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In vitro functional characterization of a panel of non-fentanyl opioid new psychoactive substances

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Abstract

The landscape of new psychoactive substances (NPS) is constantly evolving, with new compounds entering the illicit drug market at a continuous pace. Of these, opioid NPS form a threat given their high potency and prevalence. Whereas previously, the use of fentanyl and fentanyl derivatives was the main point of attention, legislations have reacted accordingly, which may have been a driving force towards the (ab)use of alternative μ-opioid receptor (MOR) agonists. In contrast to fentanyl (analogues), details on these novel non-fentanyl opioid NPS are scarce. We investigated the biological activity of a panel of 11 'alternative', newly emerging MOR agonists (2-methyl-AP-237, AP-237, bromadol, brorphine, butorphanol, isotonitazene, mitragynine, 7-OH-mitragynine, MT-45, piperidylthiambutene and tianeptine) using two closely related *in vitro* MOR activation bio-assays, monitoring either G protein (mini-Gi) or β-arrestin2 (βarr2) recruitment. Activity profiles were obtained for all tested compounds, with values for potency (EC_{50}) ranging from 1.89 nM (bromadol) to >3 μM (AP-237 and tianeptine). Bromadol, brorphine, isotonitazene, piperidylthiambutene and tianeptine had the highest efficacy (E_{max}) values, exceeding that of the reference compound hydromorphone ≥1.3-fold (βarr2 assay) and >2.6-fold (mini-Gi assay). Information on the recruitment of two distinct signaling molecules additionally enabled evaluation of biased agonism, none of the evaluated opioids being significantly biased. Taken together, this study is the first to systematically investigate the *in vitro* biological activity of a diverse panel of emerging non-fentanyl opioid NPS at

MOR. Given the known danger of (fatal) intoxications with many opioid NPS, it is important to continuously monitor and characterize newly emerging compounds.

Keywords (4-6)

New psychoactive substances (NPS), synthetic opioids, non-fentanyl opioids, characterization, μ-opioid receptor, bio-assay

Introduction

The last decade has seen a dynamic growth in the availability and use of new psychoactive substances (NPS) across the globe. By definition not controlled by the United Nations international drug control conventions of 1961 and 1971 (UNODC 2020), these newly misused substances comprise a wide variety of drugs, including synthetic cannabinoid receptor agonists, stimulants and opioids. NPS are typically labeled as “legal highs”, “research chemicals” and “not for human consumption”, promoting their allegedly legal status as easily available alternatives for traditional drugs of abuse. The appearance of over 730 NPS in Europe since 1997 has truly added a new dimension to the illicit drug market and increasingly challenges the traditional approaches to drug monitoring and control (EMCDDA 2019d; EMCDDA and Europol 2019; Peacock et al. 2019).

Although currently representing a relatively small share of Europe’s NPS market, new synthetic opioids (NSOs) form a particular menace to public health, owing to their high potency and prevalence (EMCDDA 2019d; EMCDDA and Europol 2019). Many of these newly abused opioids were once pursued by the pharmaceutical industry for their potential as narcotic analgesics. However, owing to unwanted pharmacological side effects and addiction liability, their development was often abandoned before progressing to clinical trials. Nowadays, the published patents and synthesis routes stemming from this research are being pirated by underground chemists to diversify the recreational drug market (Meyer 2016; Salle et al. 2019; Sharma et al. 2019). Other synthetic opioids, such as fentanyl and butorphanol, are effectively used in human or veterinary medicine whilst also being abused for their opioid effects. Already in the early 1980s, the first illicitly produced fentanyl analogues, including α-methylfentanyl and 3-methylfentanyl, were being sold to heroin users on the US west coast (Armenian et al. 2018; Ayres, Starsiak, and Sokolay 1981; Jannetto et al. 2019). Starting slowly, but then gradually gaining pace, the past decade has seen a new ‘wave’ of NSOs. Amongst the first to appear for online sale in this new wave were AH-7921 in 2012 and MT-45 in 2013. As from 2014, several compounds of the ‘U series’ (originally developed in the ‘70s by the Upjohn Company) became increasingly detected, with U-47700 being one of the most prevalent non-fentanyl NSOs (Blanckaert & Cannaert et al. 2020; Sharma et al. 2019). Between 2012 and 2018, however, the majority of NSOs hitting the drug market remained fentanyl analogues (Cannaert & Ambach et al. 2018; Evans-Brown, Gallegos, and Christie 2018; Jannetto et al. 2019). Being increasingly confronted with the severe risk of fatal poisoning following the use of these substances, important efforts were made to counter this trend (Bao et al. 2019; DEA 2018). The introduction of a new legislation in China in 2018 was probably the most

impactful measure, as the number of new fentanyl analogues started to decrease drastically from 2018 onwards (Bao et al. 2019; Blanckaert & Cannaert et al. 2020). However, as exemplified by the recent emergence of brorphine, which could be considered a fentanyl analogue, drug designers still manage to find loopholes in existing legislations, even when these are 'generic' and thus aiming at covering a very wide range of analogues (Verougstraete & Vandeputte et al., under review) (Belgisch Staatsblad 2017; DEA 2018).

As a response to the recent changes in (inter)national control measures targeting fentanyl analogues, there has been a trend of non-fentanyl opioids (re)appearing on the recreational drug market (e.g. 2-Me-AP-237, isotonitazene) (Blanckaert & Cannaert et al. 2020; Sharma et al. 2019). While extensive literature has been published on the pharmacology and toxicology of fentanyl, fentanyl derivatives and some of the early non-fentanyl opioids (Jannetto et al. 2019; Prekupec, Mansky, and Baumann 2017; Sharma et al. 2019; Vasudevan et al. 2020), many of these somewhat obscure, newly emerging opioids have received considerably less attention. However, the increasing amount of case reports on (sometimes fatal) intoxications involving these drugs, stresses the danger of their use. As a better insight into the pharmacology of NSOs may allow to prioritize international and legislative efforts towards controlling (variants of) these emerging drugs, we investigated the pharmacological profile of 11 non-fentanyl opioid NPS (**Figure 1**). The compounds in this panel were chosen based on their structural diversity as non-fentanyl μ-opioid receptor (MOR) agonists and their recent and/or emerging appearance on the recreational drug market (Arillotta & Schifano et al. 2020; Blanckaert & Cannaert et al. 2020; Sharma et al. 2019; Ventura, Carvalho, and Dinis-Oliveira 2018). Although brorphine could, in essence, be considered a structural analogue of fentanyl (Verougstraete & Vandeputte et al., under review), it currently falls outside the scope of generic legislations aiming at covering fentanyl derivatives (Belgisch Staatsblad 2017; DEA 2018) and was therefore included in this study as well. While several opioid receptors have been identified, the receptor of focus in this study was MOR, as this is the main target for most of the potent analgesics currently in use (Al-Hasani & Bruchas 2011; Pasternak & Pan 2011). Efficacy and potency values of the 11 compounds were studied using two closely related MOR activation assays monitoring either G protein or β-arrestin 2 (βarr2) recruitment. In addition, information on these two signaling pathways enabled the evaluation of potential biased agonism of these compounds at MOR, a concept that is now being increasingly debated for opioids (Gillis & Gondin et al. 2020).

