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Biased agonism of clinically approved μ-opioid receptor agonists and TRV130 is not controlled by binding and signaling kinetics

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HIGHLIGHTS

- Oliceridine (R)-TRV130 and buprenorphine display equal extreme biased agonism.
- Buprenorphine display high residence time compared to oliceridine (R)-TRV130.
- Signaling and binding kinetics does not alter observational bias.
- μ-opioid receptor agonists display different dependence on GRK2 and GRK5 overexpression.

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ABSTRACT

Binding and signaling kinetics have previously proven important in validation of biased agonism at GPCRs. Here we provide a comprehensive kinetic pharmacological comparison of clinically relevant μ-opioid receptor agonists, including the novel biased agonist oliceridine (TRV130) which is in clinical trial for pain management. We demonstrate that the bias profile observed for the selected agonists is not time-dependent and that agonists with dramatic differences in their binding kinetic properties can display the same degree of bias. Binding kinetics analyses demonstrate that buprenorphine has 18-fold higher receptor residence time than oliceridine. This is thus the largest pharmacodynamic difference between the clinically approved drug buprenorphine and the clinical candidate oliceridine, since their bias profiles are similar. Further, we provide the first pharmacological characterization of (S)-TRV130 demonstrating that it has a similar pharmacological profile as the (R)-form, oliceridine, but displays 90-fold lower potency than the (R)-form. This difference is driven by a significantly slower association rate. Finally, we show that the selected agonists are differentially affected by G protein-coupled receptor kinase 2 and 5 (GRK2 and GRK5) expression. GRK2 and GRK5 overexpression greatly increased μ-opioid receptor internalization induced by morphine, but only had modest effects on buprenorphine and oliceridine-induced internalization. Overall, our data reveal that the clinically available drug buprenorphine displays a similar pharmacological bias profile in vitro compared to the clinical candidate drug oliceridine and that this bias is independent of binding kinetics suggesting a mechanism driven by receptor-conformations.

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1. Introduction

G protein-coupled receptors (GPCRs) are a major drug target family through which ~30% of all medicines in the clinic mediate their action, and with ~20% of all drugs in clinical trials targeting these receptors (Hauser et al., 2017). The conventional model of GPCR activation describes that binding of agonists stabilize a receptor state that leads to activation of its cognate G protein, whereas antagonist binding...
stabilizes an inactive receptor conformation. Due to this, GPCR signaling was traditionally interpreted in a linear fashion as a switch that could either turn a given signaling pathway “on” or “off” (Costa-Neto et al., 2016). Today, increasing evidence shows that GPCR functionality is pluridimensional, meaningGPCRs can interact with multiple G proteins, β-arrestins, G protein-coupled receptor kinases (GRKs) and other effectors (Daaka et al., 1997; Schönege et al., 2017; Violin et al., 2006; Zhang et al., 2015). Ubiquitous expression of GPCRs combined with the pluridimensional signaling, can lead to activation of non-desired signaling pathways in either therapeutically relevant or non-relevant tissues upon drug administration. As such, selective activation of signaling pathways by a GPCR has in recent years been shown to hold a great potential in drug discovery (DeWire et al., 2013; Violin et al., 2014). Ligands were found to differentially regulate these different pathways through their action on a unique receptor leading to the concept of biased agonism (Costa-Neto et al., 2016).

The concept of biased agonism has opened up the possibility to design agonists that selectively activate therapeutically relevant signaling pathways, while not engaging pathways responsible for undesired effects that are mediated by the same receptor. Deciphering the contribution from individual effectors to physiological outcomes is complex, yet necessary to fully exploit the potential of biased agonism. Taking the example of the μ-opioid receptor (μ-OR), which has been extensively studied in relation to bias, it has been demonstrated that β-arrestin2 knock out in mice led to decreased side-effects such as respiratory depression and constipation, as well as reducing morphine tolerance but not dependence (Bohn et al., 2006; Raehal et al., 2005). Further, morphine reward and dependence have been shown to be driven by GRK5 but not GRK3 whereas morphine-induced locomotor activity is dependent on GRK6 (Glück et al., 2014; Raehal et al., 2009). Recently, G protein biased agonists for the μ-OR have been discovered that have analgesic properties comparable to morphine, yet with reduced side-effects such as nausea and constipation in rodents and humans (Chen et al., 2013; DeWire et al., 2013; Manglik et al., 2016; Soergel et al., 2014), and reduced respiratory depression in rodents (DeWire et al., 2013; Manglik et al., 2016). In humans, some oliceridine dose regimens have been reported to reduce opioid-induced respiratory adverse effects compared to morphine, but reduced side-effects such as nausea and constipation in rodents and humans (Chen et al., 2013; DeWire et al., 2013; Manglik et al., 2016; Soergel et al., 2014; Viscoli et al., 2019, 2016) albeit not all (Singla et al., 2017) clinical studies. Thus, the μ-OR is a promising drug target in relation to biased agonism.

Until now, most studies on biased agonism have primarily focused on comparing signaling outcomes at their individually optimal activation time for measurement. However, a recent study on the dopamine D2 receptor showed that agonist-dependent signaling bias can change dramatically with time, and that this characteristic was linked to the binding kinetics of the agonists (Klein Herenbrink et al., 2016). For the μ-OR it has also been demonstrated that expression levels of GRK2, one of the key mediators in β-arrestin recruitment and thereby receptor internalization, can influence the degree of bias (Manglik et al., 2016; Thompson et al., 2016). This has led to the question whether bias is in fact driven by kinetics and cellular background rather than conformation.

In this study, we set out to explore and compare the bias profiles of clinically relevant agonists and determine whether kinetic context is critically important when assessing their bias profiles and whether these agonists display differences in GRK2 and GRK5 sensitivity.

