TRV0109101, a G Protein-Biased Agonist of the μ -Opioid Receptor, Does Not Promote Opioid-Induced Mechanical Allodynia following Chronic Administration[§]

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ABSTRACT

Prescription opioids are a mainstay in the treatment of acute moderate to severe pain. However, chronic use leads to a host of adverse consequences including tolerance and opioid-induced hyperalgesia (OIH), leading to more complex treatment regimens and diminished patient compliance. Patients with OIH paradoxically experience exaggerated nociceptive responses instead of pain reduction after chronic opioid usage. The development of OIH and tolerance tend to occur simultaneously and, thus, present a challenge when studying the molecular mechanisms driving each phenomenon. We tested the hypothesis that a G protein-biased μ -opioid peptide receptor (MOPR) agonist would not induce symptoms of OIH, such as mechanical allodynia, following chronic administration. We observed that the development of opioid-induced mechanical allodynia (OIMA), a model of OIH, was absent

in β -arrestin1^{−/−} and β -arrestin2^{−/−} mice in response to chronic administration of conventional opioids such as morphine, oxycodone and fentanyl, whereas tolerance developed independent of OIMA. In agreement with the β -arrestin knockout mouse studies, chronic administration of TRV0109101, a G protein-biased MOPR ligand and structural analog of oliceridine, did not promote the development of OIMA but did result in drug tolerance. Interestingly, following induction of OIMA by morphine or fentanyl, TRV0109101 was able to rapidly reverse allodynia. These observations establish a role for β -arrestins in the development of OIH, independent of tolerance, and suggest that the use of G protein-biased MOPR ligands, such as oliceridine and TRV0109101, may be an effective therapeutic avenue for managing chronic pain with reduced propensity for opioid-induced hyperalgesia.

Introduction

Although opioids remain the standard for managing acute moderate to severe pain, their use for treatment of chronic conditions remains controversial (Johannes et al., 2010; Gomes et al., 2014; Trang et al., 2015). In addition to potential for dependence, long-term opioid usage may result in tolerance and opioid-induced hyperalgesia (OIH). With tolerance, opioid dosages need to be increased to maintain the same level of analgesia (Lee et al., 2011; Williams et al., 2013). In contrast, with OIH, increased opioid dose results in a paradoxical increase in pain sensation (Simonnet and Rivat, 2003; Chu et al., 2008; Lee et al., 2011). Individuals with OIH report pain responses from subthreshold stimuli (allodynia) or exaggerated pain in response to noxious stimuli (hyperalgesia). This pain can be diffuse and independent of the original site of injury (Lee et al., 2011). OIH has been observed in response to a variety of μ -opioid peptide receptor (MOPR) agonists, including morphine, oxycodone, fentanyl, remifentanyl,

and even codeine (Seymour et al., 1982; Lipman and Blumenkopf, 1989; Guignard et al., 2000; Angst and Clark, 2006; Chu et al., 2006; Eastman et al., 2014; Johnson et al., 2014; Mauermann et al., 2016). OIH is also prevalent among heroin drug users and those undergoing methadone substitution therapies (Compton et al., 2001, 2012).

The specific molecular mechanisms of OIH are currently unknown. Recent work has highlighted the role of MOPR-mediated signaling on peripheral nociceptors as a primary mechanism in the development of OIH and tolerance (Corder et al., 2017). As regulators and mediators of MOPR signaling, β -arrestins may contribute to this process and have been described with contrasting roles in the development of OIH (Rowan et al., 2014a,b). In primary sensory neurons, [D-Ala², N-Me-Phe⁴, Gly-ol⁵]-enkephalin (DAMGO) and morphine simultaneously recruit β -arrestin to MOPR and decrease β -arrestin interaction with transient receptor potential cation channel subfamily V member 1 (TRPV1), which results in increased sensitivity of TRPV1 (Rowan et al., 2014a,b). In contrast, DAMGO-mediated mechanical allodynia was prolonged in β -arrestin2 KO mice and attributed to the loss of β -arrestin-mediated regulation of NMDA receptor function (Chen et al., 2016). β -Arrestin2 knockout mice exhibit enhanced and prolonged antinociception to a single

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ABBREVIATIONS: cAMP, 3',5'-cyclic adenosine monophosphate; DAMGO, [D-Ala², N-Me-Phe⁴, Gly-ol⁵]-enkephalin; DOPR, δ -opioid peptide receptor; HEK, human embryonic kidney; KO, knockout; KOPR, kappa-opioid peptide receptor; MOPR, μ -opioid peptide receptor; MPE, maximum possible effect; NOPR, nociceptin peptide receptor; OIH, opioid-induced hyperalgesia; OIMA, opioid-induced mechanical allodynia; WT, wild type.

dose of morphine (Bohn et al., 1999) and reduced tolerance (Bohn et al., 1999, 2000, 2002), while effects on OIH were not documented. As the role of β -arrestins in the development of OIH and tolerance remain ambiguous, we sought to investigate this question further.

Previous work using β -arrestin2 knockout mice has established a role for β -arrestins in other opioid-associated adverse events such as respiratory depression and reduced gastrointestinal function and, thus, supported the rationale for the development of G protein-biased MOPR agonists as differentiated analgesics that promote robust antinociception with reduced liability for adverse events (Bohn et al., 1999; Raehal et al., 2005). G protein-biased MOPR agonists promote more effective coupling to G protein signaling pathways (associated with analgesia) than to β -arrestin recruitment (associated with opioid-induced adverse events) compared with morphine. Recently characterized G protein-biased MOPR agonists such as oliceridine (TRV130), PZM21, and mitragynine pseudoinodoxyl have shown less respiratory depression and gastrointestinal dysfunction than morphine while maintaining analgesic efficacy in rodents (DeWire et al., 2013; Manglik et al., 2016; Varadi et al., 2016). Oliceridine has also shown reductions in adverse events in humans (Soergel et al., 2014; Siuda et al., 2017). Although mitragynine pseudoinodoxyl exhibited delayed antinociceptive tolerance, the effects of biased ligands on OIH have not been reported. Therefore, the use of a G protein-biased MOPR ligand provides a pharmacological approach uniquely positioned to study the contribution of β -arrestins in the development of OIH and opioid tolerance that may have clinical implications for compounds, like oliceridine, that are in clinical development.

