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Title: Functional characterization of a novel opioid, PZM21, and its influence on behavioural responses to morphine.

Running title: Pharmacological properties of PZM21.

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Abbreviations

5-HT	serotonin
βarr2	β-arrestin-2
δ-OR	δ-opioid receptor
μ-OR	μ-opioid receptor
CPP	conditioned place preference
Cyp	cyprodime
DA	dopamine
DAMGO	D-Ala2, N-MePhe4, Gly-ol]-enkephalin
I.p.	intraperitoneal
I.th.	intrathecal
I.v.	intravenous
Morph	morphine
MPE	maximum possible effect
Oxy	oxycodone
Sal	saline

μ-opioid receptor (μ-OR)

PZM21

morphine

dopamine (DA)

serotonin (5-HT)

DAMGO

δ opioid receptor (δ-OR)

oxycodone

Keywords

PZM21, biased opioid, antinociception, reward, tolerance, self-administration, monoamines release

Bullet point summary

1. What is already known: PZM21 is a new opioid efficacious for the 'supraspinal' component of pain with reduced side effects and rewarding activity.

2. What this study adds: PZM21 presents antinociceptive effects in reflexive test and supresses morphine reward. However after repeated treatment causes tolerance and withdrawal.

3. Clinical significance: PZM21 may be a promising treatment for opioid use disorder.

ABSTRACT

Background and purpose: The concept of opioid ligands biased toward the G protein pathway with minimal recruitment of β-arrestin-2 has become a promising approach for the development of novel, efficient and potentially nonaddictive opioid therapeutics. A recently discovered biased μ-opioid receptor agonist, PZM21, was reported to be analgesic and possess reduced side effects. Here, we aimed to further investigate the behavioural and biochemical properties of PZM21.

Experimental approach: We evaluated antinociceptive effects of systemic and intrathecal PZM21 administration. Its addiction-like properties were determined using several behavioural approaches: conditioned place preference, locomotor sensitization, precipitated withdrawal and self-administration. Further, we assessed the influence of PZM21 on morphine-induced antinociception, tolerance and reward. Effects of PZM21 on striatal release of monoamines were evaluated using brain microdialysis.

Key results: PZM21 caused long-lasting dose-dependent antinociception. It did not induce reward- and reinforcement-related behaviour, however, its repeated administration led to antinociceptive tolerance and naloxone-precipitated withdrawal symptoms. Pretreatment with PZM21 enhanced morphine-induced antinociception and attenuated the expression of morphine reward. In comparison to morphine, PZM21 administration led to moderate release of dopamine and robust release of serotonin in the striatum.

Conclusion and implications: PZM21 presents antinociceptive efficacy and does not possess rewarding or reinforcing properties. However, its clinical application may be restricted, as it induces tolerance and withdrawal symptoms. Notably, its ability to diminish morphine reward implicates that PZM21 may be useful in opioid use disorder therapy.

INTRODUCTION

Although opioid analgesics are usually the first choice and most effective treatments for pain, numerous side effects, including a strong addictive potential, severely limit their clinical effectiveness (Webster *et al.*, 2011). Substantial evidence indicates that opioid-induced analgesia and adverse effects are processed by distinct cell signalling pathways: analgesia is promoted by G protein signalling, whereas multiple undesirable effects are mediated through the regulatory protein β-arrestin-2 (βarr2; Bohn *et al.*, 1999; Raehal *et al.*, 2005). Therefore, there is a growing interest in pharmacological approaches that allow separation of analgesia from opioid side effects by developing biased (functionally selective) ligands that preferentially activate G protein signalling with minimal engagement of the βarr2 signalling pathway (Brust *et al.*, 2016; Chen *et al.*, 2013; DeWire *et al.*, 2013; Groer *et al.*, 2007; Maillet *et al.*, 2015; Manglik *et al.*, 2016). The majority of the research has addressed the μ-opioid receptor (μ-OR) as a target for action of biased ligands, since μ-OR agonists are the most effective analgesics (Madariaga-Mazón *et al.*, 2017). However, the μ-OR also represents a key molecular trigger for reward processing and contributes to the development of addictive behaviour (Contet *et al.*, 2004). Thus, in addition to the exclusion of somatic side effects associated with opioid use, the main challenge for μ-OR ligands biased toward G protein signalling is reduction of opioid-induced reinforcement. At present, there is no μ-OR agonist devoid of rewarding potential.

A novel, recently discovered G protein-biased opioid analgesic, PZM21, was described as a potent, selective μ-OR agonist and was reported to inhibit the emotional reaction to thermal nociceptive stimuli (named 'affective analgesia') with reduced morphine-like side effects and addictive potential in mice (Manglik *et al.*, 2016). In the present study, we aimed to further investigate behavioural and biochemical properties of PZM21 as well as possible mechanisms underlying its action and distinct effects on antinociception and addiction-like behaviour. We show that acute treatment with PZM21 results in long-lasting dose-dependent antinociception

that is mediated by the μ-OR, but repeated administration of this compound causes the development of antinociceptive tolerance and expression of withdrawal symptoms upon naloxone administration. Furthermore, PZM21 is devoid of opioid-like reinforcing properties; however, its action is accompanied by slight, but dose-dependent release of dopamine (DA) in the striatum. Interestingly, PZM21 induces a robust increase in striatal extracellular levels of serotonin (5-HT). Furthermore, we demonstrated that pretreatment with PZM21 may influence behavioural responses to morphine in mice and notably, is able to diminish opioid reward.

METHODS

Animals. All behavioural tests were performed on adult C57BL/6J mice (25-30 g, 8 weeks old at the beginning of the experiments, RRID:IMSR_JAX:000664), apart from experiments with intrathecal drug delivery and intravenous self-administration, which were performed on Wistar (RRID:RGD_13508588) and Sprague–Dawley (RRID:RGD_70508) rats (280-350 g, 10 weeks old at the beginning of the experiments), respectively. Experiments were carried out on male rodents to compare our results with previously published data (Manglik *et al*., 2016; Hill *et al*., 2018) and to avoid the possible influence of the menstrual cycle and reproductive status of female rodents on obtained results. C57BL/6J mice and Sprague-Dawley rats were acquired from the Maj Institute of Pharmacology PAS breeding facility (Krakow, Poland); Wistar rats were obtained from Charles River (Hamburg, Germany). All animals were group housed, mice 8-10 per cage (265 × 180 × 420 mm, Ehret Labor- und Pharmatechnik GMBH & Co.KG, Germany) and rats 5 per cage (380 × 200 × 590 mm, Ehret Labor- und Pharmatechnik GMBH & Co.KG, Germany) with aspen litter (MIDI LTE E-002 Abedd, AnimaLab, Poland), under standard room temperature 22 ± 2°C, humidity 50 ± 5%, and 12/12 h light–dark cycle, with free access to food and water (standard diet, Special Diets Services, England) and environmental enrichment. Animal studies are reported in compliance with the ARRIVE

guidelines (Kilkenny *et al*., 2010) and in compliance with the guidelines made by the *British Journal of Pharmacology*. Experiments were performed according to the European Union regulations and the Directive 2010/63/EU and were approved by the II Local Bioethics Commission (permit numbers: 1213/2015, 1305/2016, 66/2017, 84/2018; Krakow, Poland). Animals were randomly assigned to treatment groups and the experimenter was blinded to drug treatment until after data analysis has been performed. The n values in the experiments were chosen based on our previous experience with similar experimental protocols. The total number of animals as well as their suffering was minimized, according to the 3R principle. The exact numbers of animals in each group used in the experiments are listed in the Supplementary Materials (**Table S5**; n in each group >5). The criteria for excluding animals from experiments and statistical analysis in the present study included: abnormal basal response in the tail flick test (higher than 6 s indicating attenuated pain sensitivity) as well as technical issues (catheter obstruction, equipment malfunction).

Drugs and reagents. PZM21 was synthesized according to the previously published procedure (Manglik *et al.*, 2016). A detailed synthesis procedure as well as data confirming high enantiomeric purity of the synthesised compound are included in the Supplementary Materials **(Fig. S1, S2, Table S1, S2**). PZM21 displayed high μ-OR affinity similar to that of the prototypic μ-OR ligand DAMGO (**Fig. S3**, **Table S3**), which is in accordance with previously described results regarding the μ-OR-mediated G protein activity of the compound (Manglik *et al.*, 2016). The δ opioid receptor (δ-OR) affinity of PZM21 was significantly lower compared to the μ-OR affinity (**Fig. S3**, **Table S3**).

