A G Protein-Biased Ligand at the \( \mu \)-Opioid Receptor Is Potently Analgesic with Reduced Gastrointestinal and Respiratory Dysfunction Compared with Morphine

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Received November 5, 2012; accepted January 7, 2013

ABSTRACT

The concept of ligand bias at G protein-coupled receptors broadens the possibilities for agonist activities and provides the opportunity to develop safer, more selective therapeutics. Morphine pharmacology in \( \beta \)-arrestin-2 knockout mice suggested that a ligand that promotes coupling of the \( \mu \)-opioid receptor (MOR) to G proteins, but not \( \beta \)-arrestins, would result in higher analgesic efficacy, less gastrointestinal dysfunction, and less respiratory suppression than morphine. Here we report the discovery of TRV130 ([(3-methoxythiophen-2-yl)methyl][2-[(9R)-9-(pyridin-2-yl)-6-oxaspiro[4.5]decan-9-yl]-ethyl]amine), a novel MOR G protein-biased ligand. In cell-based assays, TRV130 elicits robust G protein signaling, with potency and efficacy similar to morphine, but with far less \( \beta \)-arrestin recruitment and receptor internalization. In mice and rats, TRV130 is potently analgesic while causing less gastrointestinal dysfunction and respiratory suppression than morphine at equianalgesic doses. TRV130 successfully translates evidence that analgesic and adverse MOR signaling pathways are distinct into a biased ligand with differentiated pharmacology. These preclinical data suggest that TRV130 may be a safer and more tolerable therapeutic for treating severe pain.

Introduction

Morphine is the archetypal opioid (Hamilton and Baskett, 2000) and is still considered a mainstay of analgesic therapy. It elicits analgesia by stimulating the \( \mu \)-opioid receptor (MOR), a G protein-coupled receptor (GPCR) highly expressed in the central nervous system and gastrointestinal tract. While extremely efficacious at relieving pain, opioids, as a class, also elicit a wide array of adverse events including respiratory suppression, sedation, and gastrointestinal dysfunction, including nausea, vomiting, and constipation. In addition, the euphoria and physical dependence associated with opioids can lead to abuse and addiction. Furthermore, patients can develop tolerance to opioid analgesia, necessitating dose escalation and risking worsening tolerability (Schneider and Kirsh, 2010). In the postoperative setting where opioids are widely used, nausea and vomiting delay recuperation and hospital discharge, as can opioid-induced constipation, especially in cases of postoperative ileus (Marderstein and Delaney, 2008). In addition, although opioid-induced respiratory suppression is rare, risk of its occurrence limits dosing (Dahan, 2007), contributing to the 29% of patients who report postoperative pain despite opioid use (Bostrom et al., 1997). In the outpatient settings of acute and chronic pain, the same array of undesirable pharmacologies exists. Constipation lowers the quality of life for over half of patients taking oxycodone for chronic pain (Anastassopoulos et al., 2011) and can be dose-limiting, as many patients would rather suffer reduced analgesia than continue opioid use and contend with serious gastrointestinal discomfort. Furthermore, respiratory suppression can be fatal in cases of overdose and is associated with nearly 15,000 deaths per year, a number that has escalated as opioid prescriptions have increased in recent years (Manchikanti et al., 2012). These adverse pharmacologic responses are elicited by all marketed opioid analgesics and have long been considered an immutable feature of strong MOR agonists. In rodents, these effects are reversible by MOR antagonists and absent in MOR knockout animals (Mathes et al., 1996; Sora et al., 1997; Kieffer, 1999), indicating they are “on-target” and cannot be improved by increasing receptor selectivity. However, numerous studies have shown that not all downstream signaling activities of a GPCR act with parallel, equivalent efficacies (Kenakin, 2011). Indeed, signal selectivity at a receptor can be targeted pharmacologically; “biased ligands” stabilize conformations that preferentially couple the receptor to particular...
intracellular pathways without coupling to others (Kenakin, 2011; Rajagopal et al., 2011). In extreme cases, a biased ligand is equivalent to a full agonist reference compound for one pathway but is inert, or even displays inverse efficacy, in another pathway stimulated by the reference ligand. Such a ligand, in a physiologic setting of an endogenous circulating agonist, can stimulate some receptor responses while inhibiting others (Violin et al., 2010; Kenakin, 2011). GPCR ligand bias has been observed between a number of intracellular signaling pathways, including coupling to different Go subunits (Spengler et al., 1993; Eason et al., 1994), and between G protein coupling and β-arrestin recruitment (Azzi et al., 2003; Gesty-Palmer et al., 2006; DeWire et al., 2007; Violin and Lefkowitz, 2007; Kim et al., 2008; Ma et al., 2009; Gesty-Palmer and Luttrell, 2011; Rosethorne and Charlton, 2011).