We hypothesized that a G protein-biased MOPR ligand may be able to uncouple the induction of OIH from the development of tolerance. Here, we compare an exemplary G protein-biased MOPR ligand, TRV0109101, to morphine, fentanyl, and oxycodone at measures of opioid-induced mechanical allodynia (OIMA), a model of OIH, and antinociceptive tolerance to assess additional differentiation between G protein-biased MOPR ligands and conventional opioids.

Materials and Methods

Chemicals. [D-Ala², N-Me-Phe⁴, Gly-o⁵]-enkephalin (DAMGO, cat. no. 1171), [D-Pen^{2,5}]-enkephalin (cat. no. 1431), (–)-U-50488 hydrochloride (cat. no. 0496), nociceptin (cat. no. 0910), isobutyl methylxanthine (cat. no. 2845), and NKH 477 (water soluble analog of forskolin, cat. no. 1603) were purchased from Tocris Bioscience (Ellisville, MO). Morphine sulfate (cat. no. M8777), oxycodone HCl (cat. no. O1378), and fentanyl citrate (cat. no. F-3886) were purchased from Sigma-Aldrich (St. Louis, MO). Cebranopadol was purchased from MedChem Express (Princeton, NJ; cat. no. HY-15536). (R)-TRV0109101 hydrochloride was synthesized at Trevena, Inc. (Chen et al., 2013).

Cell culture. Human embryonic kidney (HEK)-293 cells stably transfected to overexpress β -arrestin2 fused to a β -galactosidase fragment were purchased from DiscoverX (DiscoverX Corporation, Fremont, CA; Part# 93-0165) and the human OPRM1 gene (NM_000914.3, encoding human μ -opioid peptide receptor MOPR), mouse OPRM1 gene (NM_001039652.1, mouse MOPR), human OPRD1 gene [NM_000911.3, human δ -opioid peptide receptor (DOPR)], human OPRK1 gene [NM_000912.3, human κ -opioid peptide receptor (KOPR)], and human OPRL1 gene [NM_182647.2, human nociceptin opioid peptide (NOPR)] receptors were fused to a complementary β -galactosidase fragment using the pCMV-ProLink plasmid purchased from DiscoverX (Part# 93-0167). Cells were grown in minimum Eagle's medium (Corning, Tewksbury, MA)

with 10% fetal bovine serum (Sigma, F2442), 1% penicillin/streptomycin (Corning; 30-002-CL), and 150 μ g/ml of G418 sulfate (Corning; 30-234-CR) and 150 μ g/ml of Hygromycin B (Corning; 0-240-CR).

β -Arrestin2 recruitment. With the stable cell lines described above (MOPR, KOPR, DOPR, NOPR), β -arrestin2 recruitment to each receptor was quantified using the DiscoverX PathHunter β -arrestin recruitment enzyme complementation assay as previously described (DeWire et al., 2013). Briefly, HEK-293 cells stably coexpressing the Prolink-tagged (C terminus) opioid receptor of interest and β -arrestin2-Prolink acceptor were plated at a density of 5,000 cells per well in white, opaque 384-well plates (LIA plate; Greiner Bio One, Monroe, NC; Part# 784080) and maintained overnight in growth media at 37°C/5% CO₂. Growth media were removed and test ligand (or vehicle) in assay buffer [Ham's F-12 (Corning; 10-080-CM), 10 mM HEPES (Lonza; 17-737E), 500 μ M isobutyl methylxanthine, and 1.5 μ M NKH-477]. Cells were treated with ligand for 1 hour at 37°C followed by the addition of PathHunter detection reagent (Part# 93-001L). One hour after detection reagent addition, β -arrestin2 recruitment was detected by chemiluminescence on a PheraStar plate reader (BMG Labtech, Durham, NC) according to manufacturer's protocol. Ligand potency and efficacy was assessed by concentration-response analysis (idbs, XLfit) and expressed as a normalized percentage of a maximal response.

cAMP accumulation. With the same cell lines described for β -arrestin2 recruitment, cells were plated and cultured identically to their β -arrestin2 recruitment counterpart. To quantify G_q coupling, the inhibition of forskolin-stimulated cAMP accumulation was detected according to manufacturer's protocol using the cAMP HiRange Kit (Cisbio, Bedford, MA; Part #62 AM6PEJ) after a 30-minute incubation with ligand in assay buffer at 37°C/5% CO₂. After detection reagent addition, plates were incubated for 60 minutes at room temperature and subsequently read using time-resolved fluorescence (665 nM/620 nM) on a PheraStar plate reader, according to manufacturer's protocol. Ligand potency and efficacy was assessed by concentration-response analysis (idbs, XLfit) and expressed as a normalized percentage of a maximal response.

Receptor internalization. The PathHunter GPCR internalization assay (DiscoverX; Part# 93-0745C3) was performed according to the manufacturer's protocol. Receptor localization to the early endosome was detected by chemiluminescence using the PheraStar (BMG LabTech, Cary, NC) plate reader, according to manufacturer's instructions.

