supernatant was centrifuged at 41 000×*g* for 30 min, and the membrane pellets were re-suspended in 10 volumes/g wet weight of 10 mmol/L Tris-sucrose buffer and sonicated with a Polytron tissue grinder (10 s, low speed). The homogenate was centrifuged again for 30 min at 41 000×*g* and 4 °C. The resulting membrane pellet was re-suspended in 50 mmol/L Tris buffer with protease inhibitors to a final protein concentration that ranged from 40 to 50 μg/mL. These membrane fractions were frozen under liquid N_2 and stored at -80 °C before use in radio-ligand binding studies. Protein determination was performed as previously described[42].

Radio-ligand receptor binding

Membrane fractions were incubated with [^{3}H]DPDPE (0.1 nmol/L, 50.6 Ci/mmol), [^{3}H]DAMGO (0.1 nmol/L, 50.0 Ci/mmol), or [^{3}H]U69593 (0.1 nmol/L, 41.4 Ci/mmol) for binding to δ-, μ-, or κ-ORs, respectively. Samples were incubated for 90 min at 25 °C in a total volume of 2.0 mL 10 mmol/L Tris-HCl buffer containing 5 mmol/L $MgCl_2$ and protease inhibitors, with 15–20 μg of protein per tube. The reaction was terminated by rapid filtration through GF/C glass fiber filters (Whatman, Maidstone, UK) using a cell harvester (model M-48R; Brandel Inc, Gaithersburg, MD, USA), followed by two 5-mL rinses with ice-cold 50 mmol/L Tris-HCl buffer. Specific binding was defined as displacement of the radio-ligand by 1.0 μmol/L naloxone. Filters were counted by liquid scintillation spectrometry at an efficiency of 40% to 45%, as determined by external standards. The half-maximal inhibitory concentrations (IC_{50}) were obtained from the competition curves of the radio-ligand receptor binding assays. The apparent binding affinities (K_i) were calculated using the Cheng and Prusoff equation[43].

Opioid receptor-mediated PKAcat-eGFP redistribution assays

Cells were inoculated into 96-well plates (approximately 10 000 cells/well) and cultured as described above. After 24 h, the cells were incubated for 15 min in 200 μL F12 medium, F12 medium containing 10 μmol/L forskolin, or F12 medium containing varying concentrations of the test compounds in the presence of 10 μmol/L forskolin. Cells were then fixed with 200 μL/well 4% formaldehyde for 20 min, followed by three 200-μL rinses with PBS. Finally, 100 μL PBS containing 1 μmol/L Hoechst33342 was added for nuclear staining. Cytoplasmic fluorescence spot formation was measured using the IN Cell Analyzer 2000 Cellular Imaging and Analysis System (GE, Fairfield, CT, USA). Activity (%) was calculated relative to the positive (forskolin only) and negative (F12 medium) controls. The concentration-response data were fitted to sigmoidal curves to determine the 50% effective concentration

$$\text{Formula: activity (\%)} = \frac{\text{Drug(I–BI)×TA–Positive (I–BI)×TA}}{\text{Negative (I–BI)×TA–Positive (I–BI)×TA}}$$

(EC_{50}) values and the maximal effects (E_{max}).

I: Intensity; BI: Background intensity; TA: Total Area

Selective standard agonists (SNC80 for δ-OR, DAMGO for μ-OR and (±)U50488 for κ-OR) were used to normalize the E_{max} and EC_{50} values for all test compounds for each receptor type. The E_{max} values obtained for SNC80, DAMGO and (±)U50488 were set at 100% for their respective receptors. These values were then used to reconstruct and normalize the concentration-response curves and to obtain the EC_{50} values for all test compounds and the E_{max} values for the five mixed opioid analogs.

Blood gas and antinociceptive studies

Male albino Wistar Hannover rats weighing 200 to 300 g were anesthetized with 2% isoflurane in a 30% O_2 and 70% N_2O vehicle. The femoral artery and external jugular vein were cannulated with silastic tubing. Anesthetic gases were then discontinued, and the rats were allowed to recover in a plastic restrainer for 60 min before administration of the test compounds. Arterial blood gasses and antinociceptive responses were measured simultaneously.

Blood gas measurements were conducted as previously described[35]. Briefly, every five minutes after intravenous administration of a test compound, 0.15 mL arterial blood was drawn into a syringe with heparin. The syringe was capped, and the blood was analyzed immediately (Ph/Gas analyzer Synthesis 25 model; Instrumentation Laboratory, Lexington, MA, USA). The ED_{50} pCO_2 was defined as the dose of a compound that produced a 50% increase in the plasma pCO_2 level.

Analgesic effects were measured soon after blood was drawn, using a modified Haffner's tail-pinch protocol with a fixed pressure developed by Takagi *et al*[44]. Briefly, straight Blalock artery clamps (7 cm long, branches 2.7 cm long) producing approximately 1100 grams of pressure at the center point of the branches (0.8 cm from the tip, pressure measured by a spring scale) were used for this test. The test was performed with the rat in a plastic cage. A clamp was placed on the tail (1 inch from the tip) and was left in place until an escape response occurred (*ie*, tail-flick, clamp-biting or vocalization) or for a maximum time of 20 s. The escape response time was recorded using a stopwatch. The basal response time for each rat was determined to be approximately 2 s. Rats with unusual response times were excluded from the experiment. To evaluate analgesic effects, the response time for each individual rat was measured and recorded before and after the injection (iv) of the test compounds. The potency of compounds was determined based on the recorded response times after compound administration, rather than the partial analgesia (response time 2 to 6 s) and complete analgesia (response time >6 s) classification adopted by Takagi *et al*[44]. The latency of the escape response was calculated and converted into a percent maximal possible effect score, as described below. The determination of dose-response relationships and statistical analyses were performed using the Prism program.