Evidence from knockout (KO) mice suggests that opioids signal through distinct MOR pathways, indicating that ligand bias might elicit differential MOR pharmacology. In β-arrestin-2 KO mice, morphine analgesia was enhanced and prolonged, with reduced desensitization, compared with morphine in wild type littermates (Bohn et al., 1999). Similar findings were noted in both mice and rats following injection of β-arrestin-2 interfering RNAs to specific brain regions (Li et al., 2009; Yang et al., 2011). In contrast, morphine-induced constipation and respiratory suppression were reduced in β-arrestin-2 KO mice versus wild type animals (Raehal et al., 2005). Thus at the MOR, β-arrestins serve as negative modulators of analgesia and positive modulators of some MOR-mediated adverse effects such as constipation, tolerance, and respiratory suppression. This is consistent with the understanding that β-arrestins not only desensitize a receptor’s G protein-mediated signaling but also stimulate distinct and independent cell-signaling outcomes (Wei et al., 2003; Rajagopal et al., 2006; Gesty-Palmer et al., 2009; Violin et al., 2010; Allen et al., 2011; DeWire and Violin, 2011; Audet et al., 2012).

It remains unclear if a G protein-biased ligand at the MOR can successfully recapitulate the improved profile of morphine in β-arrestin-2 KO mice. We sought to discover and characterize such a biased MOR ligand to test this possibility in hopes of developing a safer, better tolerated, and more efficacious opioid analgesic.

**Materials and Methods**

**Drugs and Chemicals**

TRV130 hydrochloride and trifluoroacetate were synthesized by the chemistry group of Trevena Inc. as described elsewhere (Yamashita et al., manuscript in preparation). [d-Ala², NMe-Phe⁴, Gly-ol⁵]-enkephalin (DAMGO) and buprenorphine were purchased with the 12-hour light/dark cycle. Assays were approved by the Institutional C57BL/6J mice or male Sprague-Dawley rats (Hilltop Laboratory Animals, Inc., Scottsdale, PA) housed in standard conditions with a 12-hour light/dark cycle. Assays were approved by the Institutional Animal Care and Use Committee and were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted by the U.S. National Institutes of Health. Studies of respiratory suppression were performed at Calvert Laboratories (Scott Township, PA), and pharmacokinetics studies were performed at ChemPartners, Inc. (Shanghai, China), under protocols reviewed and approved by the respective Institutional Animal Care and Use Committees.

**Cell Culture and Preparation**

Human embryonic kidney (HEK)-293 cells stably transfected to overexpress β-arrestin-2 fused to a β-galactosidase fragment were purchased from DiscoveRx (PathHunter β-arrestin assay; DiscoveRx Corporation, Fremont, CA) and the human OPRM1 gene (NM_000914.3, encoding human MOR), mouse OPRM1 gene (NM_001039562.1, mouse MOR), rat OPRM1 gene (NM_001038597.2, rat MOR), dog OPRM1 gene (XM_003638781, dog MOR), human OPRD1 gene (NM_000911.3, human δ-opioid receptor (DOR)), human OPKR1 gene (NM_000912.3, human KOR (κ-opioid receptor)), and human OPRL1 gene (NM_0026472.1, human nociceptin receptor (NOP)/ORL-1) receptors were fused to a complementary β-galactosidase fragment using the pCMV-ProLink plasmid purchased from DiscoveRx. Cells were grown in minimum Eagle’s medium with 10% fetal bovine serum, 1% penicillin/streptomycin, and 150 μg/l of neomycin and 150 μg/l of hygromycin.

**β-Arrestin-2 Recruitment**

The PathHunter enzyme complementation assay (DiscoveRx) was performed according to the manufacturer’s protocol, and read for chemiluminescence on a PheraStar plate reader (BMG Labtech, Durham, NC). Briefly, when β-arrestin-2 translocates to active receptor, the complementary β-galactosidase fragments fused to receptor and β-arrestin interact to form a functional enzyme, which is detected by chemiluminescence. For all in vitro assays, data were normalized as a percentage of maximal assay responses, typically defined by DAMGO-stimulated activity in the MOR assays, unless indicated otherwise.

**Cyclic Adenosine Monophosphate Accumulation**

Receptor G protein-mediated responses were determined by measuring changes in cAMP using the cAMP–homogenous time-resolved fluorescence kit (Cusbio, Codolet, France), using the same cell lines as used to measure β-arrestin recruitment. MOR, KOR, DOR, and NOP all couple to Goi so G protein coupling was measured as inhibition of forskolin-stimulated cAMP accumulation in the presence of 1.5 μM NKH-477 (water-soluble forskolin, Tocris catalog #1603) and 500 μM 3-isobutyl-1-methylxanthine (IBMX). cAMP accumulation assays were run in parallel with β-arrestin-2 recruitment, using the same cells, drug dilutions, and assay buffers [1% dimethylosil- oxide (DMSO), P12 Ham’s buffer] to ensure accurate assay-to-assay comparisons of data. Plates were read using a time-resolved fluorescence ratio (665 nm/620 nm) on a PheraStar plate reader.

