The novel μ-opioid receptor agonist PZM21 depresses respiration and induces tolerance to antinociception

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BACKGROUND AND PURPOSE
PZM21 is a novel μ-opioid receptor ligand that has been reported to induce minimal arrestin recruitment and be devoid of the respiratory depressant effects characteristic of classical μ receptor ligands such as morphine. We have re-examined the signalling profile of PZM21 and its ability to depress respiration.

EXPERIMENTAL APPROACH
G protein (G_i) activation and arrestin-3 translocation were measured in vitro, using BRET assays, in HEK 293 cells expressing μ receptors. Respiration (rate and tidal volume) was measured in awake, freely moving mice by whole-body plethysmography, and antinociception was measured by the hot plate test.

KEY RESULTS
PZM21 (10⁻⁹ – 3 x 10⁻⁵ M) produced concentration-dependent G_i activation and arrestin-3 translocation. Comparison with responses evoked by morphine and DAMGO revealed that PZM21 was a low efficacy agonist in both signalling assays. PZM21 (10–80 mg·kg⁻¹) depressed respiration in a dose-dependent manner. The respiratory depression was due to a decrease in the rate of breathing not a decrease in tidal volume. On repeated daily administration of PZM21 (twice daily doses of 40 mg·kg⁻¹), complete tolerance developed to the antinociceptive effect of PZM21 over 3 days but no tolerance developed to its respiratory depressant effect.

CONCLUSION AND IMPLICATIONS
These data demonstrate that PZM21 is a low efficacy μ receptor agonist for both G protein and arrestin signalling. Contrary to a previous report, PZM21 depresses respiration in a manner similar to morphine, the classical opioid receptor agonist.

Abbreviations
DAMGO, D-Ala²,N-MePhe⁴, Gly-ol-enkephalin; GIRK, G protein-activated inwardly rectifying potassium channel; MPE, maximum possible effect; MV, minute volume; PZM21, 1-[(2S)-2-(dimethylamino)-3-(4-hydroxyphenyl)propyl]-3-[(2S)-1-(thiophen-3-yl)propan-2-yl]urea; Rluc, Renilla luciferase

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Introduction

Opioid-induced respiratory depression is the primary cause of death following illicit or prescription opioid misuse. Fatal opioid overdose accounted for over 140 deaths a day in the USA in 2016 (National Institutes of Health). In a significant proportion of these deaths, a prescription opioid or illicitly synthesized opioid, rather than heroin, was involved. The development of an opioid agonist with appreciable potency as an analgesic, but lacking the ability to induce respiratory depression, would be highly desirable for clinical application and to reduce opioid-related deaths.

Current therapeutically useful opioid ligands such as morphine produce their analgesic and respiratory depressant effects through activation of the μ-opioid receptor (μ receptor) (Matthes et al., 1996). The μ receptor is a GPCR that signals through activation of G\textsubscript{i}/G\textsubscript{\gamma} proteins and through arrestin signalling (Williams et al., 2013). In recent years, the concept of biased ligands for GPCRs has been developed (Kelly, 2013; Kenakin and Christopoulos, 2013; Thompson et al., 2015; Smith et al., 2018). It is now well established that agonists acting at the same orthosteric site on a GPCR may activate different signalling pathways, that is, be biased for activating G protein or arrestin signalling. The idea that a G protein-biased μ receptor agonist would have a reduced ‘on target’ side effect profile arises from the observation that in arrestin-3 (β-arrestin 2) knockout mice, morphine, the prototypical opioid agonist, showed a reduced propensity to produce respiratory depression and constipation (Rahal et al., 2005) implying that respiratory depression resulted from μ receptor coupling to arrestin-mediated signalling.

Manglik et al. (2016) reported the development of a novel μ receptor agonist, PZM21, structurally unrelated to prototypical μ receptor agonists such as morphine, which appeared to have some selectivity for G protein activation over arrestin translocation. They also reported that PZM21 had a greatly reduced propensity to produce respiratory depression. We have synthesized PZM21 and have re-examined its selectivity profile for G\textsubscript{i} activation over arrestin-3 recruitment and its ability to depress respiration in mice. We observed that PZM21 was a low efficacy agonist for both G\textsubscript{i} activation and arrestin recruitment. In contrast to Manglik et al. (2016), we observed that PZM21 significantly depressed respiration, in a manner similar to an equi-antinociceptive dose of morphine.