2. Methods and materials

2.1. Materials

All reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. All restriction endonucleases, Dulbecco’s modified eagle medium (DMEM), penicillin/streptomycin (PenStrep), dialyzed and non-dialized fetal bovine serum (dFBS and FBS), OptiMEM, Dulbecco’s phosphate buffered saline (DPBS), Hank’s balanced salt solution (HBSS), Lipofectamine 2000, pcDNA5/-S-FRT/TO vector, pOG44 Flp-Recombinase expression vector and the Flp-In™ T-REX™ 293 cell line were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Zeocin, hygromycin B, blasticidin S HCl and pluronic® F-68 non-ionic surfactant were purchased from Life Technologies (Carlsbad, CA, USA). Polyethyleneimine (PEI) was purchased from Polysciences Inc. (Warrington, PA, USA). Coelenterazine 400a was purchased from NanoLight Technology (Pinetop, AZ, USA). Tag-lite® SNAP Lumi4-Tb® was purchased from CisBio (Codolet, France). MicroScint-0, GF/C filter plates and [3H]-Naloxone were purchased from Perkin Elmer (Waltham, MA, USA). DAMGO and Gpp(NH)p were purchased from Abcam (Cambridge, United Kingdom). Buprenorphine, loperamide and morphine were obtained from Lundbeck A/S (Valby, Denmark) or purchased from Sigma-Aldrich (St. Louis, MO, USA). The pcDNA3.1(+)-GRK2 and pcDNA3.1(+)-GRK5 constructs were kindly gifts from Novo Nordisk A/S (Maaloev, Denmark).

2.2. Synthesis of TRV130

(R)- and (S)-TRV130 were synthesized in-house by a modified method to that previously reported (Chen et al., 2013) to provide racemic TRV130 followed by chiral preparative SFC to give both enantiomers with high enantiopurity (e.r. > 99:1). See supporting information for full experimental details and characterization.

2.3. Plasmids and constructs

The parental pcDNA5/FRT/TO vector was modified by removal of an MluI site using site-directed mutagenesis. For the generation of SNAP-tagged receptor, the vector was additionally modified with the inclusion of an expression cassette containing the 21 amino acid human interleukin 2 receptor alpha (hIL-2Ra) signal peptide (MDSYLLMWGLITFIMVPGCCA), FLAG-tag (DYKDDDDK) and SNAP-tag (Maurel et al., 2008) in frame with unique 3’ MluI and 5’ NotI sites as for receptor insertion. Construction of pcDNA5/FRT/TO encoding the N-terminally FLAG-tagged human μ-OR or FLAG-SNAP-tagged human μ-OR were thereafter conducted by restriction cloning. Vectors encoding BRET constructs with human Ga13, Ga42 and β-arrestins sequences were described previously (Galés et al., 2005; Namkung et al., 2016; Quoyer et al., 2013).

2.4. Cell lines and culturing

Parental Flp-In T-REX293 cells were maintained in DMEM supplied with 10% dFBS, 1% PenStrep and 100 μg/ml zeocin at 37 °C and 5% CO2 in a humidified incubator. Flp-In T-REX293 cells were transfected with the pOG44 vector encoding the Flp-Recombinase and pcDNAs/ FRT/TO encoding the FLAG-tagged human μ-OR at a ratio of 9:1 with Lipofectamine 2000 as the transfection reagent, according to the manufacturer’s instructions. Flp-In T-REX293-FLAG-µ-OR were maintained in DMEM supplied with 10% dFBS, 1% PenStrep, 15 μg/ml blasticidin S HCl and 200 μg/ml hygromycin B at 37 °C and 5% CO2. HEK293A cells were maintained in DMEM supplied with 10% FBS and 1% PenStrep at 37 °C and 5% CO2.

2.5. BRET measurements

HEK293A cells were transfected with PEI as previously described (Namkung et al., 2016) with 20 ng Ga13-RlucII or 10 ng Ga42-RlucII (BRET donor) along with 300 ng G72-GFP10 (BRET acceptor) to detect G protein activation. For β-arrestin recruitment recordings, cells were transfected with 20 ng β-arrestin1-RlucII or 20 ng β-arrestin2-RlucII (BRET donor) along with 500 ng rGFP-CAAX (BRET acceptor). In both BRET assays, 200 ng pcDNAs/FRT/TO-FLAG-µ-OR were co-transfected and the total DNA amount was adjusted to 1 μg with pcDNA3.1(+) for
G protein activation assays, 100 ng戈甲 was additionally co-transfected. Forty-eight hours post transfection, cells were washed in DPBS and incubated in assay buffer (HBSS, 1 mM CaCl₂, 1 mM MgCl₂, 20 mM HEPES, 0.01% pluronic® F-68, pH 7.4) for 30 min prior to stimulation with varying concentrations of ligand for the indicated times of kinetic measurements. Two minutes prior to BRET recordings coelenterazine 400a was added to a final concentration of 2.5 μM. All BRET measurements were performed using a Mithras LB940 Multimode Microplate Reader (Berthold Technologies, Bad Wildbad, Germany) equipped with the filters for BRET: 400/70 nm (donor) and 515/20 nm (acceptor). Raw BRET ratios were determined by calculating the ratio of light intensity emitted by GFP10 or rGFP divided with the light intensity emitted by RLucII. The BRET ratios were normalized by subtracting BRET ratios obtained with buffer-stimulation and thereafter as per cent of stimulation with maximum DAMGO concentration ((BRET_Tligand/BRETDAMGO) × 100) to all individual time points.