**Internalization Assay**

The PathHunter GPCR internalization assay (DiscoveRx) was performed according to the manufacturer’s protocol. Briefly, U2-osteosarcoma (U2-OS) cells stably expressing the human MOR with complementary pieces of β-galactosidase were genetically fused to the receptor and a component of the endocytic vesicle, respectively. When coexpressed, the two fusion proteins detect receptor localization to endosomes, detected by chemiluminescence.

**Immunoblotting**

HEK-293 cells stably expressing the human MOR were grown in minimum Eagle’s medium supplemented with 10% fetal bovine serum. After overnight serum starvation, cells were stimulated with 1 μM DAMGO, morphine, fentanyl, TRV130, or vehicle for 5 minutes. Lysates were fractionated by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with phospho-MOR serine 375 antibody per manufacturer’s instructions (Cell Signaling Technologies, Danvers, MA; catalog number 3451). The immunoreactive band was visualized by chemiluminescence.
for the MOR was visualized and quantified using GeneSnap and GeneTools software (Syngene USA, Frederick, MD).

**Radioligand Binding Assays**

Equilibrium binding of unlabeled compounds was measured by inhibition of radioligand binding ([^3]H-diperenorphine) to HEK cell membranes expressing human MOR, KOR, and DOR. Unlabeled ligand and buffer (50 mM HEPES, 5 mM MgCl₂, 1 mM EGTA and 0.05% bovine serum albumin, pH 7.2, at 23°C) containing radioligand were added to polypropylene 96-well plates (Costar Corp., Cambridge, MA). Assays were initiated by the addition of membrane (5–10 µg protein/well) suspension. The concentration of[^3]H-diprenorphine during behavioral observations. Further details are available in the Supplemental Methods. Experimenter was blind to the treatment of animals noted above. Experimenter was blind to the treatment of animals baseline response of 3 OH), which was set at a temperature that would produce a consistent placed on the tail-flick apparatus (Columbus Instruments, Columbus, OH). Filters were washed 2 times with 0.5 ml of ice-cold phosphate buffered saline pH 7.0 containing 0.01% Triton X100. Radioactivity on the filters was quantified using a MicroBeta TriLux Liquid Scintillation Counter (PerkinElmer Life and Analytical Sciences, Boston, MA). Assays were initiated by the addition of membrane (5–10 µg protein/well) suspension. The concentration of[^3]H-diprenorphine (specific activity 50–52 Ci/mmol; PerkinElmer Life and Analytical Sciences, Boston, MA), was 0.5–1 times the independently determined Kᵢ. Compounds were diluted in DMSO and tested at a final concentration of 1% DMSO. Nonspecific binding was defined in the presence of 1 µM naltrexone. Competition assays were performed at 23°C for 3–4 hours to allow adequate time for equilibrium binding. In all assays, total radioligand bound to the filter was less than 10% of the total radioligand added. The separation of bound from free radioligand was accomplished by rapid vacuum filtration of the incubation mixture over GF/B filter mats using a Brandel cell harvester (Brandel, Gaithersburg, MD). Filters were washed 2 times with 0.5 ml of ice-cold phosphate buffered saline pH 7.0 containing 0.01% Triton X100. Radioactivity on the filters was quantified using a MicroBeta TriLux Liquid Scintillation Counter (PerkinElmer Life and Analytical Sciences). Further details are available in the Supplemental Methods.

**Hot Plate Studies**

The hot plate test is adapted from that described previously (Tyers, 1980). Male C57BL/6J mice (20–25 g) or male Sprague-Dawley rats (200–250 g) were acclimated to the vivarium for 48 hours. Animals were placed individually on a heated surface (56°C for mice, 52°C for rats) and the time interval (seconds) between placement and a shaking, licking, or tucking of the hind paw was recorded as the predrug latency response. This same procedure was repeated 30 minutes after s.c. administration of compound in 10% ethanol, 10% cremophor, 80% water (10/10/80). All compounds were administered s.c. in a volume of 1 ml/kg with vehicle adjustments made for mouse dosing. The cutoff time, designed to prevent injury to the animals, was 30 seconds (with vehicle latencies of approximately 5–10 seconds). The percent maximum possible antinociceptive effect [% maximum possible effect (MPE)] was determined using the formula:

\[
\text{Percent MPE} = \left( \frac{(\text{Post drug latency} - \text{baseline latency})}{\text{baseline latency}} \right) \times 100
\]

(1)

We used the predrug latency of each animal and cutoff times as noted above. Experimenter was blind to the treatment of animals during behavioral observations. Further details are available in the Supplemental Methods.