Materials & Methods

a. Chemicals and reagents

(-)-Mitragynine **(7)** was obtained from Chiron (Trondheim, Norway). 2-methyl-AP-237 HCl **(1)**, AP-237 HCl **(2)**, *trans*-bromadol **(3)**, brorphine HCl **(4)**, isotonitazene **(6)**, 7-OH-mitragynine **(8)** and piperidylthiambutene HCl **(10)** were purchased from Cayman Chemical Company (Ann Arbor, Michigan, US). Butorphanol **(5)**, MT-45 **(9)** (as racemic mixture) and tianeptine **(11)** were kind gifts from Prof. V. Auwärter (University of Freiburg) and were originally obtained from Cerilliant (Round Rock, Texas, US), Lipomed AG (Arlesheim, Switzerland) and Cayman Chemical Company (Ann Arbor, Michigan, US), respectively. Hydromorphone **(A)** was purchased as hydrochloride salt from Fagron (Nazareth, Belgium). Fentanyl **(B)** was obtained from LGC Chemicals (Wesel, Germany). Dulbecco's modified Eagle's medium (DMEM; GlutaMAX™), Opti-MEM® I reduced serum medium, penicillin-streptomycin (5 000 U/mL) and amphotericin B (250 µg/mL) were obtained from Thermo Fisher Scientific (Pittsburg, PA, USA). Fetal bovine serum (FBS) and poly-D-lysine were supplied by Sigma Aldrich (Overijse, Belgium). The Nano-Glo® Live Cell Assay system (containing the Nano-Glo® Live Cell Substrate and Nano-Glo® LCS Dilution Buffer) was procured from Promega (Madison, WI, USA).

b. Determination of *in vitro* biological activity at the µ-opioid receptor (MOR)

The biological activity of 11 non-fentanyl opioid NPS was evaluated using two distinct, previously reported, cell-based receptor activation assays (Cannaert et al. 2018; Vasudevan et al. 2020). The sensitivity and specificity of these systems was previously evaluated using a wide range of opioids (Cannaert et al. 2018; Vasudevan et al. 2020) and the system has since been successfully applied in different studies (Blanckaert & Cannaert et al. 2020; Cannaert & Ambach et al. 2018; Cannaert et al. 2020; Gampfer et al. 2020). The assays are based on the functional complementation of a split nanoluciferase (NanoLuc Binary Technology®, Promega). In the assays, activation of human MOR, fused to one part of the nanoluciferase, leads to recruitment of either βarr2 (in the presence of co-expressed G protein-coupled receptor kinase 2, GRK2) or mini-Gi (GTPase domain of the Gαi subunit), fused to the other part. This results in functional complementation of the nanoluciferase, restoring its enzymatic activity and allowing the generation of a measurable bioluminescent signal upon addition of the substrate furimazine.

Human embryonic kidney (HEK) 293T cells stably expressing either the MOR-βarr2-GRK2 (further referred to as MOR-βarr2) or MOR-mini-Gi system were maintained in a humidified atmosphere containing 5% CO_2 in DMEM (GlutaMAX™) supplemented with 10% heat-inactivated FBS, 100 IU/mL

penicillin, 100 mg/L streptomycin and 0.25 mg/L amphotericin B. The stability of the cell lines was routinely monitored by flow cytometric analysis of co-expressed markers (Vasudevan et al. 2020). One day prior to the experiments, cells expressing either MOR-βarr2 or MOR-mini-Gi were seeded on poly-D-lysine coated 96-well plates at 5 x 10^4 cells/well. After overnight incubation, the cells were washed twice with Opti-MEM® I reduced serum medium to remove residual FBS. Next, 90 µL Opti-MEM® I was added to the washed wells. Nano-Glo® Live Cell reagent, a non-lytic detection reagent containing the cell-permeable furimazine, was prepared by 20-fold dilution of Nano-Glo® Live Cell substrate with Nano-Glo® LCS Dilution buffer. Twenty-five µL of the reagent solution was added to each well. Next, the plate was placed into a TriStar^2 LB 942 luminometer (Berthold Technologies GmbH & Co., Bad Wildbad, Germany). Luminescence was continuously monitored until stabilization of the signal (10-15 minutes). Subsequently, 20 µL of 6.75x concentrated stock solutions in Opti-MEM®/MeOH was added and luminescence was monitored for 120 minutes. All compounds were initially tested in concentrations ranging between 10 pM and 10 µM. If maximal receptor activation was not reached with 10 µM, concentrations up to 100 µM were evaluated. Hydromorphone was selected as a reference agonist based on previous studies (Blanckaert & Cannaert et al. 2020; Vasudevan et al. 2020) and appropriate solvent controls were included in all experiments. FACS-sorted (Fluorescence-Activated Cell Sorting) stably transduced cells were used, offering the advantage that this reduces assay variability, as opposed to when using transiently transfected cells. Every experiment was performed at least in triplicate (n = 3), with duplicates run for each concentration within an experiment. This approach has proven to yield robust results (Janssens et al. 2020; Vasudevan et al. 2020). Data points were excluded for the highest concentration of agonist when the signal showed a reduction of 20% or more compared to the signal obtained for the next dilution (high concentrations probably leading to cell toxicity and/or generating a higher, but narrower peak, leading to lower areas under the curve (AUC)). The complete dataset (1476 data points) was screened for outliers using the Grubbs test, which resulted in a total of ten outliers that were subsequently omitted from the dataset. The βarr2 data for brorphine **(4)** and MT-45 **(9)** were also reported in Verougstraete & Vandeputte et al. (under review) and Cannaert & Hulpia et al. (2020), respectively. Curve fitting and statistical analyses for the experiments were performed using GraphPad Prism 8 software (San Diego, CA, USA). Absolute luminescence signals were corrected for solvent controls and inter-well variability before concentration-responses (AUC values) were normalized to the maximum response of the reference compound hydromorphone (arbitrarily set at 100%). A non-linear regression model (three-parameter logistic regression) was fitted to the normalized responses, yielding measures of potency (EC_{50}) and efficacy (E_{max}), the latter relative to hydromorphone.