Maximum percent effect (MPE%)=(response time after compound administration–basal response time)/(maximum time–basal response time)×100%. (Maximum time=20 s)

The analgesic ED_{50} values of morphine and fentanyl were determined by this protocol in our previous work[34, 35] and found to be similar to their known antinociceptive potency in rats, thus confirming the validity of the protocol.

Data analysis and statistics

Pharmacological data *in vivo* were analyzed by linear regression of the linear portion of the dose-response curves, and the competition receptor binding data and concentration-response data in PKAcat-eGFP redistribution assays were fitted by nonlinear regression analysis. Correlations of *in vivo* effects (ED_{50} for antinociception and respiratory depression) with measures of receptor activity (EC_{50} for potency and K_i for binding affinity) were also analyzed by linear regression. Data were analyzed using the computer program Prism (GraphPad Software Inc, San Diego, CA, USA). All data in this study except ratio values are expressed as the mean±SEM.

Results

Binding affinity of opioid receptor triple agonists to opioid receptor subtypes

Following our reports that outlined the synthesis and pharmacology of the first nonpeptidic δ-OR agonist BW373U86[10, 13–16, 45], DPI-125, DPI-3290, DPI-130, KUST202 and KUST13T02 were identified in a structure-activity relationship study and subjected to further pharmacological testing. The binding affinities (K_i values) of these compounds are summarized in Table 1. Similar to our previous results[34, 35], these compounds exhibited mixed δ-, μ- and κ-OR ligand activities. Although there were slight differences among the different OR types, all compounds showed high affinities for δ-, μ-, and κ-ORs, with K_i values of approximately 1 nmol/L. The only exceptions were DPI-130, which had a far lower affinity for κ-ORs (K_i 21.8 nmol/L), and KUST13T02, which had a higher affinity for μ-ORs (K_i 0.26 nmol/L) and lower affinity for δ-ORs (K_i 16.5 nmol/L).

Table 1. Binding affinities for opioid receptor subtypes[a, b].

Compound	Receptor binding K_i (nmol/L)		
	δ-OR	μ-OR	κ-OR
DPI-125	0.97±0.30	0.36±0.10	1.36±0.39
DPI-3290[34]	0.18±0.02	0.46±0.05	0.62±0.09
DPI-130	0.40±0.24	1.58±0.63	21.8±4.6
KUST202	1.86±0.39	2.97±0.30	1.81±0.25
KUST13T02	16.5±0.51	0.26±0.05	1.89±0.31

[a] Radio-labeled standard OR agonists: [^{3}H]DPDPE (δ-OR), [^{3}H]DAMGO (μ-OR), or [^{3}H]U69593 (κ-OR). [b] Data represent mean±SEM from 3–4 separate experiments.

Development of PKAcat-eGFP redistribution assay for OR activation

ORs belong to the family of G-protein-coupled receptors (GPCRs)[46–48]. Upon the activation of ORs by opioids, GTP binds to pre-coupled Gα protein and cause the dissociation of the activated GTP-Gα and Gβγ subunits. These subunits then proceed to activate voltage-dependent K^+ channels and inactivate Ca^{++} channels, thus reducing membrane excitability, and reducing cyclic adenosine monophosphate (cAMP) levels by adenylyl cyclase inhibition. The activity of protein kinase A (PKA) is dependent upon intracellular cAMP levels. Using a previously described protocol[49–51], we used GFP-tagged catalytic subunits (PKAcat-eGFP) to monitor PKA activity as a measure of opioid receptor activation. In the resting inactive state, PKAcat-eGFP forms aggregates inside the cells. Upon activation by increased levels of cAMP, the eGFP-tagged catalytic subunit dissociates from PKA and disperses uniformly throughout the cytoplasm. This redistribution transition can be monitored under fluorescence microscopy and quantified to measure the activity of GPCR-coupled G_s upon activation. Forskolin activates adenylyl cyclase directly to increase cAMP levels and disperse the aggregated fluorescent subunits. The activation of ORs leads to the inhibition of adenylyl cyclase and a decrease in cAMP levels and can therefore be measured by the reappearance of aggregate spots in the presence of forskolin.

Potency of OR triple agonists in the PKAcat-eGFP redistribution assay

PKA is a ubiquitous serine/threonine protein kinase and a major mediator of intracellular cAMP signaling in eukaryotes whose activity is dependent on cellular levels of cAMP. When cytosolic cAMP level increases, two cAMP molecules bind to each PKA regulatory subunit of the R_2C_2 complex, the regulatory subunits dissociate from the catalytic subunits, and the free catalytic subunits are activated. In this assay, human δ-, μ- and κ-ORs were stably transfected into a GPCR reporter assay cell line for G_s- or $G_{i/o}$-coupled receptors that stably expresses the catalytic domain of human PKA (PKAcat) fused to eGFP. Binding of opioid agonists to these ORs causes the activation of the $G_{i/o}$ complex in intact cells, leading to the inhibition of adenylyl cyclase, a decrease in the formation of cAMP from ATP, and the reassociation of the PKA regulatory and catalytic subunits. This in turn leads to the aggregation of PKAcat-eGFP in cytoplasmic foci. In contrast, cells stimulated with forskolin generate high levels of cAMP, resulting in a diffuse distribution of PKAcat-eGFP in the cytoplasm. The redistribution of PKAcat-eGFP reflects changes in cytoplasmic cAMP concentrations in response to regulation by opioid receptors. This assay is conducted in intact cells rather than prepared membranes and may therefore provide results that are more germane to *in vivo* conditions.