**Fecal Boli Assay.**

Mice (male C57BL/6J, 20–25 g) were fasted overnight to allow an accurate measurement of colonic transit. Animals were allowed free access to water the night before the experiment. On the day of the experiment animals were weighed and compounds were administered s.c. (as in hot plate studies), 20 minutes after animals were placed individually in a testing chamber. Fecal boli were removed from each animal's test chamber and weighed hourly for four hours (Raehal et al., 2005). Because TRV130 had a weak effect in this assay, the Hill slope was fixed to 1.0 to allow curve fitting to determine the ED₅₀. 100% MPE is defined in this assay as zero grams fecal boli production, and vehicle-treated animals define the 0% effect boundary.

**Rat Blood Gas Studies**

Briefly, respiratory suppression was determined using male Sprague-Dawley rats (200–250 g) surgically prepared with carotid artery catheters. Animals were administered either vehicle, TRV130 at doses of 0.3, 0.6, 1.2, and 2.4 mg/kg s.c. or morphine 3, 6, 12, and 24 mg/kg, s.c. in a volume of 2 ml/kg prepared in the same vehicle solution as was used as in the rat hot plate, tail-flick, and incisional pain assays. Articular blood samples (approximately 0.5 ml/sample) were collected from the arterial catheter from all animals at predose, 5, 30, 120, and 240 minutes following dosing for pCO₂, pO₂ and pH measurement. Rats were observed for behavioral changes prior to dosing, and approximately 30 minutes, 2 and 4 hours postdose.

**Pharmacokinetics**

Plasma and brain levels of TRV130 were determined by ChemPartner after i.v. or s.c. (10/10/80 vehicle) dosing of the species listed in Table 2 by standard mass spectrometry bioanalytical methods. Brain levels of TRV130 were measured in mice. Over an 8-hour period, samples were taken at 0, 5, 15, 30 minutes, 1, 2, 4, and 8 hours in all species. Standard pharmacokinetic calculations were used to produce values in Table 2.

**Quantification of Ligand Bias**

We applied an “equiactive comparison” between signaling pathways previously described (Griffin et al., 2007; Rajagopal et al., 2011). Estimates of the intrinsic relative activity (RAᵢ) for both the G protein (RAᵢG) or β-arrestin signaling (RAᵢβarr) pathways are calculated...
using eq. 2. The bias ratio for efficacy of G protein signaling versus β-arrestin is determined as the ratio of these intrinsic relative activities.

\[
RA_i = \frac{E_{\text{max}}^\text{ref} \cdot EC_{50}^\text{lig}}{E_{\text{max}}^\text{lig} \cdot EC_{50}^\text{ref}}
\]

This method assumes that measured responses reflect ligand binding at equilibrium and that Hill slopes are 1.0; both of these assumptions are supported by the data presented here. In addition, we used the “equimolar” comparison of compound responses at the same ligand concentrations for both G protein and β-arrestin-2 pathways to qualitatively demonstrate bias (Rajagopal et al., 2011).

Statistics

Statistics were calculated using Prism version 5 (GraphPad Inc., San Diego, CA). Compound ED50 values were determined using a computer-assisted nonlinear regression analysis of the dose-response curve. We also measured one way analysis of variance using GraphPad Prism with a Tukey-Kramer multiple comparison posttest (GraphPad Software).

Results

**TRV130 Is a G Protein-Biased Ligand at the MOR.** To identify G protein coupling-biased ligands of the human MOR (hMOR), we screened Trevena’s internal chemical library, measuring both G protein coupling (by inhibition of forskolin-stimulated cAMP accumulation) and β-arrestin-2 recruitment to the hMOR (by enzyme complementation). Several active compounds in a related chemical series were identified and further optimized for potency, ligand bias, and selectivity, resulting in the discovery of [(3-methoxythiophen-2-yl)methyl]([2-(9R)-9-(pyridin-2-yl)-6-oxaspiro[4.5]decan-9-yl]ethyl)amine (TRV130; depicted in Fig. 1). This compound shows no structural similarity to morphine, fentanyl, or other previously described MOR agonists.