Methods

G\textsubscript{i} activation and arrestin-3 translocation assays. HEK 293 cells were cultured at 37°C in DMEM supplemented with 10% fetal bovine serum and penicillin/streptomycin. Cells were seeded onto 10 cm dishes and grown to 80% confluence before transfection. To determine the relative ability of the agonists to activate G\textsubscript{i} G proteins, a BRET\textsuperscript{2}-based assay that monitors the separation of Ga\textsubscript{11} and G\textsubscript{T2} was used. HEK 293 cells were transiently transfected with rat HA-μ receptors, Ga\textsubscript{11}-Renilla luciferase II (RlucII) and GFP10-G\textsubscript{T2}. Immediately prior to each assay, cells were resuspended in clear DMEM and then transferred to a 96-well plate at 90 μl per well. Measurements of BRET were made at 37°C. Coelenterazine 400a, at a final concentration of 5 μM, was injected 5 s prior to reading the cell plate. BRET measurements were made on a FLUOstar Omega plate reader (BMG LABTECH, Ortenberg, Germany) using the following filter set: acceptor, 515 ± 30 nm; and donor, 410 ± 80 nm filters. BRET signals were determined as the ratio of the light emitted by acceptors (GFP10) over donor (RlucII). For G\textsubscript{i} activation, BRET measurements were taken 2 min after agonist application. Agonist stimulation resulted in a rapid decrease in the BRET signal between Ga\textsubscript{11}-RlucII and GFP10-G\textsubscript{T2} in cells co-expressing the HA-tagged μ receptors. To determine the extent of agonist-induced arrestin-3 association, cells were co-transfected with human μ receptor-Rluc and arrestin-3-GFP. The extent of arrestin-3 recruitment to the receptor was assessed following a 10 min incubation with the agonists.

Animals. All animal care and experimental procedures were performed in accordance with the UK Animals (Scientific Procedures) Act 1986, the European Communities Council Directive (2010/63/EU) and the University of Bristol ethical review document. Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny et al., 2010; McGrath and Lilley, 2015). Male C57BL and CD-1 mice (Harlan Laboratories, Biester, UK) weighing approximately 30 g were group housed, four to six per cage, in an environment maintained at 22°C, on a reversed 12 h dark–light cycle with food and water available ad libitum. Experiments were performed in the dark phase to ensure that they were in their most active phase. Animals were randomly ascribed to treatment groups with the experimenter blinded to drug treatment until after subsequent data analyses had been performed. A total of 180 mice were used in the study.

Mouse respiration. We have used both C57BL mice, the strain used by Manglik et al. (2016), and CD-1 mice, the strain we have used in previous studies of opioid depression of respiration (Hill et al., 2016; Lyndon et al., 2017; Withey et al., 2017) to ensure that any responses observed were not strain-specific. Respiration was measured in freely moving mice using plethysmography chambers (EMKA Technologies, Paris, France) supplied with either air or a 5% CO\textsubscript{2} in air mixture (BOC Gas Supplies, Manchester, UK) as described previously (Hill et al., 2016). Rate and volume of respiration were recorded and averaged over 5 min periods. Breathing 5% CO\textsubscript{2} in air increases minute volume (MV) but does not induce stress in mice (Hill et al., 2016).

Data are presented both as MV and as percentage change from the pre-drug MV baseline, calculated for each mouse individually before mean data were plotted. Presenting data as percentage change from the pre-drug levels has been done to control for variation between treatment groups that may have different baseline levels of respiration. In our experience, variations in baseline respiration levels do not
influence the extent of opioid depression of respiration (Hill and Henderson, unpublished data).

**Measurement of nociception.** Male mice (CD-1) were placed on a hot plate at 52.5°C, and the latency to exhibit paw withdrawal, jumping or paw licking was measured. A maximum cut-off time of 30 s was used to prevent tissue damage. Hot plate latency was measured every 15 min. Antinociception was quantified as the percentage of maximum possible effect (% MPE), which was calculated as follows:

\[
\% \text{MPE} = \left( \frac{\text{test latency} - \text{control latency}}{30 - \text{control latency}} \right) \times 100.
\]

**Induction of tolerance.** Male mice (CD-1) received twice daily injections (at 09:00 and 21:00 h each day) of PZM21, morphine or saline at 12 h intervals for 5 days. Two separate sets of experiments were performed, one to measure tolerance to antinociception and the other to measure tolerance to respiratory depression. Each day, hot plate latency or respiration was monitored for 60 min after the first drug administration of the day.

**Experimental design and data analysis**

**In vitro experiments.** The maximum responses for PZM21, DAMGO and morphine in both Gi activation and arrestin-3 recruitment were compared using one-way ANOVA with Bonferroni’s post test.

**In vivo experiments.** Data from previous experiments where respiratory depression or antinociception was measured following acute opioid administration in naïve mice were subjected to post hoc power analyses using G*Power (version 3.1.9). Our calculations indicated that \( n = 6 \) (respiration experiments) or \( n = 8 \) (antinociception experiments) for each individual group would produce a significant result if an actual effect occurred.