Due to the heterogeneous distribution of ORs in the brain, comparison with standard agonists of each receptor type to correct for variation is a validated common practice for *in vivo* pharmacological studies. For *in vitro* studies on transfected cells, since the observed activity of an agonist can vary depending upon the receptor density on the cell surface, the use of standard agonists is also essential in assessing potency in different cell lines. The standard full agonists SNC80

(δ-OR), DAMGO (μ-OR), and (±)U50488 (κ-OR) increased the aggregation of PKAcat-eGFP in a concentration-dependent manner. The EC_{50} values and maximal effects (E_{max}), as shown in Figure 3 and Table 2, are consistent with values reported previously with GTPγ[^{35}S] binding assays[52]. The EC_{50} values are 6.30, 30.80 and 5.88 nmol/L for SNC80, DAMGO and (±)U50488, respectively (Table 2).

In general, most of our compounds showed high potency

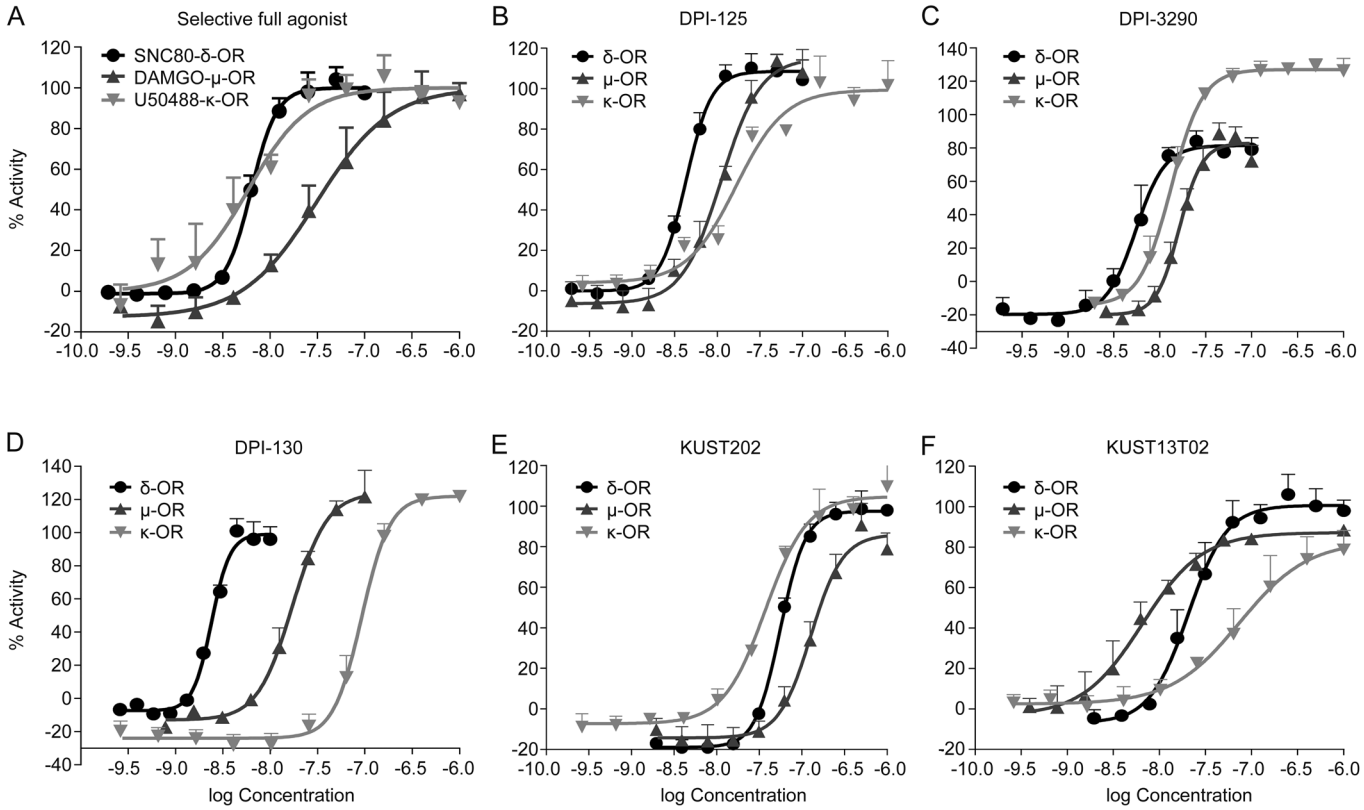

Figure 3. Concentration-dependent activities of PKA redistribution assay. ●: CHO-PKAcat-eGFP/δ-OR cell; ▲: CHO-PKAcateGFP/μ-OR cell; ▽: CHO-PKAcat-eGFP/κ-OR cell. (A)OR type selective full agonists; (B)DPI-125; (C) DPI-3290; (D) DPI-130; (E) KUST202; (F) KUST13T02. Concentration-response curves for all compounds were normalized after setting E_{max} for the standard agonists SNC80, DAMGO and (±)U50488 as 100% at their respective receptors. Each value represents the mean±SEM from at least three independent experiments performed in quadruplicate. The EC_{50} and E_{max} values were shown in Table 2.

Table 2. Receptor agonist potencies in PKA redistribution assay[a, b, c, d].

Compound	δ-OR		μ-OR		κ-OR	
	EC_{50} (nmol/L)	E_{max} (%)	EC_{50} (nmol/L)	E_{max} (%)	EC_{50} (nmol/L)	E_{max} (%)
SNC80	6.30±0.45	100.00±3.80				
DAMGO			30.80±2.53	100.00±3.67		
(±)U50488					5.88±1.57	100.00±2.34
DPI-125	4.29±0.36	108.60±6.51	11.10±3.04*	115±6.02	16.57±4.14*	99.40±8.89
DPI-3290	5.48±0.85	81.52±3.55	16.27±2.23*	83.00±4.68	13.29±1.93*	127.1±3.98
DPI-130	2.40±0.11	99.24±7.62	17.14±2.39**	124.00±17.17	92.58±8.75$^{**\#\#}$	122.00±1.46
KUST202	55.88±1.76	97.53±4.21	127.00±39.35	86.06±7.11	37.29±4.65$^{*\#}$	104.8±8.31
KUST13T02	20.64±5.44	107.80±4.56	6.54±1.71*	102.3±1.05	74.31±22.2$^{*\#}$	114.00±8.97