c. Calculation of pathway bias

Pathway bias was calculated as previously described. Also here, hydromorphone was used as an unbiased reference compound (Vasudevan et al. 2020; Winpenny, Clark, and Cawkill 2016). In a first step, Equation (1) was used to calculate the $\Delta\log(E_{max}/EC_{50})$ for every compound (A = test compound, B = reference compound).

$$\Delta \log\left(\frac{E_{max}}{EC_{50}}\right) = \log\left(\frac{E_{max,A}}{EC_{50,A}}\right) - \log\left(\frac{E_{max,B}}{EC_{50,B}}\right) \qquad \text{Equation (1)}$$

Pathway bias was subsequently calculated using Equation (2).

$$\Delta\Delta \log\left(\frac{E_{max}}{EC_{50}}\right) = \Delta\log(\frac{E_{max}}{EC_{50}})_{\beta\text{arr2}} - \Delta\log(\frac{E_{max}}{EC_{50}})_{mini-Gi} \qquad \text{Equation (2)}$$

The average $\Delta\Delta\log(E_{max}/EC_{50})$ (coined 'bias factor') was calculated from three independent experiments, each performed in duplicate, and was plotted together with the standard error of the mean (SEM) for each compound. In line with previous work from our group (Janssens et al. 2020; Pottie et al. 2020; Vasudevan et al. 2020; Wouters et al. 2020), statistical analysis was carried out by non-parametric one-way ANOVA (Kruskal-Wallis), followed by post hoc Dunn's multiple comparison test to examine significant differences between the reference hydromorphone and each of the different compounds. The choice for Dunn's multiple comparison test over Dunnett's test renders the analysis more conservative.

Results

Activation profiles were obtained for all tested compounds (**Figure 2**). Efficacies (E_{max}) and potencies (EC_{50}) were calculated and can be found in **Table 1.** For AP-237 **(2)** and tianeptine **(11)**, maximal receptor activation could not be reached in all assays due to the relatively weak agonism displayed by these compounds, with cellular toxicity at high concentrations. For butorphanol **(5)** and mitragynine **(7)**, the partial agonism (relative to hydromorphone) led to very wide 95% confidence intervals, particularly in the MOR-mini-Gi assay.

The most potent compound in terms of both βarr2 and mini-Gi recruitment was bromadol **(3)**, with an EC_{50} of 1.89 nM (95% confidence interval (CI) 1.23-2.93 nM) for βarr2 and 3.04 nM (95% CI 1.48-6.28 nM) for mini-Gi. No plateau in response was observed for AP-237 **(2)** (βarr2 and mini-Gi assays) and tianeptine **(11)** (mini-Gi assay), hence for these compounds no E_{max} and only ambiguous EC_{50} values could be derived (the latter in the µM range) in the respective assays. In both assays, butorphanol **(5)**, mitragynine **(7)** and 7-OH-mitragynine **(8)** were partial agonists compared to hydromorphone **(A)**. All other compounds were more efficacious than hydromorphone in activating MOR, the efficacy of bromadol **(3)**, brorphine **(4)**, isotonitazene **(6)**, piperidylthiambutene **(10)** and tianeptine **(11)** exceeding that of hydromorphone ≥1.3-fold and >2.6-fold in the βarr2 and mini-Gi assay, respectively. With the single exception of butorphanol **(5)**, higher E_{max} values were found in the mini-Gi recruitment assay, the difference between both assays being most pronounced (up to 3-fold) for the most potent compounds.

The highly similar set-up of the employed MOR activation assays (only differing in the nature of the recruited signal transduction molecule) also allowed the assessment of biased agonism of the studied compounds at MOR (with the exception of AP-237 **(2)** and tianeptine **(11)**, for which E_{max} values could not be derived in all assays). **Figure 3** depicts the quantitative bias plot, in which the level of bias is plotted as $\Delta\log(E_{max}/EC_{50})_{\beta arr2} - \Delta\log(E_{max}/EC_{50})_{mini\text{-}Gi} \pm SEM$. None of the studied compounds showed statistically significant biased agonism.

Discussion

This study reports the *in vitro* pharmacological characterization of 11 emerging non-fentanyl opioid NPS. Several of these have been used in the past for research purposes but were never marketed (e.g. isotonitazene, MT-45), whereas others, such as butorphanol (e.g. Beforal®/Torbugesic®) or tianeptine (e.g. Stablon®/Coaxil®), remain available as prescription drugs in some countries. Recently, these compounds have started to emerge on the recreational drug market for their opioid activity. At the time of writing, only a limited number of studies, deploying a variety of different assays (discussed further), have reported *in vitro* pharmacological data for the compounds evaluated here. In fact, to the best of our knowledge, no *in vitro* efficacy and potency values have been described before for AP-237 **(2)**, bromadol **(3)** and piperidylthiambutene **(10)**.

By reviewing the limited literature on what is already known, the findings in this study illustrate the difficulty in comparing data obtained via different assays using various reference compounds. This calls to attention the need for *in vitro* assay standardization across laboratories and the inclusion of relevant comparator compounds with known pharmacology (e.g. fentanyl). Although this *in vitro* study does not provide receptor binding affinity data and is limited to evaluating the compounds' responses at MOR, it pioneers in the systematic, comparative evaluation of a diverse panel of emerging opioid NPS by means of two highly similar, yet distinct, receptor activation assays monitoring either β-arrestin 2 or mini-Gi recruitment to activated MOR. The set-up of these assays offers several advantages over other assays. First, minimally-sized fusion proteins are involved (with two parts of a split nanoluciferase fused to the partnering molecules), aimed at minimally interfering with normal recruitment to the activated receptor. Second, a receptor-proximal event is monitored, i.e. the recruitment of either β-arrestin 2 or mini-Gi to activated MOR. This offers the advantage of minimal signal amplification, allowing to make a distinction between partial and full agonists. Assays monitoring a more downstream event, on the other hand, may lead to ambiguous results as the maximal signal may already be obtained in the absence of full receptor activation (also referred to as 'receptor reserve') (Gillis & Gondin et al. 2020; Wouters et al. 2019). These features are particularly relevant, as it was recently hypothesized that the intrinsic efficacy (E_{max}) of MOR agonists is inversely correlated with their therapeutic window (Benredjem & Gallion et al. 2019; Gillis & Gondin et al. 2020; Wolff et al. 2012). In the study by Gillis & Gondin et al. (2020), high efficacy MOR agonists such as fentanyl were found to have a very narrow index of therapeutic effect *versus* respiratory depression in *in vivo* mouse studies, this respiratory depression arguably being the most important cause of death following opioid intoxication. With this in mind, the pharmacological data obtained here may allow to direct and

prioritize scheduling efforts towards those compounds which presumably pose the highest risk to users, i.e. those with the highest intrinsic efficacy. In the set we evaluated, these are bromadol **(3)**, brorphine **(4)**, isotonitazene **(6),** piperidylthiambutene **(10)** and tianeptine (**11**), although the potency of the latter two is moderate to weak, respectively, presumably requiring high doses. Furthermore, as also discussed further, it should be stressed that *in vitro* data can never fully predict the eventual *in vivo* effects an individual might experience from using research chemicals.