PZM21 was administered to mice at doses of 20, 40 or 80 $mg \cdot kg^{-1}$, depending on the experimental schedule. For intrathecal drug delivery, PZM21 was administered at doses of 2.5, 5 and 7.5 μg and for intravenous drug self-administration PZM21 was used at doses of 0.05 and 0.5 $mg \cdot kg^{-1}$ (per infusion). Morphine (Pharma Cosmetic, Poland; 5, 10, 20 $mg \cdot kg^{-1}$) or

oxycodone (Mundipharma, Poland; 0.06 mg·kg^{-1} (per infusion) were used as positive control treatments. As a negative control physiological saline was used. Cyprodime (Tocris, USA; 10 mg·kg^{-1}) and naloxone (Merck, Poland; 4 mg·kg^{-1}) were used as a selective μ-OR antagonist and nonselective opioid antagonist, respectively. All the drugs were dissolved in saline and administered intraperitoneally (i.p.) in a volume of 10 ml/kg, intrathecally (i.th.) in a volume of 5 μl/administration or intravenously (i.v.) in a volume of 0.0125 ml/infusion. Drugs used for anesthesia: ketamine (7.5 mg·kg^{-1} in mice and 100 mg·kg^{-1} in rats), xylazine (1 mg·kg^{-1} in mice and 10 mg·kg^{-1} in rats) and pentobarbital (60 mg·kg^{-1} in rats) were supplied by Biowet-Pulawy (Poland). The chemicals used for HPLC were purchased from Merck (Poland).

Intrathecal catheter implantation and drug delivery. The intrathecal (i.th.) drug administrations were achieved through implanting catheters according to the method described by Yaksh and Rudy (1976) under pentobarbital (60 mg·kg^{-1}) anesthesia, as reported previously (Rojewska *et al*., 2014). Briefly, a polyethylene catheter (PE 10, Intramedic, Clay Adams, Becton Dickinson and Company, Rutherford, USA) was sterilized by flushing with 70% ethanol and then sterile water prior to insertion. Rats were placed on a on a stereotaxic table (David Kopf Instruments, USA) and an incision was made in the atlantooccipital membrane. Then the catheter was carefully introduced through the atlantooccipital membrane to the subarachnoid space at the rostral level of the spinal cord lumbar enlargement (L4–L6), flushed with 10 μl of sterile water, and tightened with the tip. After implantation, the animals were allowed to recover for a minimum of 7 days and received enrofloxacin (KRKA, Slovenia) 0.1 ml s.c./rat once daily for 2 days. The i.th. injections were performed using a 50 μl Hamilton syringe with a 30 1/2-gauge needle; 5 μl was injected per animal, followed by 10 μl of saline.

Antinociception assessment. Tail flick. Tail flick was performed using a tail flick apparatus (Ugo Basile, Italy), and a light beam was used as a thermal nociceptive stimulus. The light beam was applied to the dorsal side of animals' tail, and the time latency to tail withdrawal or

shaking was recorded. To avoid tissue damage, a cut-off latency was set at 9 s. Responses were expressed as a percentage of the maximum possible effect (% MPE), calculated according to the formula: $[(T_1 - T_0)/(T_2 - T_0)] \times 100$, where T_0 and T_1 are the tail-flick latencies before and after drug injection, respectively, and T_2 is the cut-off time. To study for the influence of PZM21 on morphine-induced antinociception, the compound was administered 30 min prior to morphine and tail flick test was performed as described above.

Hot plate. The hot plate test was conducted using a hot plate analgesia meter (COTM, Poland). The mice were placed on the plate, which was preheated to 52.5°C, and the time latency to the first sign of spinally-mediated withdrawal reflexes (later described as a paw flinching) was measured. Moreover, we have measured the time latency to the first sign of complex behaviour (including licking/biting of the paw and/or jumping). A maximum exposure time was set at 30 s (cut-off) to avoid tissue damage. Both types of responses were expressed as % MPE.

Tolerance to thermal antinociception. To assess the development of tolerance to thermal antinociception induced by treatment with morphine or PZM21, the animals received injections with the drugs for 7 consecutive days and were tested in the tail flick assay 1 hour after drug administration. To study for the influence of PZM21 on the development of tolerance to morphine antinociception, mice were pretreated with PZM21 30 min prior to morphine and then tail flick test was performed.

Conditioned place preference test (CPP). The CPP procedure was conducted as previously described (Szklarczyk *et al*., 2012). Briefly, a CPP apparatus (Med Associates, USA) consisted of three different compartments. The CPP procedure began on day 0 with 5 min of acclimatization to the apparatus. On day 1 (preconditioning test), mice were allowed to freely explore the whole apparatus for 20 min, and time spent in each compartment was measured. The procedure was unbiased, so that no significant differences in compartment preference were found within each group during preconditioning test. During the conditioning days (days 2–

11), mice were injected with morphine or PZM21 (day 3, 5, 7, 9, 11) or saline (day 2, 4, 6, 8, 10) and immediately placed in the respective compartment for 60 min. To study the effects of PZM21 on morphine-induced CPP, separate groups of mice were subjected to the procedure, and 30 min prior to the morphine injection, they received a pretreatment with PZM21. The postconditioning test was performed on day 12 and was the same as the preconditioning one. The difference between the times spent in the drug- and vehicle-paired compartments during the postconditioning session was considered to be a measure of CPP (CPP score).

Locomotor sensitization. The measurement of locomotor sensitization lasted for 6 days and was performed using custom-made activity chambers. Each day, all animals were first injected with saline, placed in the chambers for 2 hours, received the appropriate injection (saline, morphine or PZM21) and were placed back in the boxes for an additional 2 hours. To study for the influence of PZM21 on sensitization to morphine-induced hyperlocomotion, separate groups of mice received PZM21 injections 30 min prior to morphine. The expression of sensitization was tested after 8 days-incubation period. All animals were habituated to the chambers for 2 hours 1 day before the onset of the experiments.

Naloxone-precipitated withdrawal. For naloxone-precipitated withdrawal mice were chronically (at 8:00 and 16:00 for 5 consecutive days) treated with saline, morphine or PZM21, similarly to method described by Abdel-Zaher *et al*. (2006). On the sixth day, three hours after the final saline or drug administration, all animals received 4 $mg \cdot kg^{-1}$ naloxone and immediately after the injection each animal was placed in a transparent acryl cylinder (20 cm in diameter, 50 cm in height) for 15 min to observe jumps, which were considered as a manifestation of withdrawal.

Intravenous drug self-administration. A self-administration procedure was performed as previously described (Solecki *et al*., 2013). Briefly, rats were anesthetized with ketamine (100 $mg \cdot kg^{-1}$) and xylazine (10 $mg \cdot kg^{-1}$) and implanted with a silastic catheter in the external jugular

vein. For catheter implantation, a guide cannula (C313G, Plastics One Inc., USA), attached to silastic tubing (0.025 inner diameter, Bio-Sil, Bio-Rad, USA) and Marlex mesh via dental cement, was inserted subcutaneously between the shoulder blades and exited the skin via a dermal biopsy hole (3 mm). The other end of the tubing was threaded under the skin, inserted 3 cm into the right jugular vein, and then sutured securely to the underlying muscle tissue. After the catheter implantation, rats underwent 7 days of surgical recovery during which the catheters were flushed with 0.3 ml of saline and 0.2 ml of heparin solution (Braun, Germany) in order to prevent occlusion. All animals were given an anti-inflammatory and analgesic drug Tolfedine 4%, 1 ml·kg^{-1}, i.p. (Vetoquinol Biowet, Poland) and 5 ml of glucose to prevent dehydration during post-surgery recovery. For the first three days after the operations, animals were treated with antibiotics added to the drinking water (Sul-Tridin 24%, Biowet-Pulawy, Poland). Self-administration training was preceded by 2–3 days of food restriction to ~90 % of free feeding levels. Rats were trained under a fixed ratio 1 schedule of reinforcement during which each active lever press led to intravenous drug infusion and conditioned stimulus (CS) cue presentation (tone + stimulus light for 6 s) in standard operant chambers (Med Associates, USA). Each active lever response was followed by a 20-s time out during which lever pressing had no programmed consequences. Similarly, inactive lever presses had no programmed consequences. Each rat underwent 2 hours daily training sessions for 10 consecutive days.

Drug seeking under extinction conditions. Drug seeking under extinction conditions was performed as previously described (Solecki *et al*., 2013, 2018). After drug self-administration training, rats underwent 3 days of forced abstinence in their home cages without access to drug or drug-associated contextual and discrete cues. This forced withdrawal period did not include extinction to better model the medical detoxification experienced by many people with substance use disorders that occurs without behavioural extinction training. On withdrawal day 3, animals were placed in operant chambers for 2 h, and active lever presses led to the discrete

CS presentation (i.e., 6 s tone and light) with no drug delivery. A 20-s timeout followed the CS termination, during which time responses were recorded but had no programmed consequences. Inactive lever presses had no programmed consequences. In such testing settings, drug seeking (i.e., active lever responding) was driven by both contextual cues and discrete CS presentation contingent upon active lever presses.