[a] EC_{50} and E_{max} of tested compounds were derived from the curves in Figure 2 by using CHO-PKAcat-eGFP/δ-OR, CHO-PKAcat-eGFP/μ-OR, and CHO-PKAcat-eGFP/κ-OR cells. [b] Positive controls are the standard agonists: SNC80 for δ-OR, DAMGO for μ-OR, and (±)U50488 for κ-OR. [c] EC_{50} and E_{max} were estimated and normalized after setting E_{max} for the standard agonists as 100% for their corresponding receptor. [d] Data represent mean±SEM from at least three independent experiments performed in quadruplicate. Student's *t*-test was used. $^{*}P<0.05$, $^{**}P<0.01$ compared with EC_{50} at δ-OR of the same compound. $^{\#}P<0.05$, $^{\#\#}P<0.01$ compared with EC_{50} at μ-OR of the same compound.

for each receptor, and the E_{max} values before normalization were greater than 70% of that for the forskolin control. However, the EC_{50} values were significantly different. The activity of DPI-3290 was consistent with our previous results measuring OR-mediated inhibition of tension development in the vas deferens of mice[34]. The EC_{50} values for DPI-3290 for δ-, μ- and κ-opioid receptors were 5.48, 16.27, and 13.29 nmol/L, respectively (Figure 3C, Table 2); these are lower than or similar to values for the corresponding standard agonists. The EC_{50} values for DPI-125 for δ-, μ- and κ-opioid receptors were 4.29, 11.10 and 16.57 nmol/L (Figure 3B, Table 2), respectively, similar to the activities of DPI-3290. Treatment with DPI-125 caused aggregation of PKAcat-eGFP in the redistribution assays for all three ORs. DPI-130 activities were lowest for κ-ORs, with EC_{50} values of 2.40, 17.14 and 92.58 nmol/L for δ-, μ- and κ-opioid receptors, respectively (Figure 3D, Table 2).

The percent maximal responses induced by the compounds ranged from 80% to 130% (Table 2). The potency of a compound is proportional to its binding affinity and activation efficacy at a particular receptor and negatively related to the EC_{50} values of the agonist concentration-response curves from the PKA redistribution assay (*ie*, lower EC_{50} values reflect higher potency and *vice versa*). The potency of DPI-3290 was highest for δ-ORs (EC_{50} 5.48±0.85 nmol/L), followed by κ-ORs (EC_{50} 13.29±1.93 nmol/L) and μ-ORs (EC_{50} 16.27±2.23 nmol/L) (Figure 3C, Table 2). The difference in potency between κ- and μ-ORs was not significant. The potency of DPI-130 was highest for δ-ORs (EC_{50} 2.40±0.11 nmol/L), intermediate for μ-ORs (EC_{50} 17.14±2.39 nmol/L) and lowest for κ-ORs (EC_{50} 92.58±8.75 nmol/L) (Figure 3D, Table 2). Similarly, the potency of DPI-125 was highest for δ-ORs (EC_{50} 4.29±0.36 nmol/L), intermediate for μ-ORs (EC_{50} 11.10±3.04 nmol/L), and lowest for κ-ORs (EC_{50} 16.57±4.14 nmol/L) (Figure 3B, Table 2). The difference in potency between κ-ORs and μ-ORs was not significant. In contrast, KUST13T02 exhibited potent activity for μ-ORs (EC_{50} 6.54±1.71 nmol/L) but less potent activity for δ-ORs (EC_{50} 20.64±5.44 nmol/L) and much weaker activity for κ-ORs (EC_{50} 74.31±22.2 nmol/L) (Figure 3F, Table 2). KUST202 showed relatively weak activities at all three receptors, with a maximum potency for κ-ORs (EC_{50} 37.29±4.65 nmol/L) (Figure 3E, Table 2).

OR triple agonist-mediated antinociception and hypercapnia in rats

A tail-pinch test was used in this study, as it allows experiments on the antinociception and respiratory depression effects of opioid compounds to be conducted simultaneously in the same animal[34, 35]. This test was reported by Takagi *et al*[44] as a modification of the original Haffner's test[53]. In our experiments, a straight Blalock artery clamp was placed on the tail one inch from the tip to provide a noxious stimulus. The chosen clamps produced pressures of approximately 1100 g at the center point of the branches. The basal response time to this stimulus was recorded for each animal and was approximately 2 s. The response time increased under the influence of opioids, depending upon the potency of the compound and the dose administered to the animals. The cut-off maximum time was set at 20 s to avoid tissue damage. The maximum percent effect (MPE%) was then calculated, and the determination of dose-response relationships and statistical analyses were performed using the Prism program.

As expected, the tested compounds presented the same characteristics as typical narcotic analgesics because of their strong binding affinity and high activity for μ-ORs. Table 3 shows that the antinociceptive ED_{50} values of DPI-125 and DPI-3290 were estimated as 0.050±0.005 and 0.050±0.007 mg/kg (iv) in the rat tail-pinch test, much better than that of morphine (ED_{50} 2.01±0.0005 mg/kg) under the same conditions. DPI-130 exhibited slightly weaker antinociceptive effects (ED_{50} 0.08±0.007 mg/kg), while KUST202 showed the weakest effects (ED_{50} 0.16±0.02 mg/kg). KUST13T02 showed the highest potency at μ-ORs in the PKA redistribution assay and potent *in vivo* antinociception activity (ED_{50} 0.004±0.0003 mg/kg).

Table 3. Opioid receptor subtype potency ratios and respiratory safety ratios[a, b, c].