The minimal methodological differences between both employed MOR activation assays additionally rendered this study highly suitable to assess biased agonism at MOR. In line with recently published work studying a diverse panel of NSOs (mainly fentanyl analogues) (Vasudevan et al. 2020), none of the evaluated compounds showed statistically significant biased agonism (compared to hydromorphone). For a thorough discussion on the highly debated concept of opioid agonism at MOR, the reader is referred to recent work by Gillis & Gondin et al. (2020) and by Vasudevan et al. (2020).

As is the case for many of the newly abused synthetic opioids, 2-methyl-AP-237 **(1)** was originally patented in the 1980s for its analgesic activity (Furlan 1985). The first evidence of 2-methyl-AP-237 appearing on the NPS market dates from the first half of 2019 (NFL Ljubljana 2019a). It was formally notified to the EU Early Warning System (EWS) in April 2019 (EMCDDA 2019a) and was found in seized material in the US later that year (Krotulski, Fogarty, and Logan 2019a). So far, in 2020, 2-methyl-AP-237 has been identified 3 times in the US (Krotulski, Mohr, and Logan 2020). A recent *in vitro* study reported an EC_{50} value of 568 nM using AequoScreen® (Perkin Elmer) recombinant CHO-K1 cells expressing human MOR (Aklagarmyndigheten 2019; personal communication with Prof. Dr. Henrik Green). We found about four times lower potencies for 2-methyl-AP-237 (EC_{50} = 2229 nM for both βarr2 and mini-Gi) corresponding with a 68-156 times lower potency than that of fentanyl. One month after the formal notification of 2-methyl-AP-237, also a desmethyl derivative, AP-237 **(2)**, was notified to the EU EWS (EMCDDA 2019b) and was later seized in the US (Krotulski, Fogarty, and Logan 2019b). Also known as bucinnazine, AP-237 has been evaluated for its analgesic activity in several studies since the early 1970s (Carrano et al. 1975; Carrano, Kimura, and McCurdy 1975; Irikura et al. 1968; Nishimura et al. 1970), including one study indicating its potential for dependence (Tao and Wang 1986). Since then, it has been used primarily as an analgesic in China (Tao and Wang 1986; Zang 1999). To the best of our knowledge, this study is the first to report *in vitro* biological activity data for AP-237 at MOR. With EC_{50} values in the micromolar range, it is estimated to be 3.5 to 13 times less potent than 2-methyl-AP-237. Yet another analogue in this series, *para*-methyl-AP-237, was very recently identified

in the US (Krotulski, Fogarty, and Logan 2020). It remains to be evaluated whether the low potencies observed for 2-methyl-AP-237 and its desmethyl derivative also become apparent for this compound, as well as for other analogues in this series that are likely to appear.

Bromadol **(3)**, also referred to as BDPC (*trans*-4-(*p*-bromophenyl)-4-(dimethylamino)-1-phenethylcyclohexanol), was amongst the most potent and efficacious compounds studied here. Using a standard mouse hot plate assay, Liu et al. (2003) previously suggested that the analgesic potency of bromadol may be around 2.9 times that of fentanyl (Sharma et al. 2019). The herein reported *in vitro* potencies for βarr2 and mini-Gi recruitment echo this, as the EC_{50} values for bromadol were 7.6- and 10.8-fold lower than those obtained for fentanyl using the same respective assays. However, as discussed further, it remains difficult to directly compare *in vivo* analgesic potency to *in vitro* potency values. Interestingly, the *in vivo* antinociceptive potency reported by Liu et al. (2003) was greater than what may be expected based on opioid receptor binding affinity data (Sharma et al. 2019). Although determination of binding affinity was outside the scope of the current study, the high *in vitro* efficacy and potency at MOR reported here, may provide an explanation for this.

Brorphine **(4)** has been reported to circulate in the American Midwest since the second half of 2019 (NFLIS 2019) and has been a topic in online drug user discussions for quite some time. Very recently, brorphine was identified in Belgium in a powder and in the serum of a patient seeking medical help for detoxification (Verougstraete & Vandeputte et al., under review). Kennedy et al. (2018) previously evaluated brorphine within a series of MOR agonists with purported high signaling bias. Using commercially available βarr2 enzyme fragment complementation (PathHunter®, DiscoverX) and (^{35}S)GTPγS assays, the authors reported EC_{50} values of 182 ± 42 nM and 4.8 ± 0.41 nM for βarr2 and G protein recruitment, respectively (Kennedy et al. 2018). Following operational analysis with DAMGO as an unbiased reference compound, these authors concluded that brorphine showed a certain degree of bias towards G protein recruitment (Kennedy et al. 2018). Interestingly, in our study, the pharmacological profile of brorphine was markedly different. Not only was brorphine not significantly biased compared to hydromorphone, it was also found to be more potent at recruiting βarr2 – with an EC_{50} of 31.1 nM approaching the potency of fentanyl - than G protein (EC_{50} = 106 nM for mini-Gi). In contrast to the assays used by Kennedy et al. (2018), our assays are maximally similar and do not differ in terms of signal amplification – as also suggested by Gillis & Gondin et al. (2020), this may allow a better assessment of intrinsic bias.