Brain microdialysis and analytical procedure. Mice were anesthetized with ketamine (7.5 $mg \cdot kg^{-1}$) and xylazine (1 $mg \cdot kg^{-1}$), and a vertical microdialysis probe was implanted into the striatum using the coordinates (from Bregma): AP +1.0, L +1.8 and V -3.8. On the next day, the probe inlets were connected to a syringe pump (BAS, IN, USA), which delivered aCSF composed of [mM]: NaCl 147, KCl 2.7, $MgCl_2$ 1.0, $CaCl_2$ 1.2; pH 7.4 at a flow rate of 1.5 µl/min. After 1 h of the washout period, three basal dialysate samples were collected every 20 min, the animals were injected with the appropriate drugs as indicated in figure captions, and fraction collection continued for 240 min. At the end of the experiment, the mice were sacrificed by decapitation under isoflurane anesthesia, brains were removed and histologically examined to validate the probe placement. The DA and 5-HT content of the dialysate fractions was analysed by high-performance liquid chromatography (HPLC) with coulochemical detection. Chromatography was performed using an Ultimate 3000 System (Dionex, USA) and a coulochemical detector, Coulochem III (model 5300, ESA, USA), with a 5020 guard cell, 5014B microdialysis cell and Hypersil Gold-C18 analytical column (3 x 100 mm). The mobile phase was composed of 0.1 M potassium phosphate buffer adjusted to pH 3.6, 0.5 mM EDTA, 16 $mg \cdot l^{-1}$ 1-octanesulfonic acid sodium salt, and 2% methanol. The flow rate during analysis was set at 0.7 $ml \cdot min^{-1}$. The applied potential of the guard cell was +600 mV, while those of the microdialysis cells were as follows: E1=-50 mV and E2=+300 mV with a sensitivity set at 50 $nA \cdot V^{-1}$. The chromatographic data were processed by Chromeleon v. 6.80 (Dionex, USA) software and run on a personal computer. All obtained microdialysis data were presented as a

percent of the basal level assumed to be 100% to allow comparison of the magnitude of DA and 5-HT release.

Statistical analysis. For statistical analysis, GraphPad Prism 7.0 (GraphPad Prism Software Inc., USA, RRID:SCR_002798) and Statistica (12.5, Stat-Soft, Poland, RRID:SCR_014213) were used. Group sizes listed in the **Table S5** present the number of independent samples/animals. Statistical analyses of behavioural data were performed using unpaired Student's t-test (μ-OR antagonism in hot plate test), one-way ANOVA (conditioned place preference experiments, antinociception in hot plate test and naloxone-precipitated withdrawal), two way ANOVA (drug seeking in self-administration experiments), two-way repeated measures ANOVA (antinociception and μ-OR antagonism in tail flick test, antinociceptive tolerance, locomotor sensitization test and microdialysis experiments) or three-way repeated measures ANOVA (drug self-administration procedure) followed by Bonferroni post hoc tests where appropriate (performed only when F achieved $p<0.05$ and there was no significant variance inhomogeneity). Data are presented on graphs as the mean ± SEM. Statistical significance was set at the $p<0.05$. Statistically significant differences are marked with the symbols * and #. The data and statistical analyses comply with the recommendations on experimental design and analysis in pharmacology (Curtis *et al.,* 2018).

Nomenclature of targets and ligands. Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding *et al*., 2018) and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander *et al*., 2017).

RESULTS

Antinociceptive effects of PZM21

Antinociceptive efficacy of PZM21 was assessed using two thermal antinociceptive tests: tail flick (mice and rats) and hot plate (mice). Administration of 10 mg·kg^{-1} morphine (i.p.), used as a positive control, resulted in antinociception (treatment × time interaction: $F_{4,56}$=17.49, p<0.05, **Fig. 1A**). Treatment with PZM21 (i.p.) exerted an antinociceptive effect in the tail flick test at all of the tested doses: 20, 40 and 80 mg·kg^{-1} (treatment × time interaction: $F_{12,120}$=7.08, p<0.05, **Fig. 1B**), compared to saline. PZM21-induced antinociception was dose-dependent and lasted up to 8 hours. Pretreatment with a selective μ-OR antagonist, cyprodime (10 mg·kg^{-1}, i.p.), prevented the antinociception induced by 40 mg·kg^{-1} PZM21 in the tail flick test (treatment × time interaction: $F_{4,72}$=7.65, p<0.05, **Fig. 1C**). In the hot plate test, we have distinguished two types of reaction: paw flinching and licking of the paw or jumping. The obtained data indicate that only morphine (10 mg·kg^{-1}) induced a significant effect on the paw flinching reflex ($F_{4,42}$=2.56, p<0.05) when compared to saline. PZM21 did not affect this reaction at any of the tested doses. At the same time, both PZM21 (at a dose of 80 mg·kg^{-1}) and morphine (10 mg·kg^{-1}) increased the latency to the second type of reaction (paw licking/jumping) ($F_{4,42}$=5.63, p<0.05) in the hot plate test (**Fig. 1D**). Pretreatment with cyprodime attenuated the effects induced by 40 mg·kg^{-1} PZM21 in hot plate test (t_{19}=2.10, p<0.05 and t_{19}=2.53, p<0.05 for two types of reactions, **Fig. 1E**). Therefore, we demonstrated that antinociceptive effects of PZM21 are mediated by μ-OR. What is more, i.th administration of PZM21 (2.5, 5 and 7.5 μg) caused dose-dependent antinociception in tail flick test in rats (treatment × time interaction: $F_{12,88}$=3.1, p<0.05, **Fig. 1F**), which shows that this compound is effective for the reflexive, spinally-mediated component of pain reaction.

Influence of PZM21 on addiction-like behaviour in mice

The influence of PZM21 on addiction-like behaviour in mice was investigated in CPP and locomotor sensitization tests. Moreover, we measured the development of antinociceptive tolerance and naloxone-precipitated withdrawal symptoms after chronic PZM21 administration in order to assess its potential to cause physical dependence. Treatment with PZM21 did not induce CPP at any of the tested doses (20, 40 and 80 mg·kg^{-1}, i.p.), whereas morphine-treated animals (10 mg·kg^{-1}, i.p.) developed a significant preference to the drug-associated compartment ($F_{4,45}$=9.16, p<0.05, **Fig. 2A**). Interestingly, the obtained data suggest that treatment with PZM21 at a dose of 80 mg·kg^{-1} led to a drug-induced aversion (t_{18}=2.626, p<0.05, compared to saline group, **Fig. 2A**). Repeated 6-day administration of PZM21 did not influence animals' locomotor activity at any of the doses, while treatment with morphine induced locomotor sensitization (treatment × time interaction: $F_{20,210}$=2.37, p<0.05, **Fig. 2B**). Morphine-treated animals showed increased expression of sensitization measured after 8-day incubation period, while only a slight increase in locomotor activity was observed in the group treated with 80 mg·kg^{-1} PZM21 (treatment × time interaction: $F_{4,42}$=30.16, p<0.05, **Fig. 2B**). On the other hand, chronic administration of 80 mg·kg^{-1} of PZM21 as well as 10 mg·kg^{-1} of morphine resulted in the occurrence of naloxone-induced jumps, considered as physical signs of withdrawal ($F_{4,51}$=35.98, p<0.05, **Fig. 2C**). Moreover, repeated treatment with both 40 mg·kg^{-1} and 80 mg·kg^{-1} PZM21 resulted in the development of antinociceptive tolerance measured by the tail flick test (treatment × time interaction: $F_{9,99}$=3.12, p<0.05, **Fig. 2D**). Therefore, our results indicate that PZM21 is devoid of opioid-like rewarding properties, however it induces physical dependence.

Assessment of reinforcing properties of PZM21 in intravenous self-administration paradigm in rats

PZM21, similar to saline, did not induce intravenous self-administration during 10-day training sessions in rats (**Fig. 3A-C**), in contrast to robust oxycodone self-administration, measured as a number of drug infusions (treatment × time interaction: $F_{(27, 279)} = 2.7$, $p<0.05$, **Fig. 3A**). Essentially, rats in the saline and PZM21 groups did not differentiate between active and inactive levers throughout training, whereas oxycodone self-administering rats presented more responses on active in comparison to inactive lever, starting from day 5 of training (treatment × lever × time interaction: $F_{(27, 558)}=2.88$, $p<0.05$, **Fig. 3B-C**). Finally, only rats which self-administered oxycodone, but not saline or PZM21, demonstrated drug seeking behaviour, expressed as presses on previously active lever after 3 days of abstinence (treatment × lever interaction: $F_{(3, 58)}=10.81$, $p<0.05$, **Fig. 3D**). Thus, our data indicate that PZM21 does not act as a reinforcer and it does not induce craving upon drug abstinence.