In HEK cells, TRV130 displays G protein-coupling efficacy comparable to morphine (71 and 92%, respectively, of the full agonist DAMGO) and higher potency than morphine (8 nM versus 50 nM), but TRV130 displays approximately 14% of the efficacy of morphine for β-arrestin-2 recruitment (Fig. 2). Morphine itself is a partial agonist in the β-arrestin-2 assay, as DAMGO has 8.8-fold higher efficacy and fentanyl has 4.8-fold higher efficacy than morphine (Supplemental Table 1). This is consistent with the potency and efficacy of morphine in a bioluminescence resonance energy transfer assay (Molinari et al., 2010). When we applied a mathematical model for the quantification of ligand bias, the ratio of intrinsic relative activities proposed by Ehlerdt and colleagues (Griffin et al., 2007) and later applied to G protein- versus β-arrestin-ligand bias (Rajagopal et al., 2011), we find TRV130 possesses approximately 3-fold preference for the G pathway over β-arrestin-2 relative to morphine and fentanyl (Supplemental Table 2). The low β-arrestin-2 efficacy of TRV130 leads to an uncertain EC50, precluding a statistical comparison of this bias; however, bias is also reflected qualitatively in an “equimolar” comparison of G protein and β-arrestin responses for TRV130 and morphine (Supplemental Fig. 6) (Rajagopal et al., 2011). Thus, TRV130 appears unique compared with widely used opioid analgesics: It is a strong agonist like morphine but fails to robustly engage β-arrestin-2 recruitment. Similar in vitro results for the potencies and efficacies of TRV130 with respect to this set of compounds were observed in G protein and β-arrestin-2 coupling assays for the mouse, rat, and dog MORs (Table 1). In addition, TRV130 bound with high affinity to human, mouse and rat MOR species orthologs (Table 1).

In general, β-arrestins desensitize GPCRs to reduce G protein signaling while promoting receptor internalization and stimulating G protein-independent signaling (DeWire and Violin, 2011). In accord with its reduced efficacy for β-arrestin recruitment, TRV130 exhibited minimal receptor internalization in a highly sensitive enzyme complementation assay of receptor endocytosis (Fig. 3A). Morphine caused significant internalization of the MOR in this assay, at a level approximately 40% that of the maximal responses of fentanyl and DAMGO, consistent with modest ability of morphine to promote receptor internalization (Haberstock-Debic et al., 2005). Similarly, TRV130 caused significantly less receptor phosphorylation at serine 375 on the carboxy-terminal region of the MOR than did several other strong opioids (Fig. 3B). This again is consistent with the lack of β-arrestin engagement by TRV130, since carboxy-terminal receptor phosphorylation is a major driver of β-arrestin coupling to receptor (Tohgo et al., 2003). Interestingly, morphine was a full agonist for the phosphorylation of this serine on the receptor, consistent with literature reports (Doll et al., 2011), and further supporting that compared to morphine, TRV130 selectively fails to engage the G protein-coupled receptor kinase/β-arrestin/receptor internalization axis of receptor function.

Naloxone shifted the EC50 of TRV130-evoked G protein coupling in a concentration-dependent manner consistent with a competitive mechanism of action (Fig. 4A). Fitting the data to a modified Gaddum-Schild competitive interaction model (Lazareno and Birdsall, 1993; Lew and Angus, 1995) supported this conclusion. In the same manner, TRV130 competitively displaced the EC50 of DAMGO-stimulated β-arrestin-2 recruitment (Fig. 4B), again consistent with a competitive interaction and a binding site for TRV130 that is shared with classic opioid ligands. Consistent with this finding, kinetic radioligand binding studies revealed a residence time of approximately 2 minutes for TRV130 at the hMOR, which is similar to the residence times of morphine and fentanyl (Supplemental Table 3).

We also measured TRV130 activity at the human KOR, DOR, and NOP and found that the compound is remarkably selective for the hMOR (Table 1). TRV130 is approximately 400-fold selective for the MOR over the KOR, DOR, and NOP receptors in our cell-based assays. In contrast, morphine is only 10-fold selective for the MOR over the KOR and DOR opioid subtypes. In addition, TRV130 had more than 130-fold
selectivity for the hMOR over human a2C, D2S, D3, 5-hydroxytryptamine 1a, and σ receptors in radioligand displacement studies, and weak binding (defined as < 50% inhibition of binding at 10 μM) to 120 other receptors, channels, and enzymes (Supplemental Table 4 and Supplemental Methods). Thus TRV130 is a potent, selective, and G protein-biased ligand of the MOR.