Butorphanol **(5)** is available on the market in several countries, be it for human (e.g. Beforal®) or veterinary (e.g. Torbugesic®) use. Therapeutic indications include moderate to severe migraine and anesthesia (WHO 2006). Reports of butorphanol abuse are generally related to misuse of the prescription drug (e.g. excessive prescription refill, doctor shopping) (WHO 2006). It was first notified as an NPS to the EU EWS in December 2013 after a seizure of the powder in Denmark (Blanckaert 2011; EMCDDA and Europol 2013). The potency (low-nM range; comparable to fentanyl) of this partial agonist in our MOR-mini-Gi assay is in line with findings previously obtained using (^{35}S)GTPγS and forskolin-stimulated cAMP accumulation assays in rat and murine MOR, respectively (Emmerson et al. 1996; Gharagozlou et al. 2003). It is important to note that, having mixed agonist/antagonist properties, butorphanol intake might precipitate withdrawal symptoms in individuals maintained on higher efficacy opioids (WHO 2006).

For isotonitazene **(6)**, the pharmacological parameters found in the MOR-βarr2 assay (EC_{50} = 6.64 nM and E_{max} = 159%) are in line with previously published values from our group (Blanckaert & Cannaert et al. 2020). Isotonitazene was found to be two times more potent than fentanyl and almost twice as efficacious in recruiting mini-Gi. A recent cluster of deaths in the US in which this opioid was identified, can likely be attributed to the very high potency and efficacy of this compound (Krotulski, Papsun, et al. 2019, 2020; Krotulski and Logan 2019). Whereas a translation from *in vitro* data to the *in vivo* biological effect remains subject to complex pharmacodynamic and pharmacokinetic processes, it is interesting to note that isotonitazene concentrations reported in these deaths (0.4-9.5 ng/mL or 0.97-23 nM in blood) are roughly in the same range as the *in vitro* EC_{50} values found here (6.64 nM for βarr2; 16.3 nM for mini-Gi). However, it must be stressed that it is difficult to link systemic concentrations of centrally active drugs with those in the central nervous system – a critical parameter is e.g. passage through the blood-brain barrier (Kalvass et al. 2007). Furthermore, in the context of opioid use, the aspect of tolerance greatly hampers the interpretation of *in vivo* drug concentrations. In the cases reported by Krotulski et al., a history of heroin use was reported among several of the individuals, requiring opioid tolerance to be considered when aiming at interpreting the reported concentrations. In addition, other variables must also be taken into account, such as metabolic stability, conversion to potentially active metabolites and poly-drug use.

Mitragynine **(7)** and 7-OH-mitragynine **(8)** are the primary psychoactive alkaloids in the tropical plant *Mitragyna speciosa*, colloquially known as kratom. Whereas kratom has a long history of traditional use in Southeast Asia, its worldwide recreational misuse has been on the rise in the recent years (DEA 2019; Ventura et al. 2018). Our mini-Gi data confirm the observation by others that mitragynine is less potent than its metabolite 7-OH-mitragynine, suggesting an important role for this metabolic conversion in the *in vivo* effects induced by mitragynine (Kruegel & Gassaway et al. 2016, Kruegel & Uprety et al. 2019; Takayama 2004). This, combined with other factors, additionally complicates the interpretation of mitragynine concentrations in forensic casework (Kruegel & Uprety et al. 2019; Papsun et al. 2019). The *in vitro* pharmacology of mitragynine at MOR has furthermore been reported to be highly species-dependent. For example, Kruegel & Gassaway et al. (2016) showed that mitragynine (but not 7-OH-mitragynine) acts as a competitive antagonist at murine MOR, whereas weak partial agonism was observed at human MOR. Our results at human MOR support this, as both mitragynine and its 7-OH metabolite are partial agonists when compared to hydromorphone (E_{max} < 100%; **Table 1**). In terms of potency at human MOR, however, Kruegel & Gassaway et al. (2016) reported lower EC_{50} values for mitragynine and 7-OH-mitragynine using a G protein bioluminescence resonance energy transfer (BRET) assay (EC_{50} = 339 ± 178 nM and 34.5 ± 4.5 nM, respectively) than those observed with our MOR-mini-Gi assay (EC_{50} = 1089 nM for **(7)** and 191 nM for **(8)**). These apparent discrepancies may be the result of interassay differences. Interestingly, the authors additionally reported "extremely weak" β-arrestin recruitment (even in the presence of GRK2, known to enhance coupling to β-arrestins) upon MOR activation by mitragynine and 7-OH-mitragynine in a BRET assay, stating strong qualitative bias in favor of G protein signaling (Kruegel & Gassaway et al. 2016). Using our MOR-βarr2 assay, the level of β-arrestin 2 recruitment was sufficient for both compounds to allow quantification of a possible bias (as compared to mini-Gi recruitment). No significant difference was found when compared to the unbiased reference hydromorphone. This clearly underscores the difficulty in comparing results obtained with different assays and highlights the importance of a comparative evaluation of a diverse set of compounds, using the same set-up, as applied in this study. In the current study, mitragynine and its 7-hydroxy metabolite are between 5-54 times less potent than fentanyl, depending on the studied pathway.

MT-45 **(9)** was initially studied as an analgesic in the 1970s (Haruki et al. 1975; Natsuka et al. 1975, 1987) and was first detected in Sweden in late 2013. Following Sweden's ban on AH-7921, 28 deaths had been associated with MT-45 use in a nine-month period between November 2013 and July 2014 (EMCDDA 2015). In one death in which MT-45 was the only substance involved, a blood concentration of 802 ng/mL (2300 nM) was reported (Logan et al. 2017). Taking into account the aforementioned

caveats, this concentration approaches the estimated *in vitro* EC_{80} value of MT-45 (2100 nM for βarr2 and 2896 nM for mini-Gi). In another case, a lower concentration of MT-45 was found in a decedent's blood (520 ng/mL or 1492 nM), albeit in combination with a therapeutic etizolam concentration (Papsun et al. 2016). The *in vitro* potency (EC_{50}) of MT-45 was previously reported to be 124 ± 24 nM in a (^{35}S)GTPγS assay monitoring G protein recruitment to activated murine MOR (Baumann et al. 2018). A similar potency (182 nM; 95% CI 77-426 nM) was found using a dynamic mass redistribution assay (Bilel et al. 2020). Remarkably, in a study assessing activation of the G protein pathway via monitoring of the inhibition of forskolin-stimulated cAMP accumulation, an approximately 10-fold higher value (EC_{50} = 1300 nM) was reported (Baptista-Hon et al. 2020). In the MOR-mini-Gi assay we applied, in which a receptor-proximal event is monitored, we found a somewhat intermediate potency (EC_{50} = 724 nM). The MOR-βarr2 assay deployed here, too, yielded an intermediate potency for MT-45 (EC_{50} = 525 nM). Interestingly, using another enzyme complementation assay (PathHunter®, DiscoverX) to assess βarr2 recruitment to MOR, a ~40-fold lower EC_{50} value (23 µM) was reported by Baptista-Hon et al. (2020). Noteworthy in this context is that application of this same PathHunter® assay also yielded a relatively high EC_{50} value for fentanyl (120 nM (Baptista-Hon et al. 2020)), whereas we found an approximately 8-fold lower value (EC_{50} = 14.3 nM) with the MOR-βarr2 system. Again, this stresses the importance of mentioning the applied assay format when making statements on a compound's potency and/or efficacy. In our assay systems, MT-45 is 22-37 times less potent than fentanyl. Whereas MT-45 has now been listed in Schedule I of the 1961 Convention, different analogues continue to appear on the market, stressing the importance of continued research into MT-45 and its derivatives (Baptista-Hon et al. 2020; Cannaert & Hulpia et al. 2020).