Increased monoamine release in the striatum in response to PZM21

Striatal DA and 5-HT levels following drug administration were measured for 4 h in freely moving mice. Both 40 and 80 mg·kg^{-1} (i.p.) PZM21 as well as 10 and 20 mg·kg^{-1} morphine (i.p.) markedly increased extracellular DA release (treatment × time interaction: $F_{25,275}=13.001$, $p<0.05$, **Fig. 4A**). The increase in DA release induced by 10 mg·kg^{-1} of morphine and 40 mg·kg^{-1} of PZM21 was comparable, whereas the effect of higher dose of PZM21 (80 mg·kg^{-1}) was similar in magnitude to 20 mg·kg^{-1} of morphine. The action of both drugs on DA release is also presented as the total effect expressed as the area under the curve (AUC) ($F_{4,25}=175.60$, $p<0.05$, **Fig. 4A**). Moreover, administration of PZM21 at doses of 40 and 80 mg·kg^{-1} as well as morphine (10 and 20 mg·kg^{-1}) caused an increase in 5-HT extracellular levels (treatment × time point interaction: $F_{25,275}=17.22$, $p<0.05$, **Fig. 4B**). Treatment with 40 mg·kg^{-1} of PZM21

resulted in the extracellular elevation of striatal 5-HT that was similar to 20 mg·kg^{-1} of morphine. However, higher dose of PZM21 (80 mg·kg^{-1}) produced a robust release of 5-HT, reaching above 350% of the basal level at the peak. PZM21 and morphine action on 5-HT is also presented as the total effect expressed as the AUC in the **Fig. 4B** ($F_{4,25}$=275.30, p<0.05). An additional figure presenting microdialysis probe placement is provided in the Supplementary Materials (**Fig. S5**).

Consequences of pretreatment with PZM21 on morphine-induced behaviour

We then investigated whether PZM21 may influence antinociceptive and addictive effects of morphine. For these experiments, we chose doses of 20 and 40 mg·kg^{-1} PZM21 (i.p.), as our previous data suggested a ceiling effect of PZM21 above the dose of 40 mg·kg^{-1}and these doses did not induce physical dependence or aversion. First, we determined the dose of morphine (5 mg·kg^{-1}) that induced approximately 50% of MPE in the tail flick test and assessed how pretreatment with PZM21 will influence morphine-induced antinociception. Our results show that the dose of 40 mg·kg^{-1} of PZM21 enhanced and prolonged antinociception evoked by morphine (treatment × time interaction: $F_{8,108}$=5.29, p<0.05, **Fig. 5A**). Next, we used a model of tolerance to morphine-induced antinociception and assessed the effect of PZM21 under these conditions. The obtained data showed that pretreatment with PZM21 did not influence tolerance development during repeated administration of 10 mg·kg^{-1} of morphine (treatment × time interaction: $F_{6,69}$=1, p>0.05, **Fig. 5B**). To investigate whether PZM21 modulates addictive properties of morphine we performed CPP and locomotor sensitization tests. The results obtained show that preadministration of 40 mg·kg^{-1}, but not 20 mg·kg^{-1} of PZM21, prevented the formation of morphine-induced CPP ($F_{2,34}$=6.81, p<0.05, **Fig. 5C**). Pretreatment with PZM21 did not affect the development of morphine-induced locomotor sensitization (treatment × time interaction: $F_{10,140}$=1.64, p>0.05, **Fig. 5D**), however a slight tendency toward the reduction of morphine effects was observed. Moreover, PZM21 did not influence the

expression of morphine sensitization after the 8-day incubation period (treatment × time interaction: $F_{2,28}$=2.27, p>0.05, **Fig. 5D**). Taken together, our results show that PZM21 enhances antinociceptive effects of morphine and supresses its rewarding properties.

DISCUSSION AND CONCLUSIONS

In the present study, we examined a novel G protein-biased μ-OR ligand, PZM21, as a potential nonaddictive analgesic and assessed its ability to modulate morphine-related behaviour in mice. We demonstrated that PZM21 (administered both i.p. and i.th.) efficiently exerts dose-dependent, long-lasting antinociception, and that cyprodime, μ-OR antagonist, blocks this effect. Further, we confirmed that PZM21 is a compound selective for μ-OR, while weakly interacting with δ-OR. Therefore, our results suggest that PZM21 induces antinociception by acting selectively via μ-OR signalling. Interestingly, PZM21 did not elicit maximum possible effect in the tail flick test at any of the tested doses, suggesting that increasing the dosage beyond a certain level would not enhance antinociception, which is known as a ceiling effect (Trescot *et al*., 2008). PZM21 increased the latency to paw licking/jumping reaction it the hot plate test. Therefore, our study confirmed the effectiveness of PZM21 in the 'supraspinal' component of pain processing, as was suggested by Manglik *et al*. (2016). However, our results clearly demonstrate that PZM21 action is not restricted to supraspinal central nervous system areas, because we observed the antinociceptive effect after i.th. administration of the compound in the tail flick test that is known to be a measure of spinal reflex (Deuis *et al*., 2017). Taken together, our study indicate that PZM21 is a compound with antinociceptive efficacy. Notably, G protein-biased opioid analgesics were reported to have broader therapeutic window than conventional opioids, which allows for antinociception in the absence of respiratory depression (Schmid *et al*., 2017). However, Hill *et al.* (2018) have shown that PZM21 depresses respiration, therefore a question why PZM21 induces suppression of respiration regardless of its bias toward the G protein remains to be addressed in the future studies. Opioid drugs possess

rewarding properties, which is one of the undesirable effects of their administration (Fields and Margolis, 2015). In our study, all of the tested doses of PZM21 failed to induce CPP, indicating that this compound is devoid of rewarding effects, and these results are consistent with the previous report (Manglik *et al*., 2016). However, it is possible that we did not capture PZM21-induced reward-related behaviour due to possible differences in the duration of action between morphine and the compound. Interestingly, at the highest dose (80 $mg \cdot kg^{-1}$), we observed a drug-induced aversion, indicating that under certain conditions the compound may act as an antagonist. We also showed that in contrast to morphine, PZM21 did not induce locomotor sensitization, considered as a sign of drug-induced plasticity (Marie *et al*., 2018). Furthermore, we demonstrated that PZM21 is not readily self-administered by rats and does not induce drug seeking behaviour after abstinence period, strongly suggesting no PZM21 does not present reinforcing properties at the tested doses. To our knowledge, this is the first publically-available report to assess PZM21 as a possible reinforcer. Therefore, these results show that PZM21 is devoid of opioid-like rewarding and reinforcing activity, which is unique among opioid drugs, also in biased agonists group (Soergel *et al*., 2014; Altarifi *et al*., 2017; Austin Zamarripa *et al*., 2018).

In the present study, we observed that naloxone administration-precipitated withdrawal syndrome after chronic administration of PZM21. To date, studies regarding the potential of biased opioids to induce withdrawal are limited. Kliewer *et al*. (2019) suggested, that reducing βarr2 recruitment to μ-OR might not improve the safety profile of opioids, as genetically modified mice with receptors unable to recruit βarr2 displayed typical signs of withdrawal after chronic opioid treatment. What is more, our results showed that after repeated daily administration, PZM21 caused rapid development of antinociceptive tolerance at doses of 40 and 80 $mg \cdot kg^{-1}$, which is in line with the previous report (Hill *et al*., 2018). βarr2-mediated μ-OR desensitization was previously indicated as a possible cause of tolerance to opioids

(Przewlocka *et al*., 2002; Raehal *et al*., 2005; Yang *et al*., 2011; Mori *et al*., 2017; Kliewer *et al*., 2019). However, the currently accepted idea that G protein-biased ligands should not produce tolerance has little evidence to support it and our data suggest that even opioids biased toward G protein might cause tolerance, limiting their utility as analgesics. Previous studies suggest that the development of tolerance may depend not only on long-term adaptations connected with βarr2 function but should be considered as an attribute of a particular ligand (Koch and Höllt, 2008). For example, according to some reports, tolerance to antinociceptive properties of morphine is mediated by c-Jun N-terminal kinase rather than by the action of βarr2 (Kuhar *et al.*, 2015; Marcus *et al*., 2015; Yuill *et al*., 2016). Thus, it seems that the development of opioid tolerance may be ligand-specific and involve both βarr2-dependent and independent pathways, leading to differential mechanisms of tolerance observed *in vivo*. Interestingly, it was shown that high efficacy μ-OR agonists require lower receptor reserves to maintain an analgesic effect and in turn cause lower tolerance (Stevens and Yaksh, 1989). Our results showed that PZM21 displayed high μ-OR affinity, but in terms of antinociception, presented relatively low efficacy. This observation is consistent with our data revealing that PZM21 activates G protein signalling moderately, as the maximal stimulation of receptor-mediated G protein activity is low and corresponds to a weak partial agonist activity. Therefore, a possible mechanism associated with PZM21-induced tolerance might be connected with its agonistic efficacy, however it should be further investigated why PZM21 produces such a rapid development of tolerance to antinociception, regardless of its biased agonism. Taken together, our results indicate that PZM21 does not exhibit rewarding and reinforcing properties. However, chronic treatment with this compound leads to rapid development of tolerance and causes signs of physical dependence. Thus, our report demonstrates that PZM21 differentially influences motivational and physical aspects of addictive behaviour.