2.6. TR-FRET real-time internalization

Internalization of the μ-OR was tracked with a method previously described (Roed et al., 2014). HEK293A cells were seeded at a density of 4 × 10⁵ cells/well in a 6-well plate and transfected 24 h later with 300 ng of pcDNA5/FRT/TO-FLAG-SNAP-μ-OR and varying amounts of pcDNA3.1(+)GRK2 or pcDNA3.1(+)GRK5. In all cases, the DNA amount was equalized with empty pcDNA3.1(+). Cells were transfected with Lipofectamine 2000 according to the manufacturer’s recommendations. Twenty-four hours post transfection, cells were detached and re-seeded into poly-α-lysine coated white 384-well plates at a density of 2 × 10⁴ cells/well overnight. Surface expressed FLAG-GRK2 was transferred to poly-D-lysine coated white 384-well plates and incubated in assay buffer (HBSS, 1 mM CaCl₂, 1 mM MgCl₂, 20 mM HEPES, 0.01% pluronic® F-68, pH 7.4) and incubated for 5 min with 10 μM of 100 μM fluorescein-O-acetic acid diluted in assay buffer. Thereafter the cells were stimulated with 10 μl pre-heated ligands at 2 × final concentrations of unlabeled agonists. The binding reaction was terminated by the addition of 10μM fentanyl. Radioactivity resulting from bound [³H]naloxone was measured at multiple time points after addition of fentanyl.

To determine kobs of [³H]naloxone, association was initiated by the addition of final concentrations 10 nM, 5 nM or 1 nM of [³H]naloxone to the membranes and terminated by filtration at various time points. The kobs of [³H]naloxone was derived from kobs using the k_{on} value determined from dissociation binding experiments.

Kinetic parameters of unlabeled agonists were determined by simultaneous addition of [³H]naloxone with unlabeled agonists to Flp-In T-Rex293-FLAG-μ-OR membranes. To ensure that the rate constants determined for the unlabeled agonists were independent of the ligand concentration, [³H]naloxone was mixed with three different concentrations of unlabeled agonists. The binding reaction was terminated at different time points.

2.9. Data analysis

The results were analyzed using Prism 7.0 (GraphPad Software, San Diego, CA, USA).

Concentration-response curves were fitted using the following four parameter model:

\[
Y = \text{Bottom} + \frac{(\text{Top} - \text{Bottom})}{1 + 10^{(\log(\text{EC}_{50}) - \log([A^n])})}
\]

where Bottom and Top represent the minimal and maximal asymptote of the concentration-response curve, [A] is the logarithm molar concentration of agonist and EC_{50} is the concentration of agonist required to induce 50% of the maximal response.

For quantification of ligand bias, concentration response-curves were fitted to the following form of the operational model of agonism (Kenakin et al., 2012; Klein Herenbrink et al., 2016), originally described by Black and Leff (1983):

\[
E = \text{Basal} + \frac{(E_{\text{max}} - \text{basal})}{[A]^n} \left(\frac{\frac{[A]}{K_a} + 1}{1 + \left(\frac{[A]}{K_a}\right)^n}\right)
\]

where E_{max} is the maximal possible response of the system, basal is the basal level of response, K_a the equilibrium dissociation constant of the agonist, τ represents the signalling efficacy of the agonist, and n is the slope of the function. E_{max} and n were shared between the agonists. Data for all agonists within each pathway were fit globally to determine τ and K_a. Biased agonism was then quantified by subtracting the log(r/K_a)DAMGO from log(r/K_a) of the agonists tested giving the ΔΔlog(r/K_a). The ΔΔlog(r/K_a) was calculated by subtracting the Δlog(r/K_a) of two given pathways. The agonist concentration that inhibited half of the total [³H]naloxone binding was determined using the equation:

\[
Y = \text{Bottom} + \frac{(\text{Top} - \text{Bottom})}{1 + 10^{(\log(K_{nA})})}
\]
where \( Y \) denotes the percentage of the total binding. **Bottom** and **Top** represent the minimal and maximal asymptote, \( IC_{50} \) represent the agonist concentration inhibiting 50% of the total binding. \( IC_{50} \) values obtained from the fit were used to calculate the \( K_I \) values using the equation described by Cheng and Prusoff (1973):

\[
K_I = \frac{IC_{50}}{1 + [A]/K_D}
\]

(4)

where \([A]\) represents the \([3H]\)naloxone concentration in molar and \(K_D\) represents the equilibrium dissociation constant of \([3H]\)naloxone-receptor complex in molar concentration.

The data obtained for \([3H]\)naloxone in dissociation binding were fitted to a one-phase exponential decay function and \(t_{1/2}\) was transformed into \(k_{off}\) rate constants using the following equation:

\[
k_{off} = \frac{\ln(2)}{t_{1/2}}
\]

(5)

The data obtained for \([3H]\)naloxone in association binding were fitted to a one-phase exponential association function to calculate the observed association rate \(k_{obs}\). The association rate \(k_{on}\) was calculated as described by Hill (1909):

\[
k_{on} = \frac{k_{obs} - k_{off}}{[radioligand]}
\]

(6)

where the \(k_{off}\) value was determined from dissociation binding. These rate parameters could then be used to calculate the equilibrium dissociation constant of the radioligand, \(K_D\):

\[
K_D = \frac{k_{off}}{k_{on}}
\]

(7)

Association and dissociation rates for the unlabeled agonists were calculated by fitting the data from competition kinetic experiments to a two-component exponential equation described originally by Motulsky and Mahan (1983):

\[
[RL] = \frac{N k_1 [L] \cdot 10^{-9}}{K_F - K_S} \left( \frac{k_4 - k_3}{K_F K_S} \right) e^{-k_3 t} - \left( \frac{k_4 - K_S}{K_S} \right) e^{-k_4 t}
\]

(8)

with the following variables used:

\[
K_F = 0.5(K_a + K_g + \sqrt{[(K_a - K_g)^2 + 4k_4 k_1 [L] [I] \cdot 10^{-18}})
\]

where the abbreviations used are: \(R\), receptor; \([L]\), radioligand in nanomolar; \(RL\), receptor-radioligand complex; \(I\), non-labeled competitor in nanomolar; \(k_1\), association rate of radioligand; \(k_2\) dissociation rate of radioligand; \(k_3\) association rate of non-labeled competitor; \(k_4\) dissociation rate of non-labeled competitor; \(N\), total number of receptors; \(t\), time in minutes. Fixing \([L]\), \([I]\), \(k_1\) and \(k_2\) allowed calculation of \(k_3\), \(k_4\) and \(N\).