**TRV130 Is a Rapid and Powerful Analgesic in Rats and Mice.** To assess the analgesic activity of TRV130, we ran a battery of assays in rodents. First, TRV130 was compared with morphine for antinociceptive activity in the 56°C hot plate assay in mice, which is sensitive only to highly efficacious analgesics like strong opioids, and involves both spinal and supraspinal responses (Le Bars et al., 2001). TRV130 displays an ED50 of 0.9 mg/kg and morphine displays spinal and supraspinal responses (Le Bars et al., 2001). efficacious analgesics like strong opioids, and involves both plate assay in mice, which is sensitive only to highly efficacious analgesics like strong opioids, and involves both spinal and supraspinal responses (Le Bars et al., 2001). TRV130 displays an ED50 of 0.9 mg/kg and morphine displays an ED50 of 4.9 mg/kg, with both compounds showing maximal analgesic efficacy in the assay (Fig. 5A; Supplemental Table 5). In rats, TRV130 was also robustly analgesic at 5 minutes compared with 30 minutes for morphine, but the duration of action for TRV130 and morphine were similar at approximately 90 minutes (Fig. 5B). TRV130 analgesia was also reversible in mice by administration of 3 mg/kg naloxone s.c. 15 minutes after TRV130 dosing (Supplemental Fig. 1), demonstrating that the observed analgesia is MOR-mediated, and also supported by the short residence time of TRV130 on the receptor (Supplemental Table 3). In rats, TRV130 was also robustly analgesic in several assays. In the rat 52°C hot plate, TRV130 was 10-fold more potent than morphine (ED50 of 0.32 and 3.2 mg/kg, respectively) (Fig. 6A; Supplemental Table 5). TRV130 showed similarly increased potency compared with morphine in the rat tail-flick model (Fig. 6B; Supplemental Table 5) and rat hindpaw incisional pain model (Fig. 6C).

These pharmacodynamic findings are consistent with the pharmacokinetics of TRV130, which shows rapid brain penetration (67% of plasma drug levels are achieved in brain samples, and peak brain exposure was observed at 15 minutes postdosing, unpublished data) and an exposure time-course similar to the analgesic time-course (Table 2). The pharmacokinetics of TRV130 after intravenous bolus in a variety of preclinical species showed relatively high volumes of distribution and largely monophasic clearance, similar to morphine (Table 2). This profile suggests TRV130 will be generally similar to medium-duration analgesics like morphine and hydromorphone, but with more rapid brain penetration consistent with the lipophilicity of TRV130, as reflected in calculated log P values (3.19 for TRV130 and 0.87 for morphine). Additionally, this profile suggests that TRV130 and morphine can be compared fairly in the rodent studies used here, some of which are time-sensitive. TRV130 showed extensive first pass metabolism and less than 6% oral availability in rats and mice (unpublished data).

**TRV130 Possesses An Improved Therapeutic Index of Analgesia to Constipation Relative to Morphine in Mice.** The impact of TRV130 and morphine on gastrointestinal function was evaluated in the mouse glass bead expulsion assay of colonic motility (Fig. 7, blue curves; Supplemental Table 5) and fecal bolus accumulation assay (Fig. 7, orange curves; Supplemental Table 5). These responses were then compared with the hot plate antinociceptive responses (Fig. 7, black curves). These models show that TRV130 causes less gastrointestinal dysfunction than morphine at equivalent analgesic doses. This difference is best reflected in the lack of gastrointestinal dysfunction at the

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**TABLE 1**

MOR functional and binding specificity for TRV130

<table>
<thead>
<tr>
<th>TRV130</th>
<th>cAMP</th>
<th>pEC50</th>
<th>pEC50</th>
<th>β-Arrestin-2</th>
<th>pEC50</th>
<th>pEC50</th>
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<tr>
<td>hMOR</td>
<td>8.1</td>
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<td>7.9</td>
<td>83</td>
<td>7.4</td>
<td>0.22</td>
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<td>nM</td>
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<td></td>
<td>Efficacy (%)</td>
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<td>mMOR</td>
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<td>0.06</td>
<td>0.4</td>
<td>104</td>
<td>7.9</td>
<td>0.06</td>
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<tr>
<td>rMOR</td>
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<td>4.0</td>
<td>90</td>
<td>8.0</td>
<td>0.02</td>
</tr>
<tr>
<td>dMOR</td>
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<td>0.01</td>
<td>5.0</td>
<td>92</td>
<td>8.0</td>
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<td>hNOR</td>
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<td>0.09</td>
<td>398</td>
<td>58</td>
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<td>0.03</td>
<td>1585</td>
<td>58</td>
<td>8.0</td>
<td>0.02</td>
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<tr>
<td>hDOR</td>
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<td>3162</td>
<td>46</td>
<td>8.0</td>
<td>0.02</td>
</tr>
<tr>
<td>N.M.</td>
<td></td>
<td></td>
<td></td>
<td>Efficacy (%)</td>
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<table>
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<tr>
<th>Kd high</th>
<th>Kd low</th>
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<tr>
<td>6 ± 1.7</td>
<td>551 ± 263</td>
</tr>
<tr>
<td>1 ± 0.3</td>
<td>39 ± 21</td>
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<td>18 ± 3.7</td>
<td>203 ± 26</td>
</tr>
<tr>
<td>10,000</td>
<td>N. D.</td>
</tr>
</tbody>
</table>

a, dog; h, human; m, mouse; N.D., not determined; N.Q., not quantifiable; r, rat.
TRV130 Shows an Improved Therapeutic Index of Analgesia to Respiratory Suppression Compared with Morphine in Rats.