Agonist-promoted MOPR phosphorylation. HEK293 cells stably expressing human MOPR were cultured in 6-well plates. After an overnight serum starvation, cells were stimulated with 1 μ M of morphine, fentanyl, oxycodone, or TRV0109101 for 5 minutes at 37°C. Upon removal of the stimulation media, cells were washed thrice with ice-cold phosphate-buffered saline and lysed on ice with 20 mM HEPES, 150 mM NaCl, 1% TritonX-100, and 1 CompleteMini protease inhibitor tablet (Roche, Indianapolis, IN). Lysates were rocked for 30 minutes at 4°C and cleared by centrifugation (1000 *g* for 5 minutes). Equal protein amounts were separated by SDS-PAGE and transferred to nitrocellulose using an iBlot2 Dry blotting apparatus. Agonist-promoted MOPR phosphorylation was probed by the phospho-specific antibody for MOPR pSer³⁷⁵ (Cell Signaling Technology, Danvers, MA; #3451), as per the manufacturer's instructions, and detected using a Syngene G:Box (Syngene, Frederick, MD) by chemiluminescence. The immunoreactive band representing hMOPR was quantified using LICOR Image Studio (LICOR, Lincoln, NE; v5.2) software and reported as normalized values to vehicle-treated cells.

Schild regression analysis. The competitive interaction of TRV0109101 at the human MOPR orthosteric ligand binding site was quantified via Schild analysis using the β -arrestin2 PathHunter human MOPR stable cell line. Identical culture conditions and assay reagents were used as described in the cAMP accumulation and β -arrestin2 recruitment methods above. Assay protocols were modified at the ligand addition step. After removal of growth media, cells were pretreated with competitor ligand (or vehicle) in assay buffer for 5 minutes at room temperature followed by the subsequent addition of agonist ligand in

assay buffer. After agonist addition, cells were returned to a 37°/5% CO₂ incubator, and functional responses were generated as indicated by the detection methods in the sections above. Competing concentration response curves for both competitor and agonist ligands were generated for each of the cAMP inhibition and β -arrestin2 recruitment assay protocols. Data were analyzed using global fitting to the Gaddum/Schild equation using a one site competition nonlinear regression analysis supplemented with linear regression plots of corresponding dose ratios using GraphPad Prism (GraphPad Software, San Diego, CA) (Arunlakshana and Schild, 1959; Kenakin, 2009).

In vivo. All studies were performed with 8- to 10-week-old male, C57BL/6 mice (Charles River Laboratories, Wilmington, MA) at Trevena, Inc. (King of Prussia, PA). β -Arrestin1 ($\beta arr1^{-/-}$) and β -arrestin2 ($\beta arr2^{-/-}$) knockout (KO) mice on the C57BL/6 background have been previously described (Bohn et al., 1999). In studies utilizing $\beta arr1^{-/-}$ and $\beta arr2^{-/-}$ mice, sex- and weight-matched control C57BL/6 mice (Sage Laboratories, Inc., Boyertown, PA) were used. Before the beginning of any experiments, mice were acclimated to the vivarium for at least 72 hours. During this time, mice were housed in standard conditions with 12-hour light/dark cycle, and fed ad libitum. 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 (Bethesda, MD).

In vivo mechanical allodynia. The threshold for responses to punctate mechanical stimuli (mechanical allodynia) was tested according to the frequency method with slight modifications (Bonin et al., 2014). In brief, the plantar surface of the animal hind paw was stimulated with a single von Frey monofilament (0.4 g) for approximately 1–2 seconds. If there was a withdrawal response, it was recorded as a positive response. A response was defined as a lifting or shaking of the paw upon stimulation. This was repeated 10 times for each mouse. The final measurement for each mouse is the percentage of nonresponse to stimulation for the 10 trials.

In vivo OIMA. Opioid-induced mechanical allodynia studies were designed and conducted based on previous reports (Chen et al., 2010; Hua et al., 2016). Briefly, mice were given subcutaneous injections of vehicle or μ -opioid agonists twice per day for 4 days. To induce allodynia, twice the dose corresponding to maximal efficacy in a hot plate antinociceptive assay was administered on days 1–3, while four times the maximal efficacious hot plate dose was given on day 4. On each of the 4 drug treatment days, prior to compound administration, a measurement of mechanical allodynia was obtained using the method described above. On the 5th day, animals were again tested but with no additional drug treatments. This repeat dosing paradigm resulted in a dose-dependent increase in OIMA using oxycodone, a conventional MOPR agonist (Supplemental Fig. 1). For OIMA reversal studies, animals were switched on day 6 to a different μ -opioid agonist and again tested for mechanical allodynia for the next 5 days as described above. For experiments requiring osmotic pumps (Raehal and Bohn, 2011), pumps (Alzet model 2001; Durect Corporation, Cupertino, CA) were subcutaneously implanted through a small skin incision between the shoulder blades during a surgical procedure. Before implantation, compound or vehicle was loaded into the osmotic pumps according to the manufacturer's instructions. Briefly, under light isoflurane anesthesia a small incision was made with scissors, and a small pocket was formed beneath the skin. The pump was then inserted, and the incision was closed using wound clips. To insure immediate drug delivery, osmotic pumps were submerged in 0.9% NaCl solution and incubated overnight at 37°C prior to implantation. The continuous infusion regimen aimed to administer a comparable amount of drug per day as the subcutaneous dosing regimen (average of 4 day dosing) and promote comparable analgesia (across MOPR ligands) in a hot plate assay.

To address the contribution of NOPR engagement in the development of OIH, cebranopadol (0.01 or 0.03 mg/kg s.c.; twice daily), a MOPR/NOPR-mixed agonist that lacks G protein-biased MOPR pharmacology, was also assessed in the OIMA model over 4 days of

chronic dosing. This dose range was selected to reflect doses previously used to characterize cebranopadol pharmacology (Schunk et al., 2014). Mechanical allodynia was measured as described above.