Compound	EC_{50} ratio of receptor potency			ED_{50} of tail-pinch (mg/kg, iv)[d]	ED_{50} of pCO_2 (mg/kg, iv)	Safety ratio (pCO_2: tail-pinch)
	μ/δ	κ/δ	μ/κ			
Morphine[35]	–	–	–	2.01±0.0005	4.23±0.72	2.1
Fentanyl[35]	–	–	–	0.0034±0.0002	0.0127±0.0035	3.7
DPI-125	2.587	3.862	0.670	0.05±0.005	0.72±0.21	14.4
DPI-3290[35]	2.969	2.425	1.224	0.05±0.007	0.91±0.22	18.2
DPI-130	7.142	38.575	0.185	0.08±0.007	2.15±0.80	26.9
KUST202	2.273	0.667	3.406	0.16±0.02	2.2±0.40	13.8
KUST13T02	0.317	3.600	0.088	0.004±0.003	0.034±0.006	8.5

[a] Receptor potency ratios are between OR subtype ED_{50} in PKA redistribution assay calculated from Table 2. [b] Safety ratios are between ED_{50} of opioid-mediated hypercapnia (pCO_2) and ED_{50} of opioid-mediated antinociception (tail-pinch assay) in conscious laboratory rats. [c] ED_{50} of opioid-mediated hypercapnia (pCO_2) and ED_{50} of opioid-mediated antinociception (tail-pinch assay) represent mean±SEM from 6–8 independent animals. The relationship between safety ratios and μ/δ, μ/κ or κ/δ EC_{50} ratios of receptor agonist potency were analyzed by liner regression analysis (Figure 4C) by Prism program. The relationship between the ED_{50} of antinociception (Figure 4A) or hypercapnia ED_{50} (Figure 4B) and μ-receptor potency were analyzed similarly. [d] Saline was used as the vehicle control.

The respiratory depression effects of these compounds were also measured simultaneously in conscious laboratory rats (Table 3). Consistent with our previous results[35], these compounds produced pCO_2 increases like morphine and fentanyl but at doses markedly higher than those necessary for antinociception. The ratio between the respiratory depression and antinociception activities [ED_{50} (pCO_2 increase)/ED_{50} (antinociception), respiratory safety ratio] provides a measure of the safety profile of opioid analgesics. The ED_{50} values for respiratory depression (pCO_2 increase) of DPI-125, DPI-3290, DPI-130, KUST202 and KUST13T02 were 0.72, 0.91, 2.15, 2.2 and 0.034 mg/kg, respectively, which were 14.4-, 18.2-, 26.9-, 13.8- and 8.5-fold higher, respectively, than their ED_{50} values for antinociception. The safety ratios of morphine and fentanyl are much lower (2.1 and 3.7) than those noted for these novel test compounds.

To gain further insight into the properties of these compounds, we conducted linear regression analyses on the above data. Unsurprisingly, plots of the antinociception ED_{50} values against the logarithm of potency ($logEC_{50}$) for μ-ORs in the CHO-PKAcat-eGFP/μ-OR redistribution assay showed a high positive correlation (R^2=0.955, P=0.0041 for the regression line) (Figure 4A). However, the correlation between ED_{50} values for pCO_2 increase and $logEC_{50}$ values for μ-ORs was poor (R^2=0.618, P=0.1147 for the regression line) (Figure 4B), suggesting that other factors are involved. It is well accepted that respiratory depression is mediated primarily by μ-ORs. Consistent with previous reports that δ-OR agonists can reverse μ-OR agonist-induced respiratory depression[25], these results suggest that δ-ORs play an important role as suppressors of μ-OR agonist-induced respiratory depression.

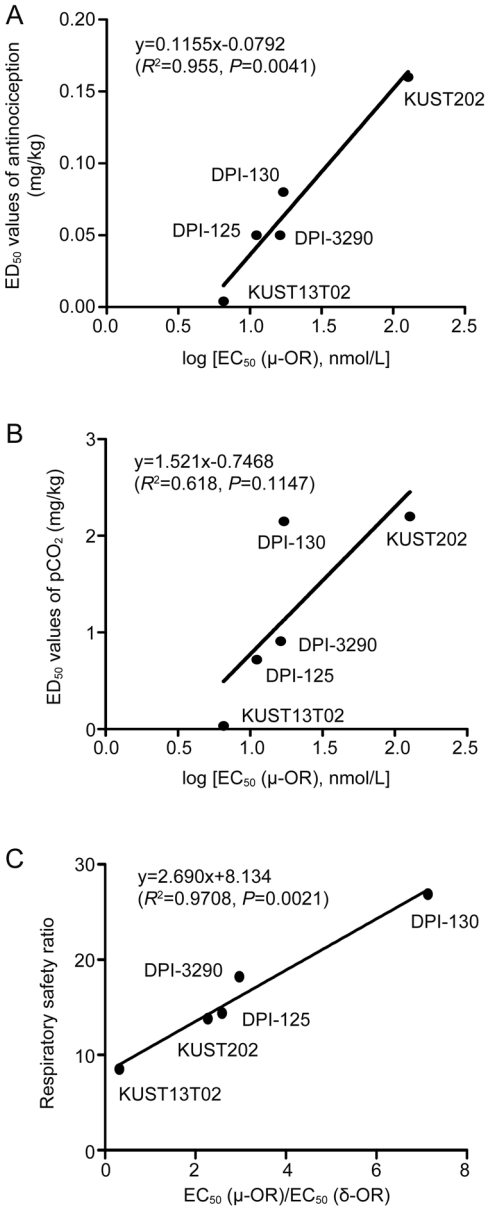

Figure 4. Correlation of ED_{50} values of antinociception and respiratory depression with receptor agonist potencies. (A) Correlation between antinociception ED_{50} values and logarithm of potency (log EC_{50}) at μ-OR. (B) Correlation between respiratory depression ED_{50} values and logarithm of potency (log EC_{50}) at μ-OR. (C) Correlation between the respiratory safety ratio and the ratio of receptor potency EC_{50} (μ-OR)/EC_{50} (δ-OR). Data were shown in Table 3. Safety ratios determined by ED_{50} values of opioid-mediated hypercapnia divided by ED_{50} values of opioid-mediated antinociception in conscious laboratory rats. Linear regression was analyzed by the Prism program.