Piperidylthiambutene **(10)** (piperidinohton) has been studied for its potential as analgesic (Adamson 1951; Adamson and Green 1950; Green 1953) and antitussive drug (Kase et al. 1955; Kimura, Ogawa, and Yabuuchi 1959), but was never marketed. Its potency at MOR (EC_{50} = 180 nM for βarr2; 443 nM for mini-Gi) is about 13-fold lower than that of fentanyl. Perhaps more reason for concern, however, is this compound's relatively high efficacy, particularly in terms of mini-Gi recruitment (E_{max} = 349% for mini-Gi; 130% for βarr2). At present, relatively little is known about the abuse of piperidylthiambutene and its structural analogues. A recent study by Arillotta & Schifano et al. (2020) used a web crawler tool to better understand trends regarding opioid use in online drug user platforms. Piperidylthiambutene was amongst the 136 non-fentanyl opioids of which open discussions among psychonauts were picked up, potentially reflecting interest in the drug (Arillotta & Schifano et al. 2020). The first formal identification of piperidylthiambutene in Europe was reported early 2019, in a powder purchased from the Internet (EMCDDA 2019c; NFL Ljubljana 2019b). Later that year, it was also seized

in the US (Krotulski, Fogarty, and Logan 2019c). In the first quarter of 2020, piperidylthiambutene has been identified in 4 different cases in the US, potentially indicating its increasing use (Krotulski, Mohr and Logan 2020). An interesting and perhaps worrying observation is that, in all 4 cases, piperidylthiambutene was identified together with the highly potent and efficacious drug isotonitazene (Krotulski, Mohr and Logan 2020).

Based on its structural similarity to tricyclic antidepressants, tianeptine **(11)** may seem the odd one out within this panel of opioid NPS. Tianeptine (e.g. Stablon®, Coaxil®) is prescribed for the treatment of depression and anxiety in several countries in Europe, Asia and Latin America. However, studies have shown that the pharmacology of tianeptine is markedly different from that of most antidepressants, the behavioral-inducing characteristics being mediated by its agonistic activity at MOR (Gassaway et al. 2014; Samuels et al. 2017). A G protein BRET and cAMP inhibition assay resulted in EC_{50} values of 194 ± 70 nM and 151 ± 45 nM, respectively, at human MOR (Gassaway et al. 2014). Interestingly, the estimated EC_{50} value obtained in our MOR-mini-Gi assay was at least 50-fold higher, indicating a lower potency than initially reported. Despite this low potency (> 200 times less potent than fentanyl), the unregulated use of tianeptine as a research chemical appears to be on the rise (El Zahran et al. 2018). When sold as dietary supplement, its use might even be a blind spot for early warning systems (Evans-Brown and Sedefov 2018; Griffiths, Evans-Brown, and Sedefov 2013). However, as several case reports highlight the danger of the potentially fatal misuse of tianeptine (Bakota et al. 2018; Dempsey et al. 2017; Proença et al. 2007; Rushton et al. 2020), increasing awareness is of critical importance.

Conclusion

Following strengthened control measures targeting fentanyl and fentanyl analogues, non-fentanyl opioids are increasingly (re)appearing on the illicit market. This study is the first to systematically evaluate the *in vitro* biological activity of a diverse panel of emerging non-fentanyl opioid NPS at MOR. Pharmacological profiling of such novel substances is crucial to make a realistic estimation of the potential danger their use might bring along. Considering the high potencies and efficacies of many compounds in the studied panel, intensive monitoring and proactive control measures remain of paramount importance.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Fig. 1 Structures of the studied non-fentanyl opioid new psychoactive substances: **(1)** 2-methyl-AP-237; **(2)** AP-237; **(3)** bromadol; **(4)** brorphine; **(5)** butorphanol; **(6)** isotonitazene; **(7)** mitragynine; **(8)** 7-OH-mitragynine; **(9)** MT-45; **(10)** piperidylthiambutene; and **(11)** tianeptine. The grey panel shows **(A)** hydromorphone, the reference compound in this study, and **(B)** fentanyl, for comparison.