Differences between drug treatments and saline controls over time were analysed using two-way ANOVA with Bonferroni’s post test. Changes in groups over time with repeated measurements were analysed using two-way repeated measures ANOVA with Bonferroni’s post test to analyse drug effect over time. Statistical significance and F (dfn, dfd) values are given in the figure legends. GraphPad Prism 7 was used for all statistical analyses. All data are displayed as mean ± SEM. The data and statistical analyses comply with the recommendations on experimental design and analysis in pharmacology (Curtis et al., 2015).

**Materials.** PZM21 hydrochloride was synthesized from enantiopure (S)-1-(thiophen-3-yl)propan-2-amine and enantiopure (S)-4-(3-amino-2-(dimethylamino)propyl) phenol (both supplied by Astatech, Bristol, Pennsylvania). The synthesis followed the methods reported by Manglik et al. (2016). Analysis by NMR, mass spectrometry and HPLC confirmed identity and purity of the sample. Reverse phase HPLC (Kinetix C18 column) and chiral analysis (Chiralpak AS column) demonstrated it to be identical to a sample of S,S PZM21 formate kindly provided by Dr Gmeiner (Friedrich-Alexander-Universität, Erlangen-Nürnberg, Germany) and confirmed >98% of the desired isomer (S,S PZM21). Data using both batches of PZM21 (i.e. those synthesized at the University of Bath and at Friedrich-Alexander-Universität) are reported in this paper.

DAMGO (Tocris Bioscience, Avonmouth, UK), morphine hydrochloride (Macfarlan Smith, Edinburgh, UK), naloxone hydrochloride (Sigma-Aldrich, Dorset, UK) and PZM21 hydrochloride were dissolved in deionized water (in vitro experiments) or sterile saline (in vivo experiments). PZM21 formate was dissolved in saline/1% DMSO. All doses of drug were administered in 0.1 mL vehicle.

**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**

**μ receptor signalling**

In HEK 293 cells expressing μ receptors, PZM21 (10^{-9} – 3 \times 10^{-3} M) produced concentration-dependent Gi activation (Figure 1A and Table 1). We compared the responses evoked by PZM21 to those evoked by two well-characterized μ receptor ligands, DAMGO (high efficacy agonist) and morphine (lower efficacy agonist) in the same assay. Examination of the maximum response produced by each agonist (an indication of agonist efficacy) (Table 1) revealed that under our assay conditions, PZM21 and morphine were partial agonists for Gi activation with significantly lower efficacy than DAMGO.

In the arrestin-3 recruitment assay PZM21 (10^{-9} – 3 \times 10^{-3} M) produced a small but measurable amount of arrestin recruitment (Figure 1B). The maximum responses to PZM21 and morphine were lower than that of DAMGO whereas PZM21 and morphine were not significantly different.

**Acute antinociception and respiratory depression**

In the mouse (CD-1) hot plate assay PZM21 (40 mg·kg\(^{-1}\) i.p.) increased latency for nociceptive behaviour for the duration of the 60 min observation period following drug administration (Figure 2A). The peak antinociceptive response induced by PZM21 (40 mg·kg\(^{-1}\) i.p.) was similar to that induced by morphine (10 mg·kg\(^{-1}\) i.p.) when compared by two-way ANOVA with Bonferroni’s comparison \( [F = 2.65 (dfn = 1, dfd = 98) \quad P > 0.05] \) (Figures 2A and 5A).

In contrast to Manglik et al. (2016), we observed that in C57/BL mice breathing air PZM21 (40 mg·kg\(^{-1}\) injected s.c.) caused significant respiratory depression (Figure 2B) that was rapid in onset, the peak effect being attained 10–15 min after injecting the drug, and persisted for the duration of the 30 min post injection observation period.
The decrease in MV caused by 40 mg·kg\(^{-1}\) PZM21 was similar in amplitude to that observed following administration of 10 mg·kg\(^{-1}\) morphine (Figure 2B). To confirm that the respiratory depressant effect of PZM21 that we had observed was not due to the presence of any impurity, we obtained a sample of PZM21 from Dr Peter Gmeiner who synthesized the PZM21 used in the Manglik et al. (2016) study; we observed that 40 mg·kg\(^{-1}\) (i.p.) of this sample of PZM21 also depressed respiration (Figure 2C).