Positive correlation between the μ-/δ-OR EC_{50} potency ratio and the respiratory safety ratio

It is well known that analgesia and respiratory depression are mainly mediated *via* μ-ORs. The receptor binding affinities and potencies reported above provide further insight into the *in vivo* effects of the tested compounds. The OR selectivity of these compounds, *ie*, the ratios of potency between OR subtypes (μ/δ, κ/δ and μ/κ EC_{50} ratios), are shown in Table 3 and plotted against the respiratory safety ratios. Figure 4C shows an excellent positive correlation for the safety ratio with the μ/δ EC_{50} ratio (R^2=0.97, P=0.0021 for the regression line) but not with the κ/δ and μ/κ EC_{50} ratios (data not shown). These results explain why the compounds with a higher potency at δ-ORs than at μ-ORs are safer drugs, suggesting that the safety of opioid analgesics could be improved by combining them with δ-OR agonists or using δ- and μ-OR mixed agonists instead.

On the other hand, Figure 5 shows that both antinociceptive effects (Figure 5A, R^2=0.8769, P=0.0191) and respiratory depression (Figure 5B, R^2=0.9362, P=0.0070) are correlated with the binding affinity at μ-ORs but not that at δ-ORs or κ-ORs (data not shown). Figure 5C shows that the safety ratio is correlated with the μ/δ affinity ratio (R^2=0.8821, P=0.0178). However, the correlation (R^2) between antinociception and binding affinity (Figure 5A) is weaker than that between antinociception and μ-OR potency (Figure 4A), and the P-value is also much larger, reflecting a lower significance. Similarly, the observed correlation and significance in the regression analyses were lower for the affinity ratios (Figure 5C) than for the potency ratios (Figure 4C). This suggests that for these mixed

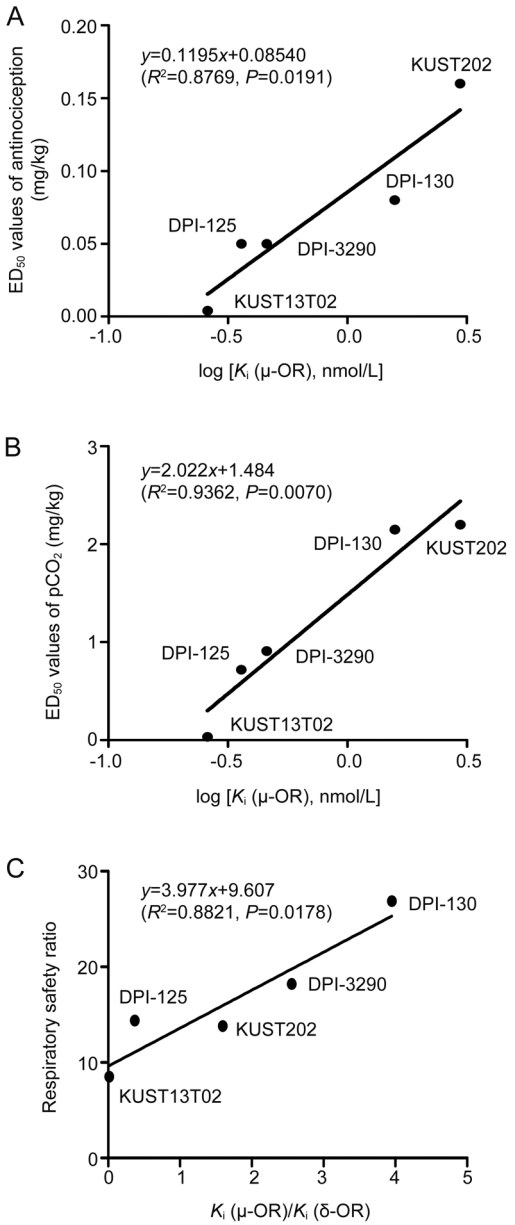

Figure 5. Correlation of ED_{50} values of antinociception and respiratory depression with receptor binding affinities. (A) Correlation between antinociception ED_{50} values and logarithm of receptor binding affinity ($logK_i$) at μ-OR. (B) Correlation between respiratory depression ED_{50} values and logarithm of receptor binding affinity ($logK_i$) at μ-OR. (C) Correlation between the respiratory safety ratio and the ratio of receptor binding affinity K_i (μ-OR)/EC_{50} (δ-OR). Data of receptor binding affinity were shown in Table 1. Safety ratios determined by ED_{50} values of opioid-mediated hypercapnia divided by ED_{50} values of opioid-mediated antinociception in conscious laboratory rats. Linear regression was analyzed by the Prism program.

agonists receptor potency is a more specific measure of respiratory safety than binding affinity.

Reinforcing effect of DPI-125 in rhesus monkeys

The reinforcing effects of DPI-125 were evaluated using a self-administration model in alfentanil-trained and alfentanil-maintained adult male rhesus monkeys, which is widely accepted for testing the abuse potential of various substances, including opioids and cocaine[39–41]; these evaluations were performed through the College on the Problems of Drug Dependence (CPDD) (see supplementary information). Four doses of DPI-125 were evaluated in four rhesus monkeys. Each monkey was tested at least twice at each dose. The dose-effect curve (mean±SEM) of DPI-125 self-administration was aggregated across all four monkeys and plotted as a percentage of the alfentanil-maintained response. DPI-125 was self-administered by all four monkeys in the study. The rates of response for DPI-125 were high across a dose range approximately 30-fold higher than that required to engender a contingent response with alfentanil. The maximal rate of response for DPI-125 at 0.01 mg/kg each injection peaked at approximately 70% of the alfentanil maximum positive control. At a higher DPI-125 dose of 0.03 mg/kg each injection, the rate of response dropped close to that of the vehicle control, yielding an inverted U-shaped curve. This comparison indicates that DPI-125 is 30-fold less potent and 30% less effective than alfentanil in terms of reinforcing effects, despite their approximately equal antinociceptive potency in rats[54].