In vivo hot plate. The hot plate test is adapted from that described previously (Tyers, 1980). Animals were placed individually on a heated surface (56°C) and the time interval (seconds) between placement and a shaking, licking, or tucking of the hind paw was recorded as the predrug latency. This same procedure was repeated 30 minutes after subcutaneous administration of compound in 10% ethanol, 10% Kolliphor EL, 80% sterile water for injection (10:10:80), or saline as appropriate. All compounds were administered subcutaneously in a volume of 1 ml/100 g, with volume adjustments made for mouse dosing. The cutoff time, designed to prevent injury to the animals, was 30 seconds (with vehicle latencies of approximately 5–15 seconds). The percent maximum possible antinociceptive effect [% maximum possible effect (MPE)] was determined using the formula:

$$\text{Percent MPE} : ((\text{Post drug latency} - \text{baseline latency}) / (30 - \text{baseline latency})) \times 100$$

The predrug latency of each animal and cutoff times noted above was used, and the experimenter was blinded to the treatment of animals during behavioral observations.

In vivo pharmacokinetics. Pharmacokinetics studies were performed at ChemPartner Co. (Shanghai, China) under protocols reviewed and approved by their Institutional Care and Use Committee. Pharmacokinetic profiles of TRV0109101 were determined in mice, rats, dogs and cynomolgus monkeys by several routes of administration (Supplemental Methods and Supplemental Table 3). Plasma (all species) and brain levels (mouse) of TRV0109101 were determined using liquid chromatography tandem mass spectrometry bioanalytical methods. Pharmacokinetic parameters were calculated using WinNonlin v6.2 software (Pharsight Corporation, Mountain View, CA); data are presented in Supplemental Table 3.

Statistical analyses. All statistical analyses were calculated using GraphPad Prism software. Unless otherwise indicated, all values are reported as means $\pm$ S.E.M., and statistical significance was determined using one-way analysis of variance with Dunnett's post hoc analyses to vehicle or two-way analysis of variance with Bonferroni post hoc analyses to vehicle. For behavioral studies, a minimal sample size of 8 in each group will have greater than 99% power to detect a difference in means, assuming a standard deviation of 20, using a two-group *t* test with a 0.05 two-sided significance level.

Results

β -Arrestins and opioid-induced mechanical allodynia. To test the contributions of β -arrestin1 and β -arrestin2 to the development of opioid-induced mechanical allodynia (OIMA) as a model of OIH, chronic minipump infusion of vehicle (saline, *n* = 8) or oxycodone (25 mg/kg/day, *n* = 8) was tested in wild-type (WT), $\beta arr1^{-/-}$, and $\beta arr2^{-/-}$ mice (Fig. 1). The use of implanted osmotic minipumps promoted consistent drug exposure and mitigated the potential pronociceptive effects of opioid withdrawal, a common pitfall in studying and diagnosing OIH. Mechanical allodynia was measured using 0.4 g von Frey filaments during 7 days of chronic minipump infusion (Fig. 1, A–C). In WT mice, significant mechanical hypersensitivity developed following 2 days of oxycodone infusion and lasted throughout the dosing paradigm (Fig. 1A), an effect that was not seen in $\beta arr1^{-/-}$ (Fig. 1B) or $\beta arr2^{-/-}$ animals (Fig. 1C). In conjunction, animals were tested 5 minutes following testing of mechanical allodynia in the hot plate model (56°C) and response latencies recorded (Fig. 1, D–F). In all strains tested, mice developed antinociceptive tolerance to oxycodone

Fig. 1. WT, but not β -arrestin (β arr)1-KO and β arr2-KO mice, exhibit opioid-induced mechanical allodynia, whereas all develop antinociceptive tolerance. Mechanical allodynia was measured over 7 days in WT (A), β arr1-KO (B), and β arr2-KO (C) mice in response to chronic minipump infusion (7 days) with vehicle (saline, $n = 8$) or oxycodone (25 mg/kg/day, $n = 8$). Five minutes after testing for mechanical allodynia, thermal (56°C) hot plate antinociceptive responses in WT (D), β arr1-KO (E), and β arr2-KO (F) mice were measured. Data are means $\pm$ S.E.M. [*** $P < 0.001$ via two-way repeated-measures analysis of variance (ANOVA) with Bonferroni post tests to vehicle].

throughout the dosing paradigm. Similar results were obtained in a different cohort of β arr1 $^{-/-}$ and β arr2 $^{-/-}$ animals to morphine (48 mg/kg/day; $n = 15$), oxycodone (25 mg/kg/day; $n = 8$) and fentanyl (3.2 mg/kg/day; $n = 8$) in both mechanical allodynia and hot plate nociceptive responses (Supplemental Fig. 2, A–D).

TRV0109101 in vitro pharmacology. Because β -arrestins appear to play a prominent role in opioid-induced mechanical allodynia, decreasing agonist-promoted β -arrestin recruitment may be an avenue for generating differentiated MOPR analgesics with reduced liability for the development of OIH. Previously, we are able to pharmacologically decouple MOPR-mediated analgesia from conventional opioid-induced adverse events such as respiratory depression and constipation using the G protein-biased MOPR ligand oliceridine (TRV130) in rodents (DeWire et al., 2013). The aligned effect of genetic and pharmacologic interdiction of MOPR/ β -arrestin pharmacology suggests that

a G protein-biased MOPR ligand may be able to deliver robust analgesia while reducing the risk of the development of OIH.