In a search for possible mechanisms underlying the lack of reward-associated behaviour in PZM21-treated animals, we measured extracellular monoamine levels in the striatum using brain microdialysis in mice. A single systemic administration of PZM21 slightly but dose-dependently enhanced the extracellular release of DA. Our data show that administration of 40 $mg \cdot kg^{-1}$ of PZM21 induces similar release of DA to 10 $mg \cdot kg^{-1}$ of morphine and effect of 80 $mg \cdot kg^{-1}$ of PZM21 is comparable to that evoked by treatment with 20 $mg \cdot kg^{-1}$ of morphine. Thus, the doses of both drugs that produced similar release of striatal DA had different effects on reward-related memory measured in CPP paradigm. Namely, 10 $mg \cdot kg^{-1}$ of morphine induced strong preference toward drug-associated compartment, whereas 40 $mg \cdot kg^{-1}$ of PZM21 did not present rewarding properties. The enhancement of DA-dependent neurotransmission within the striatum after opioid administration was commonly associated with their addictive properties (Di Chiara and Imperato, 1988; Spanagel *et al*., 1990; Barik *et al*., 2010). However, it was also proposed that DA is dispensable for morphine-induced reward measured in the CPP paradigm and for heroin self-administration (Pettit *et al*., 1984; Hnasko *et al*., 2005; Borgkvist *et al*., 2007). Thus, the hedonic properties of opioids may only partly depend on DA release within the striatal circuity. On the other hand, an enhancement of striatal DA release is strongly related to opioid-induced hyperlocomotion and locomotor sensitization (Saito, 1990; Murphy *et al*., 2001). We showed that PZM21 treatment does not influence locomotor activity. In agreement with our results, a previous study in mice revealed that βarr2 knockout may reduce the expression of some DA-dependent behaviours such as locomotor activity (Bohn *et al*., 2003). Interestingly, we demonstrated a robust, dose-dependent increase in 5-HT striatal release following PZM21 treatment. The ratio of brain 5-HT to DA was reported to underlie the analgesic effectiveness of opioids (Major and Pleuvry, 1971) as well as the abuse potential of drugs, since 5-HT neurons were shown to provide an inhibitory influence over mesolimbic DA neurons (Rothman *et al*., 2008; Navailles and De Deurwaerdère,

2011). Therefore, we propose that PZM21, through the modulation of 5-HT release, might inhibit some DA-related behaviours such as locomotor sensitization. Furthermore, 5-HT-dependent neurotransmission may enhance and prolong PZM21-induced antinociception; however, this hypothesis needs to be examined in further studies.

Lastly, we investigated whether PZM21 may influence some outcomes of morphine administration. Pretreatment with PZM21 had no effect on the development of antinociceptive tolerance to morphine, but it enhanced morphine-induced antinociception. This observation suggests that PZM21 might be used in pain relief alone or in combination with other opioid drugs. On the other hand, PZM21 prevented the formation of morphine-induced CPP. One possible explanation for PZM21-mediated suppression of morphine reward is that it directly antagonized the morphine effects at the μ-OR due to its high affinity for this receptor. Interestingly, our studies on PZM21 suggest that it shows partial agonist characteristics. The use of partial agonists of μ-OR, such as buprenorphine or nalbuphine, appears to be a successful strategy for the attenuation of opioid-induced reward and treatment of opioid use disorder (Tao *et al*., 2006; Abdel-Ghany *et al*., 2015; Nielsen *et al*., 2016; Robinson *et al*., 2017). Hence, antirewarding properties of PZM21 may be dependent on its pharmacological profile.

PZM21 was firstly described as a potent and selective μ-OR agonist (Manglik *et al*., 2016). However, our behavioural results suggest that it acts as a partial agonist/antagonist of μ-OR, as it displays a ceiling effect in antinociceptive tests, presents a tendency toward inducing aversion at high doses and interacts with morphine, modulating some effects of its administration. Notably, recently published Ca^{2+} imaging study have shown that PZM21 actually is a partial μ-OR agonist, because, when compared to DAMGO, it induces smaller activation of G protein-coupled inwardly rectifying potassium channels and smaller inhibition of a nociceptive channel - transient receptor potential melastatin (Yudin and Rohacs, 2019). Therefore, our study provide a novel insight into pharmacological properties of this compound

that differ from these described in the original publication (Manglik *et al*., 2016) and in many aspects are more consistent with reports by Hill *et al*. (2018) and Yudin and Rohacs (2019).

Taken together, present study revealed new characteristics of PZM21. The drug does not induce rewarding and reinforcing effects, indicating that biased signalling could be an attractive direction for future pharmacological studies of novel opioid-based therapeutics. However, our results point out that PZM21 does not evoke a very potent antinociception when compared to morphine and produces antinociceptive tolerance, suggesting that it may not be sufficient in clinical pain management, especially under chronic pain conditions. Further studies are required in order to assess the effects of PZM21 under different pain conditions and effects of its co-administration with other opioid therapeutics. It is especially worth considering PZM21 as a pharmacological tool for the modulation of morphine effects, especially for diminishing reward-related behaviour and therefore, PZM21 may be considered a potential treatment for opioid use disorder.

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AUTHOR CONTRIBUTIONS

L.K. and R.P. designed the study and wrote the manuscript. R.B., A.B. and Sz.B. performed the synthesis and analysed enantiomeric purity of PZM21. L.K., U.S., L.W., W.S., W.M. and B.P. planned, performed and analysed behavioural experiments. K.G. and A.W. conducted

brain microdialysis and analysed the obtained data. F.Z. and S.B. performed and analysed binding experiments. All authors read and accepted the manuscript.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the *BJP* guidelines for Design & Analysis, and Animal Experimentation, and as recommended by funding agencies, publishers and other organizations engaged with supporting research.

REFERENCES

Abdel-Ghany, R., Nabil, M., Abdel-Aal, M., and Barakat, W. (2015). Nalbuphine could decrease the rewarding effect induced by tramadol in mice while enhancing its antinociceptive activity. Eur. J. Pharmacol. *758*: 11–15.

Abdel-Zaher, A.O., Hamdy, M.M., Aly, S.A., Abdel-Hady, R.H., and Abdel-Rahman, S. (2006). Attenuation of morphine tolerance and dependence by aminoguanidine in mice. European Journal of Pharmacology *540*: 60–66.

Alexander, S.P.H., Kelly, E., Marrion, N.V., Peters, J.A., Faccenda, E., Harding, S.D., et al. (2017). The Concise Guide to Pharmacology 2017/18: G protein-coupled receptors. Br J Pharmacol *174 (Suppl 1)*: S17–S129.

Altarifi, A.A., David, B., Muchhala, K.H., Blough, B.E., Akbarali, H., and Negus, S.S. (2017). Effects of acute and repeated treatment with the biased mu opioid receptor agonist

TRV130 (oliceridine) on measures of antinociception, gastrointestinal function, and abuse liability in rodents. J. Psychopharmacol. (Oxford) *31*: 730–739.

Austin Zamarripa, C., Edwards, S.R., Qureshi, H.N., Yi, J.N., Blough, B.E., and Freeman, K.B. (2018). The G-protein biased mu-opioid agonist, TRV130, produces reinforcing and antinociceptive effects that are comparable to oxycodone in rats. Drug and Alcohol Dependence *192*: 158–162.

Barik, J., Parnaudeau, S., Saint Amaux, A.L., Guiard, B.P., Golib Dzib, J.F., Bocquet, O., et al. (2010). Glucocorticoid receptors in dopaminoceptive neurons, key for cocaine, are dispensable for molecular and behavioral morphine responses. Biol. Psychiatry *68*: 231–239.

Bohn, L.M., Gainetdinov, R.R., Sotnikova, T.D., Medvedev, I.O., Lefkowitz, R.J., Dykstra, L.A., et al. (2003). Enhanced Rewarding Properties of Morphine, but not Cocaine, in βarrestin-2 Knock-Out Mice. J. Neurosci. *23*: 10265–10273.

Bohn, L.M., Lefkowitz, R.J., Gainetdinov, R.R., Peppel, K., Caron, M.G., and Lin, F.T. (1999). Enhanced morphine analgesia in mice lacking beta-arrestin 2. Science *286*: 2495–2498.

Borgkvist, A., Usiello, A., Greengard, P., and Fisone, G. (2007). Activation of the cAMP/PKA/DARPP-32 signaling pathway is required for morphine psychomotor stimulation but not for morphine reward. Neuropsychopharmacology *32*: 1995–2003.

Brust, T.F., Morgenweck, J., Kim, S.A., Rose, J.H., Locke, J.L., Schmid, C.L., et al. (2016). Biased agonists of the kappa opioid receptor suppress pain and itch without causing sedation or dysphoria. Sci Signal *9*: ra117.