The residence time (\(t_R\)) of the unlabeled agonists could then be calculated using the equation:

\[
t_R = \frac{1}{k_{off}}
\]

(9)

3. Results

3.1. Buprenorphine and the TRV130 enantiomers are apparent extreme G protein biased agonists

To compare the bias profiles of clinically relevant agonists, we chose agonists with different pharmacological profiles both in vivo and in vitro. The agonists selected for the study were (\(\alpha\)-Ala(2)-meph(4)-gly-ol(5)) enkephalin (DAMGO), loperamide, morphine, buprenorphine, and both enantiomers of TRV130 (Fig. 1). DAMGO, a degradation-resistant \(\mu\)-OR selective analogue of the endogenous agonist met-enkephalin (Handa et al., 1981) was used as the reference agonist in all studies. Loperamidade, which is used in the clinic as an anti-diarrheal medication, was selected as it exhibits different bioavailability compared to other opiates and thereby non-analgesic effects (Regnard et al., 2011; Schinkel et al., 1996). Morphine, buprenorphine and TRV130 were selected due to their very different analgesic and pharmacological profiles in vivo and in vitro. Morphine, is a full agonist with respect to its analgesic effects but is also known to be a partial agonist in \(\beta\)-arrestin2 recruitment to the \(\mu\)-OR (DeWire et al., 2013; McPherson et al., 2010; Molinari et al., 2010). Buprenorphine is a potent partial agonist in \(G\) protein recruitment/activation at the \(\mu\)-OR, with distinct pharmacological properties such as a bell-shaped analgesic dose-response curve and a ceiling effect with respect to respiratory depression (Dahan et al., 2006, 2005; Lutfy et al., 2003). Further, buprenorphine has been shown to be biased against \(\beta\)-arrestin2 recruitment (McPherson et al., 2010; Molinari et al., 2010). TRV130 was selected since it is a biased \(\mu\)-OR clinical candidate having improved analgesic properties and reduced side-effects compared to morphine in rodents (DeWire et al., 2013) and to some degree in humans (Singla et al., 2017; Soergel et al., 2014; Viscusi et al., 2019, 2016). However, the FDA has recently evaluated...
and rejected the New Drug Application and requested additional safety data (Trevena Inc., 2018).

To keep the assay conditions consistent and to avoid bottlenecks in terms of inhibitors in second messenger assays, we focused on the two major pathways from the μ-OR, Gαi2 and GαoA activation as well as β-arrestin1 and β-arrestin2 recruitment, all of which are known to be activated/recruited by the μ-OR (Chakrabarti et al., 1995; Groer et al., 2011; Saidak et al., 2006). DAMGO, buprenorphine, morphine, loperamide and oliceridine ((R)-TRV130) all potently activated both Gαi2 (Fig. 2A) and GαoA (Fig. 2B) proteins, whereas (S)-TRV130 was 90-fold less potent compared to oliceridine for both G proteins (Fig. 2A and B). In β-arrestin recruitment, DAMGO behaved as a full agonist both in β-arrestin1 and β-arrestin2 recruitment, whereas morphine and loperamide behaved as partial agonists (Fig. 2C and D). From the concentration responses of buprenorphine, oliceridine and (S)-TRV130 in β-arrestin1 and β-arrestin2 recruitment, no meaningful fit could be obtained as none of these agonists reached a maximum plateau within the possible concentration range applied, indicating that they are very weak agonists, if at all, for these pathways.

3.2. Signaling measurement kinetics does not influence biased agonism at the μ-OR

Conventionally quantification of biased agonism has been calculated at single time points to which the optimal signaling responses are obtained within the given signaling pathway. Consequently, the time point of measurement can vary greatly from seconds to hours. Analytical methods to describe bias assume that binding equilibrium has been established. However, the assumption of equilibrium must be considered when applying these models on data from dynamic biological systems (Lane et al., 2017). If the data are obtained under non-equilibrium conditions, this can have a great impact on the apparent ligand potency. Accordingly, it has been reported that kinetics can be important for the observed bias of the selected agonists at time-resolved measurement of G protein activation and β-arrestin recruitment after agonist stimulation. DAMGO behaved as a full agonist for both Gαi2 and GαoA activation, as well as β-arrestin1 and β-arrestin2 recruitment and possessed a stable potency over time (Fig. 3A–D and Table 1). In contrast, buprenorphine behaved as a partial agonist to all time points in Gαi2 activation (Fig. 3E and Table 1) and at time points up to 20 min in GαoA activation (Fig. 3F and Table 1). In both cases, the potency of buprenorphine increased dramatically over time. Buprenorphine did not recruit the β-arrestins at any time point (Fig. 3G and H and Table 1) and therefore no formal bias could be quantified. Yet, given the difference in potency between the G protein and β-arrestin assays, for DAMGO (8.75 vs 6.39) and that the efficacy of buprenorphine toward Gαi2 and GαoA was at least 75% of that of DAMGO, one would expect to see a measurable β-arrestin response starting at least at concentrations of 1 μM. Yet no response could be detected even at 100 μM, strongly arguing in favor of bias that we qualify as apparent bias. The potency of morphine and loperamide remained constant in all pathways and both behaved as partial agonists in recruitment of the β-arrestins over the time course (Fig. 3I–L and Table 1, respectively). Like for buprenorphine, the TRV130 enantiomers behaved as partial agonists in Gαi2 activation to all time points (Fig. 3Q and U and Table 1), but only at early time points in GαoA activation (Fig. 3R and V and Table 1). Further, the TRV130 enantiomers induced similar extremely biased responses of β-arrestin1 and β-arrestin2 recruitment over the time course (Fig. 3S and T and Fig. 3X and Y and Table 1). In contrast to buprenorphine, both the TRV130 enantiomers remained stable in potency for both G proteins within the time course (Fig. 3Q–R and Fig. 3U–V and Table 1). No meaningful fits could be obtained for buprenorphine and the TRV130 enantiomers in β-arrestin recruitment at any time point, as these agonists failed to give a maximal plateau within the concentrations applied. Therefore, the apparent extreme bias of these agonists was preserved irrespective of the time point of validation.