Through previously reported lead optimization studies, we identified several structurally related G protein-biased MOPR agonists including [2-[(9R)-9-(pyridin-2-yl)-6-oxaspiro[4.5]decan-9-yl]ethyl]-(thiophen-2-ylmethyl)amine (R-19; TRV0109101) (Fig. 2A) (Chen et al., 2013; DeWire et al., 2013). Like oliceridine, TRV0109101 does not share any structural similarity to conventional opioid receptor modulators and is chemically distinct from recently identified G protein-biased MOPR ligands, PZM21 and mitragynine pseudoindoxyl. TRV0109101 promotes potent (10 nM) and robust G_{α_i} activation (approximately 81% compared with morphine) while exhibiting significantly reduced agonist-promoted β -arrestin2 recruitment (23%) (Fig. 2B, Supplemental Table 1). Because G protein receptor kinase-mediated receptor phosphorylation contributes to β -arrestin/receptor interaction

Fig. 2. TRV0109101 is a potent and MOPR-selective G protein-biased agonist. [2-[(9R)-9-(pyridin-2-yl)-6-oxaspiro[4.5]decan-9-yl]ethyl]-(thiophen-2-ylmethyl)amine, TRV0109101 (A), promoted inhibition of cAMP accumulation (B, orange) with a notable reduction in β -arrestin recruitment (B, teal). (C) TRV0109101 exhibits marked selectivity for MOPR (orange) over KOPR (yellow), DOPR (blue), and NOPR (green). Data are means $\pm$ S.E.M. for at least 3 independent experiments.

Fig. 3. TRV0109101 promotes less receptor phosphorylation and internalization than conventional opioids and is functionally competitive with other MOPR ligands. (A) In HEK293 cells stably overexpressing hMOPR, TRV0109101 exhibited significantly reduced agonist-promoted receptor phosphorylation at Ser³⁷⁵ compared with morphine. (B) TRV0109101 promoted reduced internalization compared with morphine and other MOPR agonists. Data are means $\pm$ S.E.M. for at least three independent experiments (* P < 0.05, via one-way ANOVA with Bonferroni's multiple comparison test post tests). (C) Functional competition analysis demonstrates that a MOPR-antagonist, naloxone, can competitively inhibit TRV9101-promoted inhibition of cAMP production. (D) Conversely, TRV0109101 is able

and β -arrestin function (receptor endocytosis), we monitored agonist-promoted hMOPR phosphorylation and receptor internalization. As expected of a G protein-biased MOPR ligand, TRV0109101 exhibited markedly lower G protein receptor kinase-mediated hMOPR phosphorylation at serine 375 and hMOPR internalization compared with conventional opioids (Fig. 3, A and B). Furthermore, we studied the nature of TRV0109101 interaction with the receptor in reference to other MOPR ligands by functional competition analysis. Thus, if TRV0109101 is functionally competitive with the MOPR antagonist naloxone, the EC₅₀ of TRV0109101 will increase as the antagonist concentration is titrated higher, whereas the maximal response to TRV0109101 remains the same. Indeed, increasing concentrations of naloxone exhibited a functionally competitive relationship with TRV0109101-promoted $G\alpha_i$ activity (Fig. 3C). Concordantly, increasing concentrations of TRV0109101 was functionally competitive with DAMGO-promoted β -arrestin2 recruitment (Fig. 3D). Schild analysis of these data suggests that TRV0109101 operates through the common orthosteric ligand binding site used by conventional MOPR ligands with an approximate apparent K_D of 70 nM (Figs. 3, C and D). TRV0109101 is MOPR selective (over NOPR, DOPR, and KOPR) (Fig. 2C, Supplemental Tables 1 and 2) and can activate MOPR across species (Supplemental Table 1).

TRV0109101 in vivo pharmacology. Similar to oliceridine (TRV130), TRV0109101 promoted robust analgesia in multiple models of acute pain, such as hot plate [mouse (ED₅₀ = 1.1 mg/kg) and rat (ED₅₀ = 0.3 mg/kg)], tail flick [rat (ED₅₀ = 0.09 mg/kg)], and incisional pain (rat) (Fig. 4A, Supplemental Figs. 3 and 4) (DeWire et al., 2013). TRV0109101 administration exhibited a broad therapeutic window between analgesia and inhibition of gastrointestinal function, as measured by colonic propulsion [mouse (ED₅₀ = 9.4 mg/kg, 8.5 $\times$ greater than hotplate ED₅₀)] and fecal boli accumulation [mouse (ED₅₀ = 6.3 mg/kg, 5.7 $\times$ greater than hotplate ED₅₀)] assays (Fig. 4, B–D). The magnitude of this therapeutic window was greater than that reported for morphine and comparable to the window previously observed for oliceridine (DeWire et al., 2013).

TRV0109101 pharmacokinetics. To understand the relationship between drug exposure and antinociceptive efficacy, pharmacokinetic profiling of TRV0109101 was conducted following a single intravenous, subcutaneous, and oral administration in male C57BL/6 mice. In addition to mouse, the pharmacokinetic profile of TRV0109101 was determined in rats, dogs, and cynomolgus monkeys by various routes of administration (Supplemental Methods and Supplemental Table 3). In mice, TRV0109101 was rapidly absorbed following a subcutaneous dose, with a mean C_{max} of 1560 ng/ml measured 30 minutes postdose and a $t_{1/2}$ of 0.8 hours; peak brain exposure was also observed 30 minutes postdose (Supplemental Table 3, Supplemental Fig. 5). Data from mouse pharmacokinetic studies were used to inform dosing regimens, drug administration routes, and experimental protocols. For example, considering the T_{max} , all thermal antinociceptive assays after subcutaneous administration were performed at 30 minutes postdose. Additionally, because the $t_{1/2}$ of TRV0109101 is estimated at

to effectively interdict DAMGO-promoted β -arrestin2 recruitment with an apparent K_D of 70 nM. Data are means $\pm$ S.E.M. for at least three independent experiments.

Fig. 4. TRV0109101 exhibits a robust therapeutic window between analgesia and inhibition of gastrointestinal output. Mice were injected subcutaneously with vehicle (gray) or TRV0109101 (blue) at the indicated doses and analyzed by: (A) a 56°C hot plate assay (vehicle: $n = 8$; TRV0109101: $n = 4-8$); (B) a 4-hour fecal boli accumulation assay ($n = 8$); or (C) a glass bead colonic motility assay ($n = 6-8$). (D) These same data are then expressed as %maximum possible effect (MPE) and fit with nonlinear regression for ED₅₀ determination. Data are means $\pm$ S.E.M. (* $P < 0.05$, *** $P < 0.001$ via one-way ANOVA with Dunnett's post test to vehicle group).