Chen, X.-T., Pitis, P., Liu, G., Yuan, C., Gotchev, D., Cowan, C.L., et al. (2013). Structure-activity relationships and discovery of a G protein biased μ opioid receptor ligand, [(3-methoxythiophen-2-yl)methyl]({2-[(9R)-9-(pyridin-2-yl)-6-oxaspiro-[4.5]decan-9-yl]ethyl})amine (TRV130), for the treatment of acute severe pain. J. Med. Chem. *56*: 8019–8031.

Contet, C., Kieffer, B.L., and Befort, K. (2004). Mu opioid receptor: a gateway to drug addiction. Curr. Opin. Neurobiol. *14*: 370–378.

Curtis, M.J., Alexander, S., Cirino, G., Docherty, J.R., George, C.H., Giembycz, M.A., et al. (2018). Experimental design and analysis and their reporting II: updated and simplified guidance for authors and peer reviewers. Br J Pharmacol *175.7*: 987-993.

Deuis, J.R., Dvorakova, L.S., and Vetter, I. (2017). Methods Used to Evaluate Pain Behaviors in Rodents. Front Mol Neurosci *10*:.

DeWire, S.M., Yamashita, D.S., Rominger, D.H., Liu, G., Cowan, C.L., Graczyk, T.M., et al. (2013). A G Protein-Biased Ligand at the μ-Opioid Receptor Is Potently Analgesic with Reduced Gastrointestinal and Respiratory Dysfunction Compared with Morphine. J Pharmacol Exp Ther *344*: 708–717.

Di Chiara, G., and Imperato, A. (1988). Drugs abused by humans preferentially increase synaptic dopamine concentrations in the mesolimbic system of freely moving rats. Proc. Natl. Acad. Sci. U.S.A. *85*: 5274–5278.

Fields, H.L., and Margolis, E.B. (2015). Understanding opioid reward. Trends Neurosci. *38*: 217–225.

Groer, C.E., Tidgewell, K., Moyer, R.A., Harding, W.W., Rothman, R.B., Prisinzano, T.E., et al. (2007). An Opioid Agonist that Does Not Induce μ-Opioid Receptor—Arrestin Interactions or Receptor Internalization. Mol Pharmacol *71*: 549–557.

Harding, S. D., Sharman, J. L., Faccenda, E., Southan, C., Pawson, A. J., Ireland, S., et al. (2018). The IUPHAR/BPS guide to PHARMACOLOGY in 2018: Updates and expansion to encompass the new guide to IMMUNOPHARMACOLOGY. Nucl Acids Res *46*: D1091–D1106.

Hill, R., Disney, A., Conibear, A., Sutcliffe, K., Dewey, W., Husbands, S., et al. (2018). The novel μ-opioid receptor agonist PZM21 depresses respiration and induces tolerance to antinociception. Br. J. Pharmacol. *175*: 2653–2661.

Hnasko, T.S., Sotak, B.N., and Palmiter, R.D. (2005). Morphine reward in dopamine-deficient mice. Nature *438*: 854–857.

Kilkenny, C., Browne, W., Cuthill, I. C., Emerson, M., and Altman, D. G. (2010). Animal research: Reporting in vivo experiments: The ARRIVE guidelines. British Journal of Pharmacology *160*: 1577–1579.

Kliewer, A., Schmiedel, F., Sianati, S., Bailey, A., Bateman, J.T., Levitt, E.S., et al. (2019). Phosphorylation-deficient G-protein-biased μ-opioid receptors improve analgesia and diminish tolerance but worsen opioid side effects. Nat Commun *10*:.

Koch, T., and Höllt, V. (2008). Role of receptor internalization in opioid tolerance and dependence. Pharmacol. Ther. *117*: 199–206.

Kuhar, J.R., Bedini, A., Melief, E.J., Chiu, Y.-C., Striegel, H.N., and Chavkin, C. (2015). Mu opioid receptor stimulation activates c-Jun N-terminal kinase 2 by distinct arrestin-dependent and independent mechanisms. Cell. Signal. *27*: 1799–1806.

Madariaga-Mazón, A., Marmolejo-Valencia, A.F., Li, Y., Toll, L., Houghten, R.A., and Martinez-Mayorga, K. (2017). Mu-Opioid receptor biased ligands: A safer and painless discovery of analgesics? Drug Discov. Today *22*: 1719–1729.

Maillet, E.L., Milon, N., Heghinian, M.D., Fishback, J., Schürer, S.C., Garamszegi, N., et al. (2015). Noribogaine is a G-protein biased κ-opioid receptor agonist. Neuropharmacology *99*: 675–688.

Major, C.T., and Pleuvry, B.J. (1971). Effects of α-methyl-p-tyrosine, p-chlorophenylalanine, L-β-(3,4-dihydroxyphenyl)alanine, 5-hydroxytryptophan and diethyldithiocarbamate on the analgesic activity of morphine and methylamphetamine in the mouse. Br J Pharmacol *42*: 512–521.

Manglik, A., Lin, H., Aryal, D.K., McCorvy, J.D., Dengler, D., Corder, G., et al. (2016). Structure–based discovery of opioid analgesics with reduced side effects. Nature *537*: 185–190.

Marcus, D.J., Zee, M., Hughes, A., Yuill, M.B., Hohmann, A.G., Mackie, K., et al. (2015). Tolerance to the antinociceptive effects of chronic morphine requires c-Jun N-terminal kinase. Mol Pain *11*:.

Marie, N., Canestrelli, C., and Noble, F. (2018). Role of pharmacokinetic and pharmacodynamic parameters in neuroadaptations induced by drugs of abuse, with a focus on opioids and psychostimulants. Neuroscience & Biobehavioral Reviews.

Mori, T., Kuzumaki, N., Arima, T., Narita, M., Tateishi, R., Kondo, T., et al. (2017). Usefulness for the combination of G-protein- and β-arrestin-biased ligands of μ-opioid receptors: Prevention of antinociceptive tolerance. Mol Pain *13*: 1744806917740030.

Murphy, N.P., Lam, H.A., and Maidment, N.T. (2001). A comparison of morphine-induced locomotor activity and mesolimbic dopamine release in C57BL6, 129Sv and DBA2 mice. J. Neurochem. *79*: 626–635.

Navailles, S., and De Deurwaerdère, P. (2011). Presynaptic control of serotonin on striatal dopamine function. Psychopharmacology (Berl.) *213*: 213–242.

Nielsen, S., Larance, B., Degenhardt, L., Gowing, L., Kehler, C., and Lintzeris, N. (2016). Opioid agonist treatment for pharmaceutical opioid dependent people. Cochrane Database Syst Rev CD011117.

Pettit, H.O., Ettenberg, A., Bloom, F.E., and Koob, G.F. (1984). Destruction of dopamine in the nucleus accumbens selectively attenuates cocaine but not heroin self-administration in rats. Psychopharmacology (Berl.) *84*: 167–173.

Przewlocka, B., Sieja, A., Starowicz, K., Maj, M., Bilecki, W., and Przewlocki, R. (2002). Knockdown of spinal opioid receptors by antisense targeting beta-arrestin reduces morphine tolerance and allodynia in rat. Neurosci. Lett. *325*: 107–110.

Raehal, K.M., Walker, J.K.L., and Bohn, L.M. (2005). Morphine side effects in beta-arrestin 2 knockout mice. J. Pharmacol. Exp. Ther. *314*: 1195–1201.

Robinson, S.A., Erickson, R.L., Browne, C.A., and Lucki, I. (2017). A role for the mu opioid receptor in the antidepressant effects of buprenorphine. Behav Brain Res *319*: 96–103.

Rojewska, E., Popiolek-Barczyk, K., Jurga, A.M., Makuch, W., Przewlocka, B., and Mika, J. (2014). Involvement of pro- and antinociceptive factors in minocycline analgesia in rat neuropathic pain model. Journal of Neuroimmunology *277*: 57–66.

Rothman, R.B., Blough, B.E., and Baumann, M.H. (2008). Dual dopamine/serotonin releasers: potential treatment agents for stimulant addiction. Exp Clin Psychopharmacol *16*: 458–474.

Saito, H. (1990). Inhibitory and stimulatory effects of morphine on locomotor activity in mice: biochemical and behavioral studies. Pharmacol. Biochem. Behav. *35*: 231–235.

Schmid, C.L., Kennedy, N.M., Ross, N.C., Lovell, K.M., Yue, Z., Morgenweck, J., et al. (2017). Bias Factor and Therapeutic Window Correlate to Predict Safer Opioid Analgesics. Cell *171*: 1165-1175.e13.

Soergel, D.G., Subach, R.A., Burnham, N., Lark, M.W., James, I.E., Sadler, B.M., et al. (2014). Biased agonism of the μ-opioid receptor by TRV130 increases analgesia and reduces on-target adverse effects versus morphine: A randomized, double-blind, placebo-controlled, crossover study in healthy volunteers. Pain *155*: 1829–1835.