Application of the operational model on the concentration response data where meaningful fits could be obtained and the bias factors
Fig. 3. Kinetic concentration-responses of μ-OR agonists. Responses induced by increasing concentrations of (A-D) DAMGO, (E-H) buprenorphine, (I-L) morphine, (M-P) loperamide, (Q-T) oliceridine ((R)-TRV130) and (U-Y) ((S)-TRV130) in HEK293A cells transiently expressing the human FLAG-μ-OR were determined at various time points between 2 and 60 min. The effects of the agonists were measured with BRET-based sensors and the data were normalized to the respective time point of DAMGO within each pathway. The values are expressed as mean ± SEM of three to four independent experiments performed in duplicate.
The data were fitted to a four-parameter model of agonism to determine Emax and pEC50 values. No fit could be obtained for buprenorphine, oliceridine (R)-TRV130 and (S)-TRV130 in β-arrestin recruitment. Emax of the agonist-induced responses compared to DAMGO were compared using one-way ANOVA followed by Dunnett’s multiple comparisons test.

### Gαi2 Activation

|          | 2min | 5min | 10min | 20min | 40min | 60min |
|----------|------|------|-------|-------|-------|-------|
|          | Emax | SEM  | pEC50 | SEM   | Emax  | SEM  | pEC50 | SEM   |
| DAMGO    | 98   | 1    | 8.65  | 0.04  | 98    | 1    | 8.61  | 0.03  |
| Buprenorphine | 74  | 3    | 8.35  | 0.07  | 77    | 2    | 8.32  | 0.05  |
| Morphine | 96   | 3    | 8.14  | 0.06  | 100   | 1    | 8.09  | 0.04  |
| Oliceridine (R)-TRV130 | 85  | 1    | 8.45  | 0.05  | 85    | 2    | 8.48  | 0.05  |
| (S)-TRV130 | 78  | 2    | 6.49  | 0.05  | 87    | 1    | 6.49  | 0.04  |

### GαoA Activation

|          | 2min | 5min | 10min | 20min | 40min | 60min |
|----------|------|------|-------|-------|-------|-------|
|          | Emax | SEM  | pEC50 | SEM   | Emax  | SEM  | pEC50 | SEM   |
| DAMGO    | 99   | 1    | 8.90  | 0.02  | 98    | 1    | 8.88  | 0.02  |
| Buprenorphine | 93  | 1    | 8.64  | 0.03  | 91    | 2    | 8.98  | 0.04  |
| Morphine | 103  | 1    | 8.24  | 0.06  | 107   | 2    | 8.42  | 0.04  |
| Oliceridine (R)-TRV130 | 89  | 1    | 8.53  | 0.02  | 89    | 1    | 8.57  | 0.03  |
| (S)-TRV130 | 95  | 1    | 6.66  | 0.03  | 94    | 1    | 6.74  | 0.04  |

### β-Arrestin1 Recruitment

|          | 2min | 5min | 10min | 20min | 40min | 60min |
|----------|------|------|-------|-------|-------|-------|
|          | Emax | SEM  | pEC50 | SEM   | Emax  | SEM  | pEC50 | SEM   |
| DAMGO    | 99   | 3    | 6.39  | 0.08  | 100   | 1    | 6.17  | 0.03  |
| Buprenorphine | 30  | 6    | 6.90  | 0.69  | 19    | 1    | 6.29  | 0.16  |
| Morphine | 59   | 2    | 6.49  | 0.10  | 61    | 3    | 6.74  | 0.08  |
| Oliceridine (R)-TRV130 | nd  | nd   | nd    | nd    | nd    | nd   | nd    | nd    |
| (S)-TRV130 | nd  | nd   | nd    | nd    | nd    | nd   | nd    | nd    |

### β-Arrestin2 Recruitment

|          | 2min | 5min | 10min | 20min | 40min | 60min |
|----------|------|------|-------|-------|-------|-------|
|          | Emax | SEM  | pEC50 | SEM   | Emax  | SEM  | pEC50 | SEM   |
| DAMGO    | 99   | 2    | 6.43  | 0.06  | 101   | 1    | 6.21  | 0.03  |
| Buprenorphine | 25  | 2    | 6.46  | 0.17  | 21    | 1    | 6.28  | 0.16  |
| Morphine | 65   | 1    | 6.39  | 0.10  | 66    | 1    | 6.74  | 0.08  |
| Oliceridine (R)-TRV130 | nd  | nd   | nd    | nd    | nd    | nd   | nd    | nd    |
| (S)-TRV130 | nd  | nd   | nd    | nd    | nd    | nd   | nd    | nd    |

*a: P ≤ 0.05 compared to DAMGO, b: P ≤ 0.01 compared to DAMGO, c: P ≤ 0.001 compared to DAMGO.*
3.3. Buprenorphine display significant higher receptor residence time compared to the other agonists

Characterization of the binding parameters of [3H]naloxone was determined to obtain the kinetic as well as the equilibrium binding parameters of the unlabeled agonists. The kinetic constants \( k_{on} \) and \( k_{off} \) of [3H]naloxone were determined from association and dissociation binding (Supplementary Figs. S1A and B) and were calculated to be \( 13.4 \times 10^7 \text{M}^{-1}\text{min}^{-1} \pm 0.9 \times 10^7 \text{M}^{-1}\text{min}^{-1} \) and \( 1.196 \text{min}^{-1} \pm 0.2 \text{min}^{-1} \), respectively. Using these constants, the dissociation constant \( pK_d \) was calculated to be 8.05 ± 0.03, which was consistent with data from saturation binding (\( pK_d \): 8.07 ± 0.04) (Supplementary Fig. S1C). To further confirm the kinetic parameters of [3H]naloxone, \( k_{on} \) and \( k_{off} \) were plotted against the [3H]naloxone concentration used in the association kinetic experiments. If the system follows the law of mass action, \( k_{on} \) should increase in a linear manner with increasing radioligand concentration (Fig. 3.4. μ-OR agonists display different dependency on GRK2 and GRK5 overexpression