0.8 hours (subcutaneous) opioid-induced mechanical allodynia was measured approximately 12 hours after subcutaneous dosing to reduce any antiallodynic contribution of MOPR activation.

In summary, the pharmacokinetic profile of TRV0109101 supports the antinociceptive pharmacodynamics observed in rodents. Unbound brain concentrations of TRV0109101 were not determined; however, the estimated free plasma concentrations at minimum efficacious doses in the rodent models exceed the potency at the mouse and rat MOPR receptor.

TRV0109101 and opioid-induced mechanical allodynia.

To test whether a G protein-biased MOPR agonist induces OIMA, mice were chronically infused (minipump) with either vehicle (saline, $n = 30$), morphine (48 mg/kg/day, $n = 8$), oxycodone (25 mg/kg/day, $n = 12$), fentanyl (3.2 mg/kg/day, $n = 8$), or TRV0109101 (20 mg/kg/day; $n = 8$) over 7 days. Mechanical allodynia was measured using 0.4 g von Frey filaments during 7 days of chronic minipump infusion. Significant mechanical allodynia developed following 2 days of oxycodone, morphine, and fentanyl infusion, but not after TRV0109101 (Fig. 5A). This allodynia lasted throughout the dosing paradigm. In conjunction, 5 minutes after testing mechanical allodynia animals were tested in the hot plate model (56°C) and response latencies were recorded (Fig. 5B). In this assay, mice developed antinociceptive tolerance to oxycodone, morphine, fentanyl, and TRV0109101, at the doses tested, throughout the experimental paradigm.

Fig. 5. Continuous TRV0109101 administration does not produce opioid-induced mechanical allodynia but does promote antinociceptive tolerance to thermal stimuli. (A) Mechanical allodynia was measured over 7 days in mice in response to a chronic minipump infusion (7 days) with vehicle (saline, $n = 30$), morphine (48 mg/kg/day, $n = 8$), oxycodone (25 mg/kg/day, $n = 12$), TRV0109101 (20 mg/kg/day; $n = 8$), or fentanyl (3.2 mg/kg/day, $n = 8$). (B) Five minutes after testing for mechanical allodynia, thermal (56°C) hot plate antinociceptive responses were measured. Data are means $\pm$ S.E.M. (** $P < 0.01$, *** $P < 0.001$ via two-way repeated-measures ANOVA with Bonferroni post tests to vehicle).

In addition, we adopted a twice daily subcutaneous dosing protocol on days 1–3, followed by a double dose on day 4 based on previous work (Chen et al., 2010). Mice were dosed with either vehicle (saline or 10:10:80; $n = 16$), morphine (days 1–3: 20 mg/kg; day 4: 40 mg/kg; $n = 16$), oxycodone (days 1–3: 12 mg/kg; day 4: 24 mg/kg; $n = 16$), fentanyl (day 1–3: 0.6 mg/kg; day 4: 1.2 mg/kg; $n = 16$), or TRV0109101 (days 1–3: 20 mg/kg; day 4: 40 mg/kg; $n = 8$) (Supplemental Fig. 6). In this experiment, mechanical allodynia was measured using 0.4 g von Frey filaments during 5 days of treatment and mice treated with oxycodone, morphine, or fentanyl developed OIMA, whereas TRV0109101-treated animals did not. Taken together these data suggest that chronic opioid dosing (morphine, oxycodone, and fentanyl) leads to the induction of OIMA, whereas dosing TRV0109101, a G protein-biased MOPR agonist, does not. Tolerance, however, regardless of drug class, continued to develop suggesting that OIH and antinociceptive tolerance are governed by divergent mechanisms.

TRV0109101 reversal of opioid-induced mechanical allodynia. We next tested whether morphine-induced mechanical allodynia could be reversed by switching drug treatments. We first established OIMA by treating mice, twice daily (subcutaneous), with either vehicle (saline; $n = 12$) or morphine (20 mg/kg; $n = 12$) for 4 days (Fig. 6, Treatment A). Mechanical allodynia was measured daily, and morphine-treated animals

Fig. 6. Repeated TRV0109101 administration reverses morphine-induced mechanical allodynia. Mechanical allodynia was measured following repeated morphine administration. Treatment A: Mice were dosed twice daily (subcutaneously) with vehicle ($n = 12$) or morphine for 4 days (20 mg/kg). Treatment B: On day 5, morphine-treated mice were reassigned and dose twice daily (subcutaneously) to vehicle (saline; $n = 12$), morphine (20 mg/kg; $n = 12$), or TRV0109101 (20 mg/kg, $n = 12$) for 8 additional days. Data are means $\pm$ S.E.M. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, via two-way repeated-measures ANOVA followed by Bonferroni's post tests to vehicle).