Solecki, W., Wickham, R.J., Behrens, S., Wang, J., Zwerling, B., Mason, G.F., et al. (2013). Differential role of ventral tegmental area acetylcholine and N-methyl-D-aspartate receptors in cocaine-seeking. Neuropharmacology *75*: 9–18.

Solecki, W.B., Szklarczyk, K., Pradel, K., Kwiatkowska, K., Dobrzański, G., and Przewłocki, R. (2018). Noradrenergic signaling in the VTA modulates cocaine craving. Addict Biol *23*: 596–609.

Spanagel, R., Herz, A., and Shippenberg, T.S. (1990). The effects of opioid peptides on dopamine release in the nucleus accumbens: an in vivo microdialysis study. J. Neurochem. *55*: 1734–1740.

Stevens, C.W., and Yaksh, T.L. (1989). Potency of infused spinal antinociceptive agents is inversely related to magnitude of tolerance after continuous infusion. J. Pharmacol. Exp. Ther. *250*: 1–8.

Tao, P.-L., Liang, K.-W., Sung, W.-Y., Wu, Y.-T., and Huang, E.Y.-K. (2006). Nalbuphine is effective in decreasing the rewarding effect induced by morphine in rats. Drug Alcohol Depend *84*: 175–181.

Trescot, A.M., Datta, S., Lee, M., and Hansen, H. (2008). Opioid pharmacology. Pain Physician *11*: S133-153.

Webster, L., St Marie, B., McCarberg, B., Passik, S.D., Panchal, S.J., and Voth, E. (2011). Current status and evolving role of abuse-deterrent opioids in managing patients with chronic pain. J Opioid Manag *7*: 235–245.

Yaksh, T.L., and Rudy, T.A. (1976). Chronic catheterization of the spinal subarachnoid space. Physiology & Behavior *17*: 1031–1036.

Yang, C.-H., Huang, H.-W., Chen, K.-H., Chen, Y.-S., Sheen-Chen, S.-M., and Lin, C.-R. (2011). Antinociceptive potentiation and attenuation of tolerance by intrathecal β-arrestin 2 small interfering RNA in rats. Br J Anaesth *107*: 774–781.

Yudin, Y., and Rohacs, T. (2019). The G protein-biased agents PZM21 and TRV130 are partial agonists of μ-opioid receptor-mediated signaling to ion channels. Br. J. Pharmacol.

Yuill, M.B., Zee, M.L., Marcus, D., and Morgan, D.J. (2016). Tolerance to the antinociceptive and hypothermic effects of morphine is mediated by multiple isoforms of c-Jun N-terminal kinase. Neuroreport *27*: 392–396.

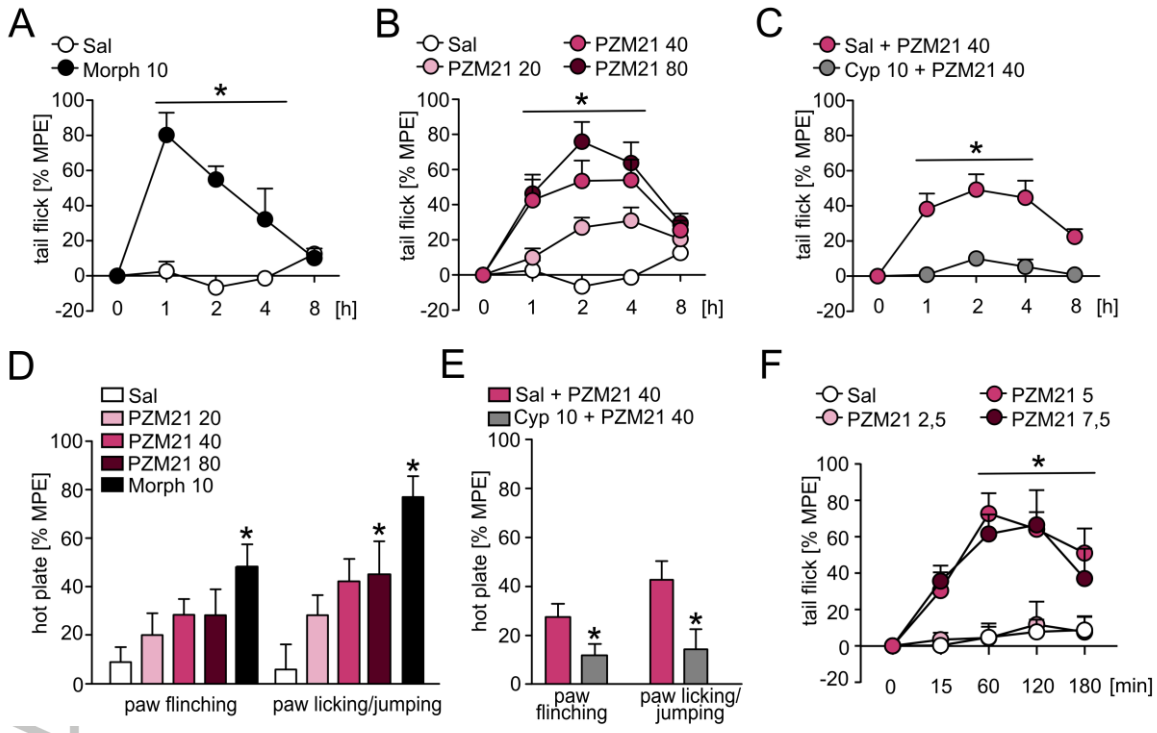

Fig. 1 Effects of PZM21 on acute thermal antinociception. [**A**] Administration of morphine (10 mg·kg^{-1}, i.p.), used as a positive control, resulted in an attenuated sensitivity to painful stimulus in the tail flick test. [**B**] Treatment with PZM21 (20, 40 and 80 mg·kg^{-1}, i.p.) caused dose-dependent antinociceptive effect measured in the tail flick test. When compared to saline, the antinociceptive effect of 20 mg·kg^{-1} of PZM21 was statistically significant 2 and 4 hours after the drug administration, while treatment with doses of 40 and 80 mg·kg^{-1} of the compound induced antinociception which lasted from 1 to 4 hours after the treatment. Morphine and PZM21 groups are compared to the same saline controls. [**C**] A selective μ-OR antagonist, cyprodime (10 mg·kg^{-1}, i.p.), administered 15 min prior to PZM21 (40 mg·kg^{-1}), prevented antinociception in the tail flick test. [**D**] PZM21 had no influence on the paw flinching reaction in the hot plate test. However, at a dose of 80 mg·kg^{-1}, it increased the latency to paw licking/jumping behaviour. Treatment with morphine significantly attenuated both types of reactions. Both responses were measured 90 min after drug administration. [**D**] Pretreatment with cyprodime attenuated the effects of 40 mg·kg^{-1} of PZM21 on both types of reaction in the hot plate test. [**E**] Intrathecal administration of PZM21 (at doses of 5 and 7.5 μg) caused

antinociceptive effect in tail flick test in rats. Data are presented as the mean ± SEM. Statistically significant effects are marked with: * $p<0.05$. **A-E:** PZM21-treated groups compared to appropriate controls. Sal – saline, Cyp – cyprodime, MPE – maximum possible effect. Numbers of animals used in experiments presented in **Table S5.**

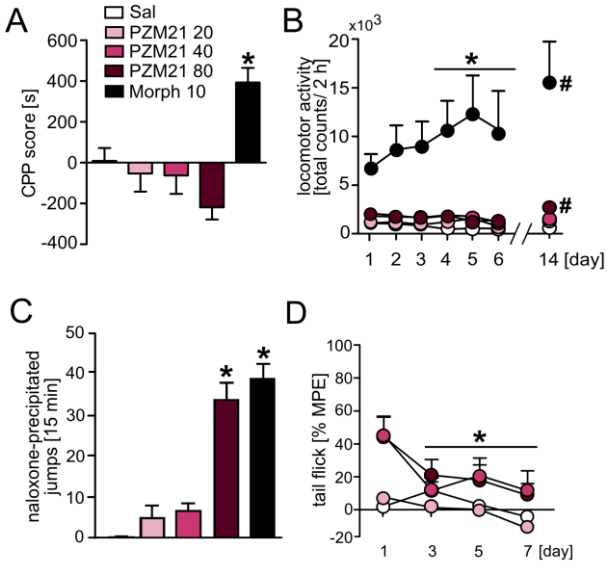

Fig. 2 Influence of PZM21 on addiction-like behaviour in mice. [**A**] In contrast to morphine (10 mg·kg^{-1}, i.p.), PZM21 (20, 40, 80 mg·kg^{-1}, i.p.) did not induce a preference toward drug-associated compartment in a CPP test at any of the tested doses. [**B**] Repeated treatment with morphine induced locomotor sensitization and expression, whereas that effect was not observed after PZM21 administration. Mice treated with 80 mg·kg^{-1} PZM21 presented a slight expression of sensitization after an 8-day incubation period. [**C**] Chronic administration of PZM21 (80 mg·kg^{-1}, but not 20 or 40 mg·kg^{-1}) as well as morphine induced naloxone-precipitated jumps, considered as a physical sign of withdrawal. [**D**] Repeated treatment with 40 and 80 mg·kg^{-1} PZM21 resulted in a decrease of antinociceptive efficacy of the compound. Tolerance was assessed using tail flick test performed on each experimental day, 1 hour after the drug administration. Data are presented as the mean ± SEM. Statistically significant effects are marked with: */# $p<0.05$. **A, C:** PZM21-treated groups compared to saline controls, **B, D**: within group effects compared to the first day of experiment are marked with *, expression of locomotor sensitization within groups compared to the last day of sensitization development are marked with #. Sal – saline, Morph – morphine, CPP – conditioned place preference, MPE – maximum possible effect. Numbers of animals used in experiments presented in **Table S5.**