Rats were used to compare TRV130 and morphine with respect to analgesia and respiratory suppression, because the larger blood volume of rats makes serial arterial blood gas measurement more feasible than in mice (Sahbaie et al., 2006). The equianalgesic doses of 3.0 mg/kg morphine and 0.3 mg/kg TRV130 were chosen, based on responses in the rat hot plate (Fig. 6A), tail-flick (Fig. 6B), and hindpaw incision assays (Fig. 6C), as a basis for comparing respiratory suppression at analgesic and supra-analgesic doses. Morphine caused a trend toward increasing pCO₂ at 3.0 and 6.0 mg/kg (Fig. 8, A and B), and caused a statistically significant increase in pCO₂ (greater than 50 mmHg, compared with 35–40 mm Hg at baseline and in vehicle-treated animals) at doses 4-fold and 8-fold over the hot plate ED₅₀ (12 and 24 mg/kg) (Fig. 8, C and D). In contrast, TRV130 did not cause this severe level of respiratory suppression even at 8-fold over the equianalgesic dose (Fig. 8, C and D). This improved therapeutic index compared with the analgesic response is illustrated in Fig. 8, E and F, as hot plate responses (in black) and peak pCO₂ measurements (in orange); hot plate response was chosen for this comparison as it, like respiratory depression, involves supraspinal processing. pO₂ and pH measures largely paralleled the changes seen in pCO₂ for both morphine and TRV130 (Supplemental Fig. 3). Behavioral notes during this study indicate that TRV130 was not nearly as sedative as morphine at equianalgesic doses, consistent with less CNS depression (Supplemental Fig. 4). These findings indicate that in rats, TRV130 has an increased therapeutic index for analgesia versus respiratory suppression and sedation.

Discussion

The data presented here demonstrate that TRV130 is a novel MOR G protein-biased ligand. The unique in vitro and in vivo profile of TRV130 suggests that it may be possible to minimize on-target MOR-mediated adverse events, which could significantly improve opioid pain management. Furthermore, the recapitulation of morphine’s improved pharmacology in β-arrestin-2 KO mice by a ligand with reduced ability to engage β-arrestin-2 demonstrates that molecular dissection of receptor signaling pathways can translate to differentiated pharmacology of biased ligands. Mechanistically, this could be the result of either a) reduced stimulation of an active, constipating, and respiratory-suppressing signaling

Fig. 3. TRV130 exhibits reduced MOR internalization and phosphorylation. Compared with morphine, TRV130 exhibits (A) reduced receptor internalization (in an enzyme complementation assay) and (B) reduced MOR phosphorylation at serine 375. Equal protein amounts were loaded into each lane. For quantification, optical densities of total hMOR (S375) immunoreactive bands were measured, normalized to the background values, and expressed as percentages of controls. *P < 0.05 versus vehicle; †P < 0.05 versus morphine and data are means ± S.E. of two to five independent experiments.

Fig. 4. TRV130 competitively binds the hMOR. (A) Levels of cAMP inhibition were monitored after 30-minute incubation with indicated concentrations of TRV130 and naloxone. Naloxone competitively shifted the cAMP inhibition curve for TRV130 (Schild slope = 0.9 ± 0.07), with an apparent Kᵦ for naloxone of 6.6 nM. (B) Levels of β-arrestin-2 recruitment were monitored after 30-minute incubation with indicated concentrations of DAMGO and TRV130. TRV130 competitively shifted DAMGO-induced β-arrestin-2 recruitment (Schild slope = 1.2 ± 0.3 and an apparent Kᵦ = 20 nM). Insets (A) and (B), data were used to calculate dose ratios and Kᵦ values for naloxone and TRV130. The graphs are representative of two to five independent experiments performed in duplicate.
mechanism of β-arrestin, or b) selective amplification of the analgesic pathway, which may be more sensitive to the acute desensitization effects of β-arrestin than the respiratory or constipating signals. The former mechanism is supported by the reduced impact of morphine on gastrointestinal motility and respiration in β-arrestin-2 knockout mice compared with wild type mice (Raehal et al., 2005); the latter mechanism is supported by the increased antinociceptive efficacy of morphine in β-arrestin-2 knockout mice compared with wild type mice (Bohn et al., 1999). However, either mechanism could explain the observed improved therapeutic index for TRV130, and neither pharmacokinetics nor receptor selectivity seem likely contributors. At the molecular level, MOR G protein coupling leads to analgesia by activation of potassium channels and inhibition of calcium channels (Altier and Zamponi, 2004; Ocana et al., 2004), but further work will be required to clarify the specific intracellular signaling pathways downstream of β-arrestins that contribute to opioid analgesia.
responses in vivo. In addition, the recently published MOR crystal structure may permit investigation of how biased and unbiased ligands may engage different conformations of the MOR to support selective coupling to G proteins versus β-arrestins (Manglik et al., 2012).