Phosphorylation of the μ-OR promotes β-arrestin recruitment and internalization. Multiple kinases such as PKC and GRKs have been shown to phosphorylate specific residues in the C-terminal of the receptor in a ligand dependent manner (Bailey et al., 2009; Doll et al., 2012; Just et al., 2013). Since different cell types could have diverse kinase abundance, overexpression of these kinases and the effect on signaling have been intensely investigated. Recently, studies have demonstrated that the ability of ligands to recruit β-arrestins can change profoundly with GRK2 overexpression, resulting in attenuation or disappearing of bias (Manglik et al., 2016; Thompson et al., 2016). To determine whether overexpression of GRK2 could increase internalization mediated by the strongly biased buprenorphine and TRV130 enantiomers and to compare the effect to the other μ-OR agonists, expression of GRK2 was titrated in HEK293A cells and the impact on μ-OR internalization was investigated.

The agonist profile of μ-OR internalization was consistent with β-arrestin recruitment, as DAMGO was a full agonist, morphine and loperamide were partial agonists and buprenorphine, olliceridine and (S)-TRV130 induced minimal internalization (Fig. 6A). In all cases, overexpression of GRK2 increased the agonist-induced internalization of μ-OR. However, the degree to which the internalization increased with GRK2 overexpression was variable for the agonists (Fig. 6B and Supplementary Fig. S4). GRK2 overexpression increased the DAMGO-induced internalization span by 53 ± 17%, whereas the morphine-induced internalization span was drastically increased by 105 ± 18%. Loperamide was the least affected by GRK2 overexpression with a span increase of 21 ± 7%. Further, the internalization span induced by the biased agonists also increased upon GRK2 overexpression, with buprenorphine-induced internalization increasing by 46 ± 10% and olliceridine ((R)-TRV130) and (S)-TRV130 by 67 ± 9% and 55 ± 12%, respectively. Both \( E_{max} \) and the span increase for buprenorphine, olliceridine and (S)-TRV130 were not significantly different, suggesting GRK2 overexpression impact these agonists equally in the system used here. Another important observation from the μ-OR internalization experiments is that GRK2 overexpression altered morphine from a low efficacy partial agonist into a full agonist, whereas the biased agonists buprenorphine and the TRV130 enantiomers were altered from being inactive into medium efficacy partial agonists (Fig. 6B). Since GRK5 has been reported to be of importance for μ-OR phosphorylation, anti-nociception, tolerance, and dependence (Doll et al., 2012; Glück et al., 2014), we also assessed the effect of titrating this kinase. In general, the agonist-induced μ-OR internalization response increased in a similar manner upon GRK5 overexpression as observed for GRK2 overexpression (Fig. 6D and Supplementary Fig. S5). However, the actual increase in μ-OR internalization was slightly less with a span increase of DAMGO-induced internalization of 34 ± 11%. Here morphine was altered from being a low-efficacy partial agonist to a high-efficacy partial agonist with a span increase of 78 ± 8%. Further, under these conditions, loperamide was least affected with an increase of 17 ± 8%. The biased agonists buprenorphine, olliceridine, and (S)-TRV130 enantiomers went from being inactive to low efficacy agonists upon GRK5 overexpression with increased responses of 28 ± 16%, 49 ± 12%, and 46 ± 26%, respectively. Again, the \( E_{max} \) and the span increase for buprenorphine, olliceridine and (S)-TRV130 were not significantly different, suggesting GRK5 overexpression, like GRK2 overexpression, impacts μ-OR internalization mediated by these biased agonists equally in this system. Ligand-independent μ-OR internalization increased slightly with increasing amounts of GRK2 and GRK5, yet this increase was not significantly different from the ligand-independent μ-OR internalization without co-transfection of GRKs (Fig. S4H and Fig. S5H). From the SNAP-tag donor signals, it could be observed that overexpression of GRK2 or GRK5 resulted in similar cell-surface expression of the μ-OR (Fig. 6C). However, further increases in GRK
μ-OR agonists display no kinetic change in bias. Concentration response curves obtained at various time points in G protein activation and β-arrestin recruitment were fitted to the operational model of agonism. The transduction coefficients log($\tau/K_A$) were calculated for each agonist in each pathway and Δlog($\tau/K_A$) values were determined with DAMGO as the reference agonist. The ΔΔlog($\tau/K_A$) values obtained in the different pathways were subtracted to calculate ΔΔlog($\tau/K_A$). The bias values were plotted in a bias web. The ΔΔlog($\tau/K_A$) values were analyzed by two-way ANOVA with all combinations of time and agonist and corrected for multiple comparison with Tukey’s test (significance $P < 0.05$).

Fig. 5. Binding kinetic parameters for μ-OR agonists. Kinetic binding parameters were determined by competition kinetic binding between the indicated agonists and [3H]-Naloxone on membranes from Flp-In T-REx293-FLAG-μ-OR cells. Kinetic traces were fitted to a two-component exponential equation to obtain (A) $k_{on}$ and (B) $k_{off}$. (C) The dissociation constant ($pK_D$) for each agonist was calculated from $k_{on}$ and $k_{off}$ and plotted against the dissociation constant ($pK_I$) obtained from equilibrium competition binding. The values are expressed as mean ± SEM from three independent experiments performed in duplicate.
overexpression were not possible in this system, as the highest amount used here already led to a slight reduction in receptor cell-surface expression.