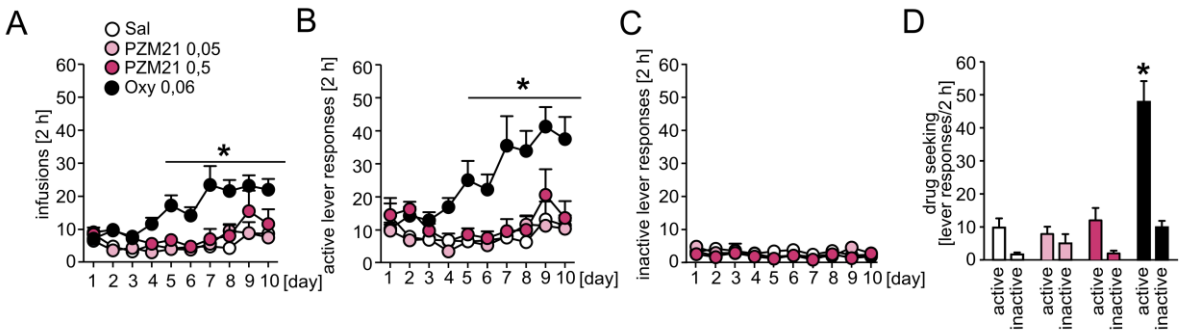

Fig. 3 Evaluation of PZM21 effects on intravenous self-administration in rats. **[A]** Rats that self-administered oxycodone (0.06 mg·kg^{-1} per infusion, i.v.), but not PZM21 (0.05 and 0.5 mg·kg^{-1} per infusion, i.v.), presented an increasing number of infusions over time. **[B]** Only rats from the oxycodone group presented an increasing number of active lever responses. [**C**] No differences between groups were observed in inactive lever presses during self-administration training. [**D**] Unlike the oxycodone group, rats in the saline and PZM21 groups did not present drug-seeking behaviour after abstinence period, as they did not discriminate between active and inactive levers and made a similar number of responses on both levers. Data are presented as the mean ± SEM. Statistically significant effects are marked with: * $p<0.05$. **A**: within group effects compared to the first day of experiment, **B-D**: comparison between active and inactive lever responses within experimental groups. Sal – saline, Oxy – Oxycodone. Numbers of animals used in experiments presented in **Table S5**.

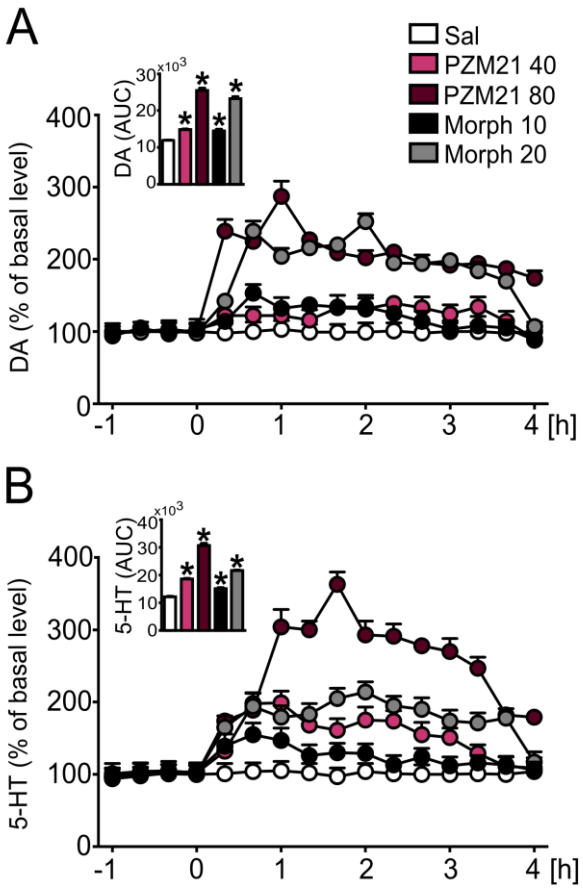

Fig. 4 Effects of PZM21 on striatal DA and 5-HT levels. [**A**] Administration of 40 and 80 mg·kg^{-1} PZM21 (i.p.) as well as 10 and 20 mg·kg^{-1} of morphine (i.p.) increased extracellular level of DA in the striatum. Basal extracellular levels were 3.71±0.51 pg in a volume of 10 µl (n=30). [**B**] All doses of PZM21 and morphine potentiated striatal 5-HT release when compared to saline. Basal extracellular levels were 0.40±0.06 pg in a volume of 10 µl (n=30). Data are presented as the mean ± SEM. Bar graphs presenting cumulative data are expressed as AUC. Statistically significant effects of each treatment compared to saline are marked with: * $p<0.05$. Sal – saline, Morph – morphine, AUC – area under the curve, DA-dopamine, 5-HT-serotonin. Numbers of animals used in experiments presented in **Table S5.**

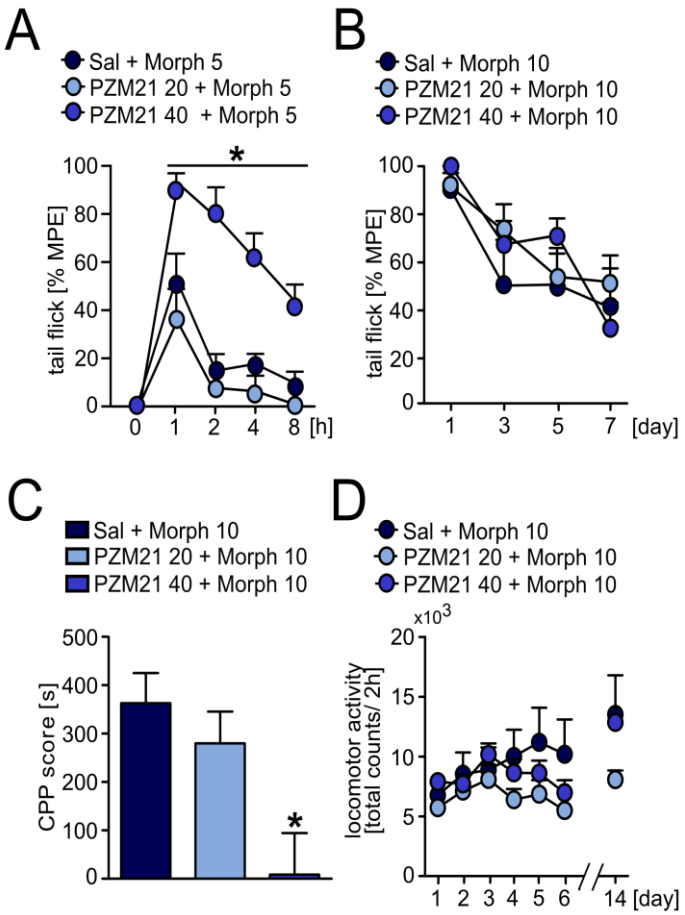

Fig. 5 Influence of PZM21 on behavioural effects of morphine. [**A**] PZM21 (at dose of 40 mg·kg^{-1}, i.p.), administered 30 min prior to morphine, enhanced antinociception evoked by 5 mg·kg^{-1} of morphine (i.p.) in the tail flick test. [**B**] Pretreatment with PZM21 had no effect on the development of tolerance to antinociception induced by 10 mg·kg^{-1} of morphine. Tolerance was assessed using tail flick test performed on each experimental day, 1 hour after the drug administration. [**C**] Preadministration of PZM21 at a dose of 40 mg·kg^{-1}, but not 20 mg·kg^{-1}, prevented the formation of conditioned response to morphine (10 mg·kg^{-1}). [**D**] Pretreatment with PZM21 resulted in a tendency toward reduced development, but not expression, of locomotor sensitization induced by repeated administration of morphine (10 mg·kg^{-1}). Data are presented as the mean ± SEM. Statistically significant effects are marked with * $p<0.05$. **A**–**D**: experimental groups compared to morphine controls. Sal – saline, Morph – morphine, MPE – maximum possible effect, CPP – conditioned place preference. Numbers of animals used in experiments presented in **Table S5.**