TRV130 shows a differentiated profile compared with morphine in several cellular readouts including β-arrestin-2 recruitment, receptor phosphorylation, and receptor internalization. Morphine effects on these endpoints can be difficult to measure in low-sensitivity assays (Whistler and von Zastrow, 1998; Sternini et al., 2000), which sometimes require overexpressed G protein-coupled receptor kinase to amplify signal (Groer et al., 2007), but is clearly detectable in the more sensitive assays used here. The fact that morphine pharmacology is altered in β-arrestin-2 KO mice, despite no apparent changes in baseline function, suggests that β-arrestin-2 engagement by morphine is meaningful, and that the more sensitive in vitro assays are measuring relevant molecular pharmacology, even if morphine is a partial agonist with respect to higher efficacy drugs such as fentanyl. Importantly, morphine is not biased with respect to fentanyl in the model used here, whereas TRV130 is biased with respect to both fentanyl and morphine. This suggests that despite higher efficacy of fentanyl than morphine for both G protein and β-arrestin responses, morphine and fentanyl, unlike TRV130, do not display functional selectivity for β-arrestin engagement at equiactive concentrations.

Quantification of ligand bias is particularly useful in systems such as overexpressed MOR, where one pathway may be selectively amplified such that partial agonists appear as biased ligands. Here we have used the “equiactive comparison” approach (Griffin et al., 2007; Rajagopal et al., 2011), which calculates intrinsic relative activity for each downstream pathway. The model we used assumes that ligands are signaling from a state of equilibrium binding, and that dose-response curves show no cooperativity; the data presented here indicates that both of these assumptions appear reasonable for TRV130, morphine, and fentanyl. By this calculation, TRV130 is approximately 3-fold more biased toward G protein signaling than morphine and fentanyl (Supplemental Table 2).

The profile of TRV130 both in vitro and in vivo is inconsistent with functional selectivity arising from unbiased partial efficacy. In our assays, TRV130 shows robust, morphine-like G protein coupling, with efficacy of 83% of morphine, whereas the well-described partial agonist buprenorphine displays efficacy of only 52% of morphine (Supplemental Table 1). In vivo, buprenorphine behaves as a partial agonist in both the analgesia and colonic motility assays (Supplemental Fig. 5), except with more glass bead retention than analgesia. Morphine exhibits full efficacy in each of these two in vivo assays, and TRV130 has maximal efficacy for analgesia but only partial efficacy in the colonic motility and fecal production assays. This highlights the differentiation of TRV130 as a biased ligand from both a strong agonist and a partial agonist, and suggests that the functional selectivity of TRV130 in vivo does not arise from differential amplification of partial agonist signals.

The profile of TRV130 shown here, of robust analgesia with reduced CNS depression and reduced gastrointestinal dysfunction compared with morphine, should translate into an important improvement in the treatment of postoperative pain. In this setting, postoperative nausea and vomiting, constipation, and sedation caused by current opioids cause significant patient discomfort and can prolong hospital stay. The risk of respiratory suppression limits opioid dosing, leaving many patients in pain during recuperation (Dahan, 2007). Thus TRV130 may be a marked improvement over current opioids in postoperative care. These improvements could also hold great promise for chronic pain management, where constipation is a severe and often dose-limiting adverse event (Anastassopoulos et al., 2011). Chronic opioid use also increases the risk of opioid addiction and tolerance to the analgesic benefits (Schneider and Kirsh, 2010). While these facets of MOR pharmacology will be explored with TRV130 in future studies, several of the previous studies of morphine in β-arrestin-2 knockout mice suggest that TRV130 may have reduced potential for addiction and analgesic tolerance (Bohn et al., 2000; Bohn et al., 2003; Raehal and Bohn, 2011; Urs et al., 2011).
Based on the preclinical profile described here, TRV130 is now in clinical development for use as an intravenous analgesic alternative to morphine and fentanyl. Although rodent oral bioavailability of TRV130 is low, the potential for oral availability in higher species is still being explored. Based on preliminary data suggesting high skin-permeation, TRV130 is also being investigated for administration via a transdermal patch to treat chronic pain. If the differentiation versus morphine shown here successfully translates to humans, the improved therapeutic windows of analgesia to respiratory suppression and gastrointestinal effects could provide physicians a more tolerable analgesic with a higher margin of safety. This in turn would lead to more effective pain management. More broadly, this would represent the successful translation of selective signaling via ligand bias to improve the therapeutic profile of a GPCR-targeted drug. This strategy could potentially be applied to other GPCRs for which on-target adverse events limit therapeutic utility.

Acknowledgments
The authors are grateful to Ian James for critical reading of this manuscript.

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References