4. Discussion

Biased agonism has been increasingly studied over the last decade, providing compelling evidence for the potential therapeutic utility of this approach (Singla et al., 2017; Zhang et al., 2015). To the best of our knowledge, this is the first study that directly compares the bias profile of clinically relevant μ-OR agonists possessing distinct pharmacological profiles, taking into account the kinetic context and comparing the kinetic properties with the biased agonist oliceridine, which has been in clinical trial as a novel treatment for acute severe pain (Singla et al., 2017, 2019; Soergel et al., 2014; Viscusi et al., 2019, 2016). Here, we have demonstrated that DAMGO, buprenorphine, morphine, loperamide and oliceridine all potently activated Gαi2 and GαoA, whereas (S)-TRV130 was significantly less potent (90-fold compared to oliceridine). In this study, buprenorphine and oliceridine behaved as partial agonists to all timepoints in Gαi2 activation, and to distinct time points in GαoA activation with similar Emax. Although buprenorphine has been shown to be a partial agonist compared to oliceridine in inhibiting cAMP accumulation in a previous study (DeWire et al., 2013), the difference in measured efficacy between our study and DeWire et al. is likely caused by a difference in measurement point (G protein activation vs. cAMP accumulation) and/or differences in receptor or G protein expression levels, which is well-known to affect measured efficacy (Bräuner-Osborne et al., 1996). Furthermore, it should be noted that the efficacy towards the G protein/cAMP pathways was measured by assessing the specific Gαi2 and GαoA activation, and therefore it cannot be excluded that buprenorphine and the TRV130 enantiomers possess a different activation pattern (more or less partiality) for other

| Table 2 | Kinetic parameters for [3H]naloxone and unlabelled μ-OR agonists. Association (kon) and dissociation (koff) parameters and affinity constants of [3H]naloxone and unlabelled agonists from kinetic and equilibrium competition binding. Data represent mean ± SEM from three independent experiments. Association and dissociation constants kon and koff from kinetic binding and law of mass action were compared with a Student's t-test and no significant difference was found (P > 0.05). For determination of kon and koff of the unlabelled agonists the binding parameters of [3H]naloxone from kinetics were used (kon = 13.4 × 10^7 M^−1 min^−1 and koff = 1.196 min^−1). |
|---|---|---|---|---|---|---|
| | Kinetics | Equilibrium | Law of mass action |
| | kon (±SEM) /× 10^7 M^−1 min^−1 | koff (±SEM) /min | pKᵯ (±SEM) | kon (±SEM) /× 10^7 M^−1 min^−1 | koff (±SEM) /min |
| [3H]naloxone | 13.4 (±0.9) | 1.196 (±0.1) | 8.05 (±0.03) | 0.85 (±0.08) | 8.07 (±0.04) | 11.6 (±3.3) | 1.285 (±0.2) |
| DAMGO | 3.24 (±0.6) | 1.029 (±0.2) | 7.50 (±0.02) | 1.04 (±0.20) | 7.34 (±0.2) |
| Buprenorphine | 7.97 (±0.8) | 0.106 (±0.02) | 8.9 (±0.13) | 10.68 (±2.83) | 8.37 (±0.09) |
| Morphine | 1.78 (±0.08) | 1.388 (±0.1) | 7.11 (±0.09) | 0.73 (±0.06) | 7.02 (±0.2) |
| Loperamide | 4.89 (±0.8) | 0.912 (±0.09) | 7.72 (±0.06) | 1.12 (±0.13) | 7.64 (±0.2) |
| Oliceridine (R)-TRV130 | 3.25 (±0.7) | 1.763 (±0.2) | 7.25 (±0.08) | 0.59 (±0.09) | 7.29 (±0.1) |
| (S)-TRV130 | 0.0732 (±0.02) | 1.896 (±0.7) | 5.62 (±0.14) | 0.75 (±0.32) | 5.73 (±0.1) |

Fig. 6. The effect of GRK2 or GRK5 overexpression on agonist-mediated μ-OR internalization. (A) Internalization of FLAG-SNAP-μ-OR transiently transfected into HEK293A cells with no co-transfection of GRK. (B and D) The effect of titration of human GRK2 or GRK5 on agonist-induced internalization of FLAG-SNAP-μ-OR. In both (A), (B) and (D) cells were stimulated with 100 μM agonist and data represent area under the curve (AUC) from real-time internalization over a time-course of 90 min for which the buffer has been subtracted. (C and E) donor signals from the labeled SNAP-tag from the different transfection conditions, representing the number of receptors at the cell-surface. Maximum μ-OR internalization induced by oliceridine, (S)-TRV130 and buprenorphine was compared with one-way ANOVA corrected with Tukey’s test for multiple comparison and no significant difference was found (P > 0.05). Data represent mean ± SEM from three independent experiments carried out in triplicate.
G proteins of the Gαi/o family. In β-arrestin1 and β-arrestin2 recruitment, DAMGO behaved as a full agonist and morphine and loperamide behaved as partial agonists. Buprenorphine, oliceridine and (S)-TRV130 showed a very strong bias towards G protein activation, as they failed to induce significant recruitment of any of the β-arrestins.

Since previous observations have suggested that apparent bias can be highly dependent on the time point of comparison (Klein Herenbrink et al., 2016), we investigated the impact of kinetics on the signaling of the selected agonists using real-time BRET and receptor internalization as well as kinetic radioligand binding assays. In general, all the selected agonists sustained a stable concentration-dependent response of the signaling pathways investigated, with buprenorphine being the only agonist that displayed an increase in potency over time in both Gαi2 and GαoA activation. This could be explained by the low dissociation rate leading to increased receptor occupancy over time. Furthermore, buprenorphine and both TRV130 enantiomers were apparently extremely biased against β-arrestin recruitment. Application of the operational model on pathways where meaningful fits could be obtained, confirmed that there was no bias between signaling pathways for the agonists between Gαi2 and GαoA activation. Buprenorphine’s time dependent increase in potency of G protein activation were similar for both G proteins, resulting in no generation of bias between the two G protein subtypes. Buprenorphine, oliceridine and (S)-TRV130 displayed an apparent extreme bias towards G protein activation, which preclude calculation of bias factors. For the agonists where bias factors could be calculated in β-arrestin recruitment, no change in bias was observed between any of the pathways (Fig. 4). Therefore, the overall bias profile for all the selected agonists was retained over time, which allowed us to conclude that kinetic context was not of critical importance.