began to display significant OIMA by day 2 and lasted throughout this 4-day treatment period compared with vehicle. On the 5th day, after testing for OIMA, mice in the morphine treatment groups were then reassigned to one of three twice daily dosing (subcutaneous), treatment groups: vehicle (saline; $n = 12$), morphine (20 mg/kg; $n = 12$), or TRV0109101 (20 mg/kg, $n = 12$) for 7 additional days (Fig. 6, Treatment B). Following the transition to Treatment B, morphine/vehicle and morphine/morphine-treated animals maintained OIMA levels throughout the entire testing period. In contrast, the morphine/TRV0109101-treated group initially developed OIMA, while under the morphine regimen, however, this OIMA was attenuated when the treatment switched to TRV0109101. This OIMA reversal was not significantly different from vehicle/vehicle-treated animals within 24 hours of initiating TRV0109101 treatment. Additional studies were performed in a new cohort of animals using fentanyl [0.2 mg/kg, 5 days of twice daily (subcutaneous) dosing] instead of morphine to induce OIMA. In addition, we reduced TRV0109101 administration to pharmacologically relevant doses, as determined by hot plate antinociception (Supplemental Fig. 7). In these experiments, fentanyl-induced allodynia was fully reversed 1 day after (Supplemental Fig. 7, Treatment A) 1 mg/kg ($n = 8$) or 3 mg/kg TRV0109101 administration (twice daily, subcutaneous; $n = 8$). OIMA reversal was maintained throughout the dosing paradigm. (Supplemental Fig. 7A, Treatment B) that was indistinguishable from vehicle/vehicle ($n = 8$)-treated animals on days 7 and 8. Interestingly, animals initially on 3 mg/kg TRV0109101 ($n = 8$) did not develop OIMA (similar to 20 mg/kg in Fig. 6), but when switched to twice daily (subcutaneous) fentanyl treatment, developed OIMA by day 8 comparable to OIMA observed with fentanyl alone (0.6 mg/kg; Supplemental Fig. 7). Five minutes after assessing mechanical allodynia, animals were tested in the hot plate model (56°C) and response latencies were recorded (Supplemental Fig. 7B). In this assay, mice developed antinociceptive tolerance to both fentanyl and TRV0109101, at the doses tested, throughout the experimental paradigm. Although switching to TRV0109101 rescues OIMA,

cross-tolerance to different MOPR agonists is observed. Taken together these data confirm that, in mice, both morphine- and fentanyl-induced allodynia can be abrogated by switching to a TRV0109101 treatment paradigm but antinociceptive-tolerance may be regulated by a distinct mechanism.

Discussion

Previous work using β -arrestin2 KO mice has established a role for β -arrestins in opioid-associated adverse events such as respiratory depression and reduced gastrointestinal function (Bohn et al., 1999; Raehal et al., 2005). These data supported the rationale for the discovery and development of G protein-biased MOPR agonists, such as oliceridine, as differentiated analgesics that promote antinociception with reduced liability for conventional opioid-induced adverse events. Here we show that chronic administration of oxycodone, morphine, or fentanyl induce OIMA, a model of OIH, in WT, but not β arr1^{-/-} and β arr2^{-/-} mice, whereas drug tolerance still developed in all genotypes. These data suggest that, in addition to its contributions to a number of characterized opioid-associated adverse events, β -arrestins are critical regulators of the development of OIMA independent of antinociceptive tolerance. We hypothesized that a G protein-biased MOPR agonist could differentiate from conventional opioids when monitoring the development of mechanical allodynia. Indeed, TRV0109101, a G protein-biased MOPR-selective agonist and analog of oliceridine, did not promote the development of mechanical allodynia. In addition, TRV0109101 administration after induction of OIMA (using morphine or fentanyl) resulted in full rescue from established allodynia. Although previous work presented conflicting roles for β -arrestins in the development of OIH (Rowan et al., 2014a,b; Chen et al., 2016), our observations in a model of opioid-induced hyperalgesia, OIMA, provide strong support for the hypothesis that OIH is mediated, at least in part, via β -arrestin-dependent pathways and a G protein-biased MOPR agonist may be an advantageous approach to delivering robust analgesia while reducing the development of OIH.

TRV0109101 is a G protein-biased agonist among a new class of MOPR ligands that include oliceridine, PZM21 and mitragynine pseudoindoxyl. Reported G protein-biased MOPR agonists do not share structural similarity with conventional opioids and promote more effective coupling to G protein signaling pathways (associated with analgesia) than to β -arrestin recruitment (associated with opioid-induced adverse events) compared with morphine. In preclinical models of acute pain and opioid-associated adverse events, G protein-biased MOPR ligands exhibit an increased therapeutic window (compared with morphine) between analgesia and opioid-induced respiratory depression and reduced gastrointestinal function (DeWire et al., 2013; Manglik et al., 2016; Varadi et al., 2016). Although opioid-induced respiratory depression and constipation were improved for each biased ligand compared with morphine, observed effects on tolerance and abuse liability have not been consistent among this new class of MOPR modulators. These observations may be attributed to additional pharmacological target engagement independent of MOPR activity (DeWire et al., 2013; Manglik et al., 2016; Varadi et al., 2016). To date, only oliceridine has been evaluated in humans; multiple randomized controlled trials have suggested that oliceridine may have a wider therapeutic window than morphine (Soergel et al., 2014; Siuda et al., 2017). Like oliceridine,

TRV0109101 is a G protein-biased, MOPR-selective agonist that demonstrates differentiation from morphine on measures of gastrointestinal function while promoting analgesia across multiple models of acute pain. Because of a pharmacodynamic and pharmacokinetic profile similar to oliceridine, we believe that TRV0109101 can serve as a representative pharmacophore for comparable compounds currently in clinical development to further assess the differentiation of a G protein-biased MOPR agonist compared with conventional opioids.

Because morphine, fentanyl, and oxycodone were unable to promote OIMA in β arr1^{-/-} and β arr2^{-/-} mice, β -arrestins may play a fundamental role in the development of OIH and corroborate that the use of a G protein-biased MOPR agonist could avoid the development of OIH. In stark contrast to conventional opioids, TRV0109101 did not induce significant OIMA at any point during the observed time course, even when administered at supratherapeutic doses. TRV0109101 also demonstrated the ability to rapidly rescue morphine and fentanyl-induced mechanical allodynia. TRV0109101-promoted reversal of OIMA was observed after the first administered dose and accomplished sustained, full reversal throughout the dosing paradigm. Interestingly, chronic administration of TRV0109101 and subsequent switching to fentanyl resulted in the induction of mechanical allodynia. These data suggest that agonist-promoted β -arrestin recruitment appears to play a prominent role in not only the development of OIH, but also the maintenance of the condition. Thus, as a G protein-biased agonist, TRV0109101 fundamentally avoids the contributions of β -arrestins in these processes at the receptor level to promote robust MOPR-mediated analgesia with reduced OIH liability.