Discussion

One objective of the present study was to determine the potency at each OR type for the selected compounds. The availability of three cloned cell lines expressing exclusively δ-, μ-, or κ-ORs made it possible to assess potency without receptor cross-interference, which is otherwise inevitable when using tissue samples. Assessing the potencies of mixed OR agonists for each type of receptor is essential in order to understand receptor signaling crosstalk *in vivo*. The ranking order of OR subtype potencies for the tested compounds was as follows: DPI-125, δ>κ>μ within a relatively small range; DPI-3290, δ>μ>κ within a relatively small range, similar to DPI-125; DPI-130, δ>μ>κ within a much wider range than DPI-125 and DPI-3290; KUST202, κ>δ>μ, most active for κ-ORs; and KUST13T02, μ>δ>κ, most active for μ-ORs (Figure 3, Table 2). Of note, the profile of KUST13T02 was very different from those of the other four compounds. Thus, these compounds provide a broad spectrum of receptor potencies to correlate with their *in vivo* pharmacology.

The analgesic effects of opioids are known to be mainly mediated by μ-ORs. The excellent correlation between antinociception (ED_{50}) in rats and receptor potencies (EC_{50}) for μ-ORs (Figure 4A) is consistent with this concept and validates the use of the PKAcat-eGFP fluorescence redistribution imaging analysis to measure the receptor potencies of opioids. Respiratory depression is also mediated by μ-ORs at higher dosages but can be observed only *in vivo*. μ-ORs located in the rostral ventromedial medulla nucleus of the brain stem are known to be responsible for opioid-induced respiratory depression[55, 56]. The reversal of μ-OR agonist-induced respiratory depression by δ-OR agonists was shown to occur in the central nervous system[25]. The poor correlation between ED_{50} values for pCO_2 increase and EC_{50} values for μ-ORs (Figure 4B) suggests that

other ORs may be involved in the regulation of respiratory depression. The excellent correlation (Figure 4C) between the ratio of ED_{50} values for pCO_2 increase/ED_{50} values for antinociception and the μ-/δ-OR EC_{50} potency ratio confirms the regulatory role of δ-ORs in μ-OR-mediated respiratory depression.

Recent studies of OR heteromers might provide new insights into the mechanisms underlying opioid effects. The formation of OR heteromers has been well described in the literature[57–62]. These heteromers might provide the foundation for various classes of interactions between ORs, resulting in cellular responses different from those associated with a single receptor type[63–65]. However, the physiological significance of OR heteromers remain largely unclear. It is possible that some adverse effects of opioids result from direct interactions between OR subtypes *via* receptor heteromers but others result from interactions between neurons with different ORs *via* neural networks or circuits. Further investigation and evidence are required to understand the link between molecular mechanisms and physiological observations.

Regardless of the molecular details of opioid effects, the present study supports the possibility of developing an opioid analgesic with lower respiratory depression side effects by combining δ-agonist and μ-agonist properties to create opioids with greater potency for δ-ORs than for μ-ORs. This type of opioid can produce analgesic effects *via* the activation of μ-ORs and simultaneously dampen μ-agonist-induced respiratory depression *via* its activity at δ-ORs, thus providing effective analgesia with greater safety (Figure 6). The present results suggest the possibility of a ten-fold improvement in the respiratory safety profile compared with that of morphine (Table 3). With the exception of KUST13T02, the tested compounds showed greater potency for δ-ORs than μ-ORs, as well as significant improvements in the safety index, which was 4.05 (8.5/2.1) times higher for KUST13T02 and 12.8 (26.9/2.1) times higher for DPI-130 than for morphine (Table 3). Thus, mixed OR agonists clearly have potential for clinical use.

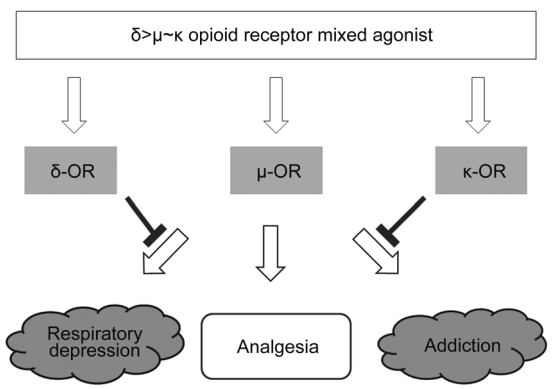

Figure 6. Hypothetic model of *in vivo* effect counteraction for mixed agonists with δ>μ~κ OR potency. Arrows and words represent effects mediated by μ-OR. T-shaped line ⊥ represents negatively regulation. The respiratory depression and addiction adverse effects of μ-OR activation are hypothetically reduced or eliminated by δ- and κ-OR activation, respectively. δ-, μ- and κ-ORs may locate in the same neuron, or in different neurons in the same/different brain areas connected through neural networks or circuit.

Furthermore, the potency ratios between μ-ORs and κ-ORs (Table 3) provide a plausible explanation for the observed reduced abuse potential of some δ-, μ-, and κ-OR mixed agonists. DPI-3290 is known to produce fewer withdrawal syndromes in rats than morphine[36]. The reinforcing effects of DPI-125 were also found to be far less potent and less effective than those of alfentanil in a rhesus monkey self-administration model[39–41] (see supplementary information).