Using kinetic competition binding we could directly compare the k_on and k_off values of the μ-OR agonists. These data showed that all the selected agonists possessed a k_on within the same order of magnitude except (S)-TRV130, which had a significantly lower association rate. The slower association rate likely accounts for the lower potency of (S)-TRV130 in Gαi2 and GαoA activation compared to the rest of the agonists. It also demonstrates that small changes in stereochemistry can have dramatic effects on the binding kinetics. However, the difference in binding kinetics between oliceridine and (S)-TRV130 did not alter the overall bias profile, indicating that the change in the 3D orientation of TRV130 around its chiral center, that influences its affinity and potency, does not affect its functional selectivity towards Gαi vs. β-arrestin. Moreover, it clearly shows that the bias of TRV130 is not driven by its potency. Calculation of k_off revealed that DAMGO, morphine, loperamide, oliceridine and (S)-TRV130 have dissociation rates within the same magnitude, whereas buprenorphine dissociates significantly more slowly from the μ-OR than the other ligands and accordingly had an 18-fold higher residence time compared to oliceridine.

Considering the differences in dissociation rates between buprenorphine and the TRV130 enantiomers in relation to their G protein biased nature, it can be concluded that bias is not necessarily driven by low k_off values. This may however be different for other receptors and/or ligands, as has been suggested for the dopamine receptor (Klein Herenbrink et al., 2016). Since binding kinetics are unlikely to be the driving force behind the bias for the agonists selected in this study, it appears likely that the bias is more related to different conformational states of the receptor. The majority of research that supports a conformation-driven mechanism of bias has relied, like this study, on indirect pharmacological data. Yet, increasing biochemical, biophysical and structural studies are appearing, which provide direct evidence of ligand-dependent GPCR and associated transducer (G protein and β-arrestin) conformational states (Furness et al., 2016; Kahsai et al., 2011; Liang et al., 2018; Rahmeh et al., 2012; Shukla et al., 2008).

Different cellular backgrounds can also result in differences in apparent bias (Gesty-Palmer et al., 2009, 2006). This could relate to differences in abundance of effector proteins. GRK2 expression has been reported to be low in HEK293 cells compared to other cell types (Atwood et al., 2011) and studies have shown that overexpression of GRK2 in HEK293 cells can affect the signaling of biased agonists (Maggič et al., 2016; Thompson et al., 2016). Therefore, we made a direct comparison of the functional consequence of the selected agonists upon GRK2 titration. These data revealed that the agonists displayed differences in GRK2-dependent potentiation of μ-OR internalization. While morphine-induced receptor internalization was increased dramatically, loperamide-induced internalization only increased slightly. In agreement with the negligible β-arrestin recruitment by buprenorphine and the TRV130 enantiomers, these agonists only induced marginal receptor internalization without GRK2 over-expression. Yet, these G protein biased agonists all increased μ-OR internalization upon GRK2 overexpression to a similar extent, which was well below the increase observed for morphine. Interestingly, these data demonstrate that despite their pronounced G protein bias, buprenorphine and the TRV130 enantiomers promote μ-OR internalization in cells with high GRK2 expression, which suggests that these biased drugs could induce receptor-internalization in tissues where GRK2 is more abundant, albeit to a lesser extent than morphine or DAMGO. Due to the observed role of GRK5 in μ-OR biology (Doll et al., 2012; Glück et al., 2014) we also assessed the impact of overexpressing this kinase. In general, GRK5 increased agonist-induced μ-OR internalization, albeit to a lesser extent than observed for GRK2. This could be explained by the relatively high abundance of endogenous GRK5 in HEK293 cells (Atwood et al., 2011).

Collectively, our study is the first to directly compare bias of clinically relevant agonists at the μ-OR and combine with a kinetic analysis approach. Overall, we show that kinetics was not of critical importance for the bias profile, which remained the same irrespective of the time point measured. Further, we demonstrate that buprenorphine and oliceridine displayed the same apparent extreme bias in vitro. Nonetheless, at this point, it cannot be ruled out that the reported beneficial clinical effects of buprenorphine, in terms of lower respiratory depression (Dahan et al., 2006, 2005; Megarbane et al., 2006) are due to its low efficacy and complex pharmacology (Khanna and Pillarisetti, 2015) rather than its biased profile (Wootten et al., 2018). A direct comparison of the physiological effects of these two agonists could give novel and valuable insights into the benefits of biased agonists in the clinic. However, it should also be kept in mind, that we have shown that the two agonists differ in the kinetic binding profile. Thus, the biggest difference observed between the agonists in this study, beside their bias profile, is the residence times, where we observed an 18-fold difference between buprenorphine and the clinical candidate oliceridine, which potentially also could lead to significantly different clinical effects (Copeland, 2016). It will therefore be important to determine if differences in therapeutic profile is caused by differences in ligand bias, ligand binding kinetics or even other parameters such as ADME properties. Finally, this study underlines that agonists can display a similar overall degree of signaling bias despite possessing different inherent binding kinetic properties.

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Author contributions

M.F.P., M.B. and H.B.-O. designed the study; T.M.W., E.M.-R., D.S.P., H.P. and D.M. synthesized and purified the TRV130 enantiomers; M.F.P., T.C.M. and F.G. carried out pharmacological experiments; S.R.F cloned the SNAP-tag expression cassette; M.F.P., M.B and H.B.-O. wrote the manuscript. All authors thoroughly read and commented on the manuscript.
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Appendix A. Supplementary data

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