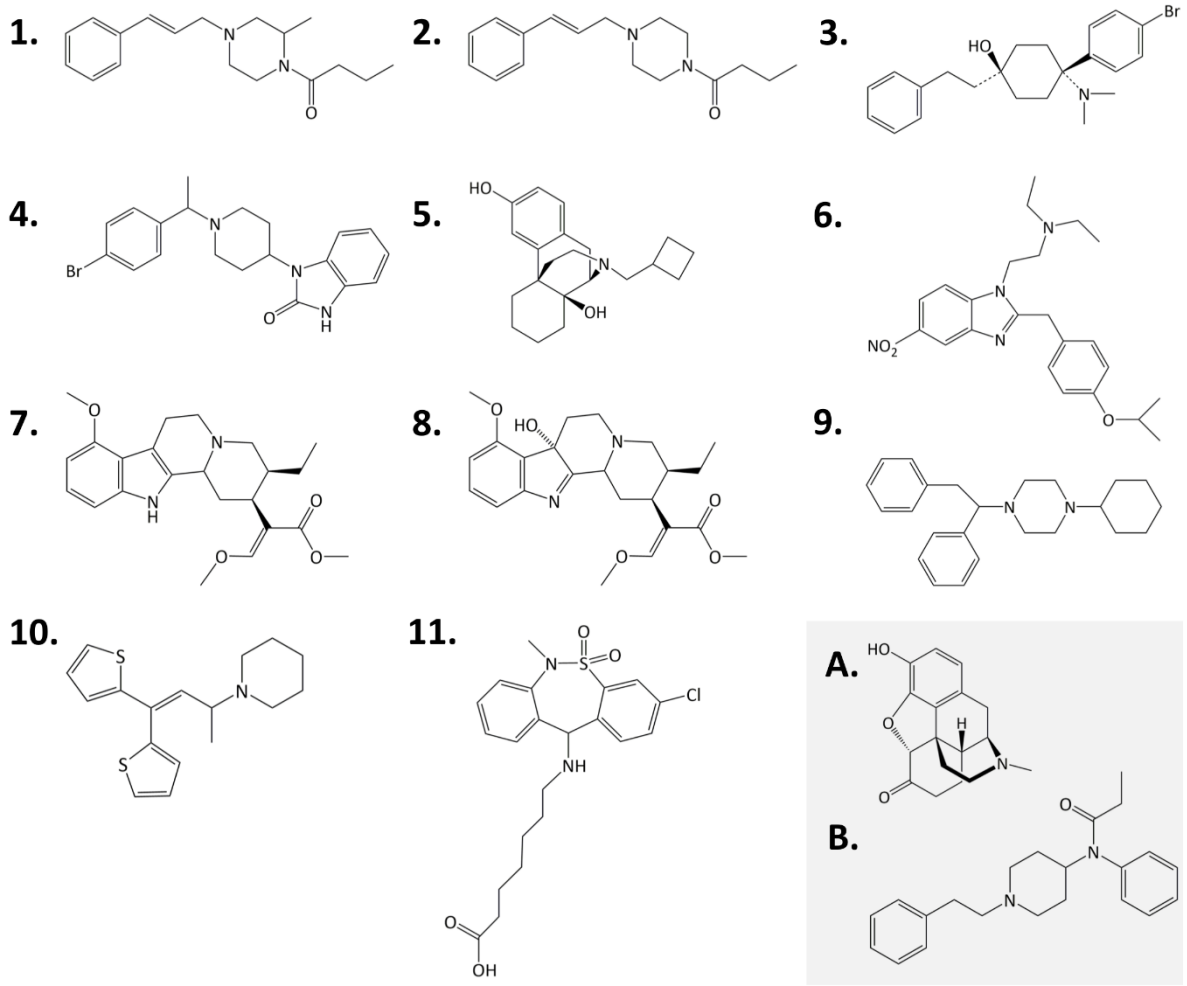
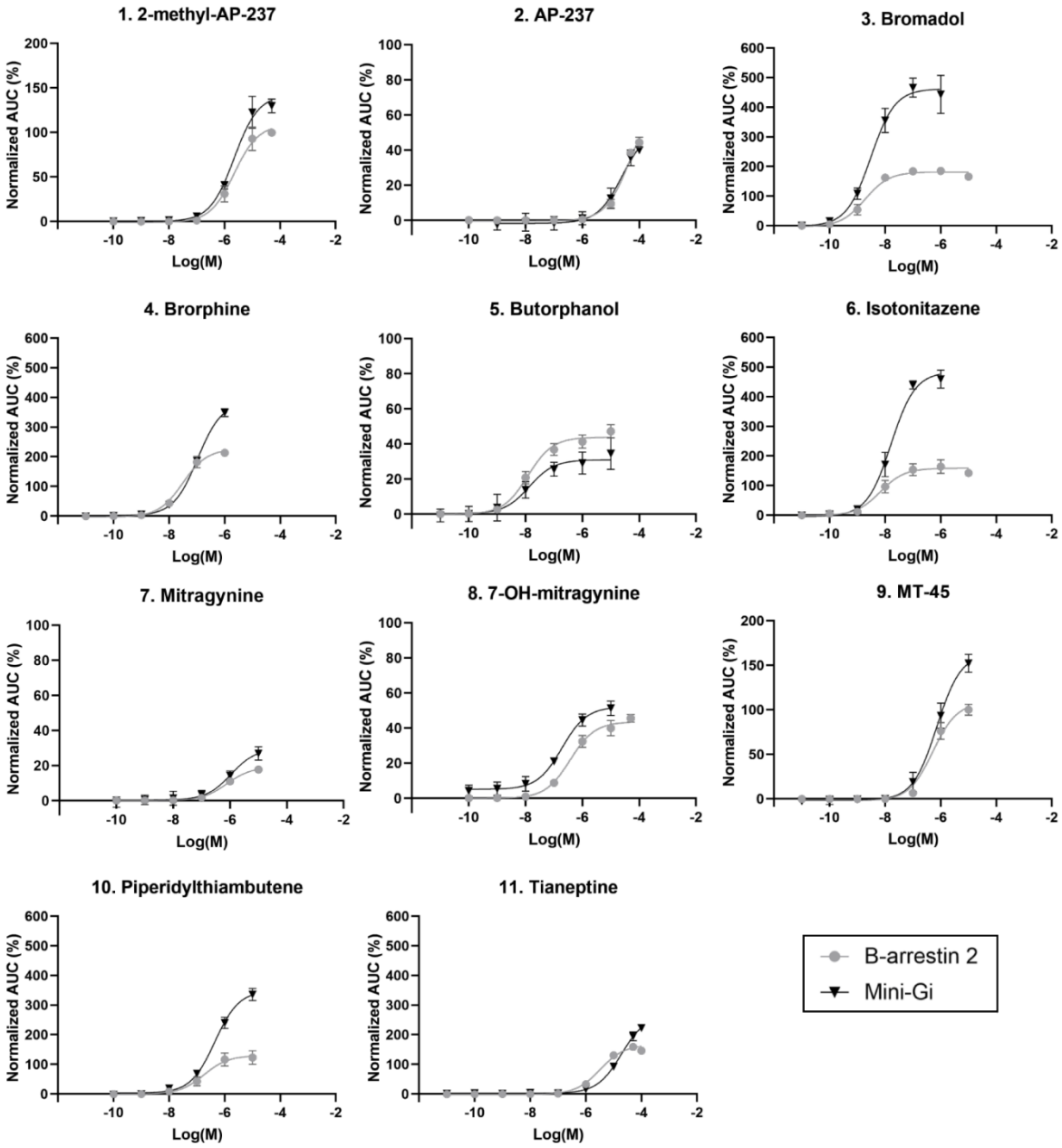


Fig. 2 Activation profiles obtained for **A.** the 11 studied non-fentanyl opioid new psychoactive substances; and **B.** the reference compound, hydromorphone, and fentanyl. Data are presented as mean receptor activation ± standard error of the mean (SEM) and are normalized to the E_{max} of hydromorphone (= 100%). Note the differences in scale of the y-axes. Black triangles, mini-Gi recruitment; grey circles, βarr2 recruitment.

2A.



2B.