Although our pharmacological (TRV0109101) and genetic (β arr1^{-/-} and β arr2^{-/-} mice) approaches suggest that β -arrestin engagement mediates the development of OIH, we have yet to study the direct contributions of NOPR engagement to the differentiated pharmacology of TRV0109101. Interestingly, cebranopadol (Schunk et al., 2014), a MOPR/NOPR-mixed agonist that lacks G protein-biased MOPR pharmacology, induced robust mechanical allodynia (comparable to oxycodone) after chronic dosing (Supplemental Fig. 8). These data, along with the congruent pharmacological and genetic evidence presented, suggest that β -arrestin recruitment to MOPR is a primary determinant in the development of opioid-induced hyperalgesia.

Although not inducing OIMA at the doses tested, TRV0109101 promoted drug tolerance comparable to conventional opioids. Interestingly, mitragynine pseudoindoxyl, a recently developed MOPR agonist/DOPR antagonist with limited activity at β -arrestin2, also displayed analgesic tolerance in the tail flick assay. In contrast to other MOPR ligands, mitragynine pseudoindoxyl-induced tolerance developed substantially slower (29 versus 5 days) than morphine when dosed twice daily (5 mg/kg, twice daily s.c.) (Varadi et al., 2016). As mitragynine pseudoindoxyl also serves as a DOPR antagonist, it is possible that this activity may contribute to observations of delayed tolerance. It will be important to examine TRV0109101 at different doses and in other models of acute pain to better examine drug tolerance among G protein-biased MOPR agonists.

A striking observation described in this work regards the critical role that β -arrestins play in the development of OIMA independent of modulating drug tolerance. This suggests that the development of OIH and opioid tolerance may operate

through fundamentally distinct mechanisms. Although previous work has suggested that morphine does not promote antinociceptive tolerance in β -arr2^{-/-} mice following once daily morphine (10 mg/kg/day s.c.) injections (Bohn et al., 2000,2002), our dosing paradigm reveals that morphine, oxycodone, and fentanyl promote tolerance independent of β -arrestin expression. Although comparable tolerance to oxycodone and fentanyl infusion was observed in β -arrestin2^{-/-} mice (Raehal and Bohn, 2011), delayed morphine tolerance was noted in β -arrestin2^{-/-} mice using a tail flick antinociceptive assay, a measure of spinal reflexive responsiveness (Bohn et al., 2002). Although β -arr2^{-/-} is believed to be involved in MOPR desensitization, it has been extensively examined in opioid tolerance, dependence and OIH (Chen et al., 2016). β -Arrestin1 is thought to play a role in MOPR ubiquitination, dephosphorylation, and resensitization (Dang et al., 2011; Groer et al., 2011; Dang and Christie, 2012; Williams et al., 2013; Raehal and Bohn, 2014), but little is known about its role in opioid-induced adverse events. As such, it is currently unclear whether the actions of β -arrestin1 and β -arrestin2 in OIH are mediated by distinct cellular mechanisms or if possible compensatory mechanisms present in knockout animals overshadow their singular contributions. Previous studies have also attributed β -arrestins with MOPR-mediated adverse events in both humans and animals, with particular emphasis on respiratory depression, gastrointestinal transit, reward reinforcement, tolerance, and now OIH (Bohn et al., 1999,2000; Raehal et al., 2005; DeWire et al., 2013; Soergel et al., 2014; Manglik et al., 2016; Varadi et al., 2016).

The clinical approach for treating opioid tolerance is markedly different than addressing OIH. Tolerance may be overcome by increasing the drug dose or by "opioid-switching" (Lee et al., 2011). "Opioid-switching" is a common clinical practice where different opioids used to manage pain are sometimes changed to maintain analgesic efficacy without subsequent opioid-induced adverse consequences including tolerance (Quigley, 2004; Mercadante and Bruera, 2006,2016). Although these protocols may improve antinociceptive tolerance, they are unlikely to improve the pain associated with OIH. Pain associated with OIH escalates with increased opioid dosage and conventional opioids, such as morphine, fentanyl and oxycodone, appear to carry a comparable liability for the development of OIH (Lee et al., 2011). Because chronic dosing of TRV0109101 did not result in OIMA and switching to TRV0109101 could reverse fentanyl or morphine-promoted OIMA, an "opioid-switching" protocol to a G protein-biased MOPR agonist, rather than a conventional opioid, could be an advantageous avenue in treating pain in patients with established OIH. Although we believe that the reversal of fentanyl or morphine-promoted OIMA using TRV0109101 is consistent with the differentiated biased ligand mechanism of action, we have not ruled out that opioid switching alone may explain this observation. Future studies monitoring a variety of opioids and dosing regimens could be useful to characterize better this phenomenon.

In conclusion, we believe that other G protein-biased MOPR agonists, under these treatment conditions, may demonstrate analogous properties to TRV0109101, including decreased liability for the development of OIH in mice compared with conventional opioids. Whether these findings translate into the clinic is yet to be determined, but it remains

an interesting area for future investigation. Broadly, this preponderance of data, along with the preclinical and clinical characterization of oliceridine, validate that the development of biased agonists to selectively target one intracellular signaling pathway over another is an important approach toward providing clinical efficacy with reduced adverse event liability.

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Authorship Contributions

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Conducted experiments: Koblish, Carr III, Siuda, Rominger, and Gowen-MacDonald.

Contributed new reagents or analytic tools: Gowen-MacDonald.

Performed data analysis: Koblish, Carr III, Siuda, Rominger, Gowen-MacDonald, and Cowan.

Wrote or contributed to the writing of the manuscript: Koblish, Carr III, Siuda, Rominger, Gowen-MacDonald, Crombie, Violin, and Lark.

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