It is worth noting that the dose-response curve of reinforcing effects for DPI-125 is similar to the results reported for nalbuphine[39], a mixed partial μ-agonist/κ-agonist that was a schedule II analgesic initially and was later removed from the list of controlled substances. When evaluated using the same model as in the present study, the reinforcing effects of nalbuphine occurred in a range of 0.0003 to 0.01 mg/kg each injection, indicating that this drug was approximately 10-fold less potent than alfentanil. The maximum effect reached was only approximately 50%, and the dose-response curve had an inverted U shape. In contrast, the reinforcing effects of traditional μ-agonist analgesics occur at much lower doses[39]. Alfentanil produces responses in a dose range of 0.00003 to 0.0003 mg/kg each injection. For highly addictive heroin, responses occur at a similar range of 0.00003 to 0.001 mg/kg each injection. The reinforcing effects of morphine are 10-fold less potent than those of alfentanil, which is consistent with its log-order lower analgesic potency compared to alfentanil. Furthermore, morphine is 40-fold less potent than DPI-125 in terms of analgesic potency (Table 3), but its reinforcing effects are approximately equivalent to those of DPI-125. Put together, these results suggest that, although DPI-125 may still be addictive, it has significantly less addiction liability than μ-agonist analgesics relative to its analgesic potency.

μ-ORs are known to mediate the development of physical and psychological dependence. Physical dependence arises from the chronic use of opioids and is precipitated by physical adaptation upon the withdrawal of opioids, whereas psychological dependence results from the euphoric effects of opioids. On the other hand, κ-agonists are known to induce dysphoria and aversive effects[26] opposite to the euphoric effects of μ-agonists[27]. Therefore, a mixed μ-OR/κ-OR agonist with an appropriate potency ratio may provide analgesia with reduced addiction potential. Indeed, κ-OR agonists and mixed μ-OR/κ-OR agonists have been shown to attenuate heroin self-administration in monkeys and rats[31–33]. Similar to the case of respiratory depression, it is also likely that the μ-OR and κ-OR signaling systems counteract each other *via* neural networks or circuits or through μ/κ-OR heteromers, resulting in a more neutral response (Figure 6).

The compounds investigated in the present study showed a broad range of μ-OR/κ-OR potency ratios (0.088 (KUST13T02), 0.185 (DPI-130), 0.670 (DPI-125), 1.224 (DPI-3290) and 3.406 (KUST202)) (Table 3). Notably, DPI-125 and DPI-3290 have the most balanced potency ratios among these compounds,

and they also showed more potent activity for κ-ORs than the other compounds (Table 2), strongly suggesting that the reduced withdrawal syndromes and reinforcing effects associated with these compounds are positively correlated with their κ-OR activity levels.

In addition, convulsion, a common adverse effect of some δ-OR selective agonists, such as BW373U86, appears to not be a problem with DPI-125. When tested in mice, the species known to be most sensitive to δ-OR agonist-induced convulsive effects, the lowest dose producing convulsion was 2–3 mg/kg iv, in one out of ten mice. This dosage is approximately 40-fold higher than the antinociceptive potency of DPI-125 (ED_{50} 0.05 mg/kg iv) and higher than the dosage that induces respiratory depression (ED_{50} 0.72 mg/kg iv). Finally, in all *in vivo* tests in rats, convulsion effects were not observed after iv administration of dosages that produced complete antinociception effects.

On the other hand, the correlation between agonist potency at each receptor subtype and the safety of the compounds suggests that comparing receptor binding affinities may not be sufficient to predict the safety of δ-, μ-, and κ-OR mixed agonists. In fact, many δ-, μ-, and κ-OR mixed ligands in our compound library did not show improved respiratory safety, which could not be predicted from their receptor binding affinity (not shown). Similarly, a large number of potential δ-, μ-, and κ-OR mixed agonists have been reported in the literature but with only binding affinity data available. Therefore, there is a need to continue searching for better δ>μ~κ mixed OR agonists based on receptor potency rather than receptor binding affinity.

In summary, the *in vitro* and *in vivo* pharmacology of five diarylmethylpiperazine compounds, including DPI-125, was assessed. Cell-based PKA fluorescence redistribution assays were used to measure potency for δ-, μ- and κ-ORs, and tail-pinch antinociception assays and blood gas measurements in rats were used to evaluate the analgesic and respiratory safety profiles. The abuse liability of DPI-125 was also evaluated in a self-administration rhesus monkey model. DPI-125 and the other four compounds are all mixed δ-, μ- and κ-OR agonists with various combinations of potencies. DPI-125 exhibited strong analgesic potency, high respiratory safety and reduced abuse tendency; these effects are presumably related to its strong δ-OR potency and balanced potencies for μ-ORs and κ-ORs. The fact that DPI-125 is safer and less addictive than traditional μ-agonist analgesics suggests that δ>μ~κ triple OR agonists may have significant clinical potential. In addition, our results emphasize the importance of further investigation of the interactions between OR signaling systems.

Acknowledgements

This study was financially supported by the Kunming University of Science and Technology (No 14078134), the Science and Technology Planning Project of Yunnan Province, China (No 2014FA002), the State Key Laboratory of Phytochemistry and Plant Resources in West China (No P2015-KF04), and the National Science and Technology Major Project of China (No 2012ZX09301003-001, 2012ZX09301003-003).

Author contribution

Shou-pu YI and Qing-hong KONG designed and performed the research, and wrote the first draft. Jie YU, Ben-qiang CUI, and Ying-fei WANG synthesized and characterized the tested compounds. Yu-lei LI and Chen-ling PAN performed the research. Guan-lin WANG, Pei-lan ZHOU, Li-li WANG, and Gang YU participated in experimental design. Ze-hui GONG, Rui-bin SU, and Gang YU revised and commented on the manuscript. Kwen-jen CHANG and Yue-hai SHEN supervised the project and wrote the manuscript with contributions from all the authors.

Supplementary information

Supplementary information is available on the website of Acta Pharmacologica Sinica.

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