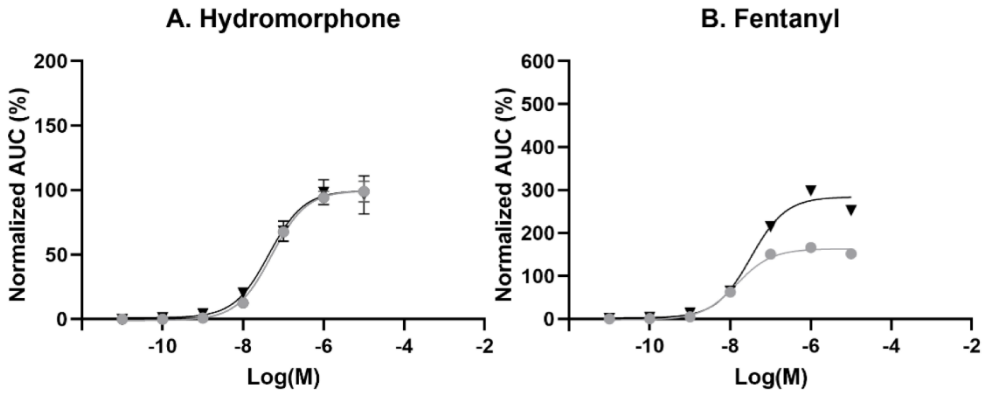


Fig. 3 Quantitative bias plot. $\Delta\log(E_{max}/EC_{50})_{\beta arr2}$ - $\Delta\log(E_{max}/EC_{50})_{mini\text{-}Gi}$ is plotted as bias factor ± SEM. A bias factor above zero indicates bias towards the β-arrestin 2 pathway, whereas a negative value implies bias towards mini-Gi recruitment. None of the tested compounds showed statistically significant biased agonism compared to the unbiased reference compound, hydromorphone **(A)**.

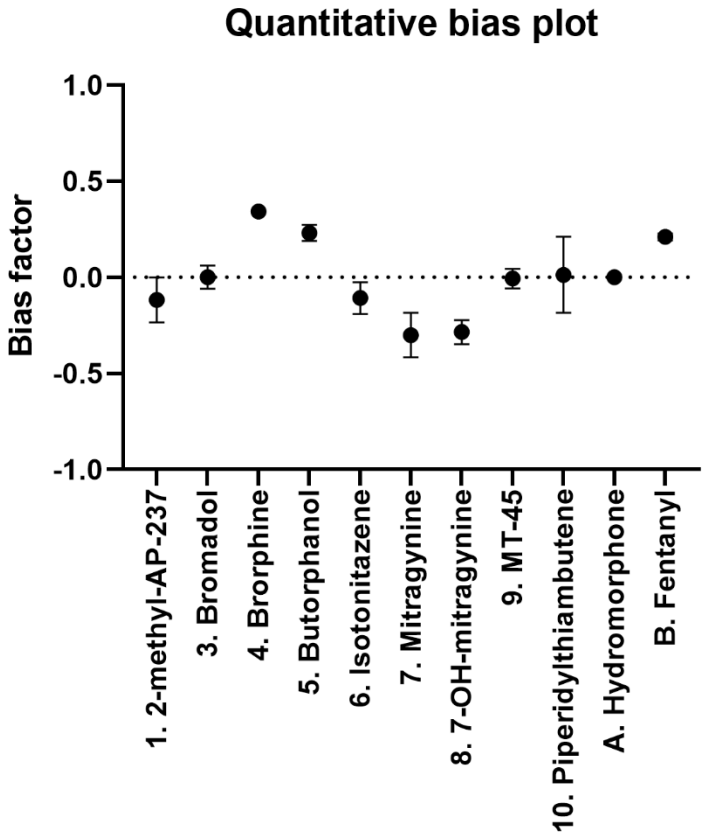


Table 1 Potency (EC_{50}) and efficacy (E_{max}, relative to hydromorphone) values (with 95% confidence intervals) as obtained in the μ-opioid receptor activation assays monitoring either β-arrestin 2 or mini-Gi recruitment. **(1)** 2-methyl-AP-237; **(2)** AP-237; **(3)** bromadol; **(4)** brorphine; **(5)** butorphanol; **(6)** isotonitazene; **(7)** mitragynine; **(8)** 7-OH-mitragynine; **(9)** MT-45; **(10)** piperidylthiambutene; **(11)** tianeptine; **(A)** hydromorphone; and **(B)** fentanyl.

	β-arrestin 2		**Mini-Gi**	
	EC50 (nM)	**Emax (%)**	**EC50 (nM)**	**Emax (%)**
1. 2-methyl-AP-237	**2229** (1267-4061)	**109** (95-124)	**2229** (1300-3932)	**142** (126-161)
2. AP-237	**> 3x10^4***	**69.3*** (58.8-85.4)	**> 8x10^3***	**52.1*** (37.7-90.0)
3. Bromadol	**1.89** (1.23-2.93)	**182** (172-192)	**3.04** (1.48-6.28)	**462** (414-512)
4. Brorphine	**31.1** (20.7-47.0)	**226** (207-246)	**106** (84.1-134)	**385** (363-408)
5. Butorphanol	**12.1** (6.66-22.9)	**43.8** (40.1-47.5)	**13.5** (1.95-104)	**30.9** (23.5-39.3)
6. Isotonitazene	**6.64** (2.84-15.0)	**159** (140-178)	**16.3** (10.6-25.5)	**484** (444-525)
7. Mitragynine	**773** (513-1148)	**19.2** (17.4-21.1)	**1089** (280-4442)	**29.7** (21.6-41.8)
8. 7-OH-mitragynine	**369** (220-612)	**43.5** (39.9-47.3)	**191** (92.4-415)	**52.2** (46.1-58.9)
9. MT-45	**525** (335-811)	**108** (97-120)	**724** (432-1176)	**163** (145-183)
10. Piperidylthiambutene	**180** (63.0-530)	**130** (105-157)	**443** (284-680)	**349** (319-381)
11. Tianeptine	**3262** (2543-4189)	**166** (157-176)	**> 1x10^4***	**266*** (243-292)
A. Hydromorphone	**51.0** (36.5-70.5)	**100** (94.0-105)	**44.0** (22.2-83.5)	**100** (89.1-111)
B. Fentanyl	**14.3** (11.3-18.1)	**163** (157-169)	**32.7** (23.3-45.8)	**284** (269-300)

(*) Maximum receptor activation seen at 100 μM. EC_{50} values to be interpreted with caution.