In mice breathing air, respiration gradually declines with time (see data for saline or saline/1% DMSO-treated mice in Figure 2B, C and for vehicle-injected mice in figure 4g in Manglik et al., 2016); animals also sometimes curl up 'asleep' which interferes with plethysmograph recording. To circumvent this, we performed experiments in which mice breathe 5% CO\(_2\) in air (see Hill et al., 2016; Withey et al., 2017). This increases MV, which in saline-treated mice remains constant for periods of up to 90 min (Figure 2D). In CD-1 mice breathing 5% CO\(_2\) in air, the respiratory depression induced by

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**Figure 1**

Opioid-induced Gi protein activation and arrestin-3 translocation in HEK 293 cells expressing recombinant \(\mu\) receptors. (A) DAMGO, morphine and PZM21 induced concentration-dependent activation of Gi, measured as the decrease in BRET signal. (B) DAMGO, morphine and PZM21 induced concentration-dependent arrestin-3 translocation measured as the increase in BRET signal. Values of EC\(_{50}\) and maximum response are recorded in Table 1. Data shown are means ± SEM, \(N = 5\) for each drug in both assays.
PZM21 (10–40 mg·kg\textsuperscript{-1} i.p.) was dose-dependent and was maintained over the 60 min period following drug administration (Figure 2D, E). Over the range of doses tested, PZM21 was approximately fourfold less potent than morphine (Figure 2E).

The decrease in MV in response to PZM21 or morphine resulted from a decrease in respiratory rate rather than a decrease in tidal volume (Figure 3). Although the depth of respiration was reduced, tidal volume was maintained because the duration of inspiration was prolonged by an apneustic compensation (see also Hill \textit{et al.}, 2016).

Administration of naloxone (1 mg·kg\textsuperscript{-1} i.p.) along with PZM21 (40 mg·kg\textsuperscript{-1} i.p.) prevented the respiratory depression induced by PZM21 (Figure 4A), demonstrating that this effect of PZM21 was mediated by opioid receptor activation. However, when the dose of PZM21 was increased to 80 mg·kg\textsuperscript{-1} (i.p.), the degree of respiratory depression also increased, but in this instance, the effect of PZM21 was not fully reversed even by a higher dose of naloxone (3 mg·kg\textsuperscript{-1} i.p.) (Figure 4B). This indicates that at high doses, the respiratory depression by PZM21 may involve a non-opioid receptor-mediated component.
Differential development of tolerance to antinociception and respiratory depression by PZM21

We have previously used a twice daily injection of opioid to study the development of tolerance to the antinociceptive (tail flick test) and respiratory depressant effects of morphine and observed that tolerance developed to the antinociceptive action of morphine but not to the respiratory depressant action (Hill et al., 2016). Here, we utilized a similar twice daily opioid injection protocol in which mice were injected at 09:00 and 21:00 each day with PZM21 (40 mg·kg⁻¹ i.p.) or morphine (10 mg·kg⁻¹ i.p.) for up to 5 days.

Repeated injection of PZM21 or morphine resulted in the development of tolerance to their antinociceptive effects as measured in the hot plate assay (Figure 5A). By the fourth day of treatment, PZM21 and morphine did not induce any antinociception. In a separate group of mice, we observed that tolerance to the respiratory depressant effect of PZM21 did not develop even after 5 days of treatment (Figure 5B). For comparison with morphine, data showing lack of tolerance development to respiratory depression with repeated morphine dosing have been recalculated from Hill et al. (2016) and included in Figure 5B.

Discussion

The main finding of this study is that the structurally novel μ receptor agonist PZM21 induces marked respiratory depression, similar to morphine. This finding is at odds with a previous study where PZM21 was reported not to induce respiratory depression (Manglik et al., 2016). In addition to our findings with the sample of PZM21 that we synthesized, we also obtained a sample of PZM21 from the original investigators and still observed respiratory depression with that sample. We observed respiratory depression in both C57BL and CD-1 mice showing that the response is not strain specific. Furthermore, we observed respiratory depression when PZM21 was injected i.p. or s.c. and when animals breathed air or 5% CO₂ in air to stabilize respiration across the observation period. We did not observe depression of respiration when mice were injected with vehicle alone. This is in contrast to Manglik et al. (2016) who observed a large effect in vehicle-injected mice that may have obscured the respiratory depressant effect of PZM21.

We have also demonstrated that repeated administration of PZM21 induced tolerance to its antinociceptive effect, similar to that seen with morphine, but with this treatment schedule, PZM21 did not induce tolerance to respiratory depression, again as seen with morphine.

PZM21 is a low efficacy agonist for both G protein and arrestin signalling. The maximum response to PZM21 compared to that of DAMGO does appear to be lower for arrestin-3 translocation than for Gᵢ activation (compare Figures 1A, B). This could indicate that PZM21 exhibits some bias for Gᵢ activation over arrestin-3 translocation. However, with low efficacy agonists such as PZM21, the error in EC₅₀ values obtained from the concentration–response curves is large (see Table 1) which makes conclusions about bias difficult. The problems of quantifying bias with low efficacy
agonists and comparing bias between different cell types and assays are increasingly being acknowledged (Thompson et al., 2015; Winpenny et al., 2016; Michel and Charlton, 2018). It should be noted that in Manglik et al. (2016), the authors were unable to formally demonstrate that PZM21 was G protein-biased, even in the presence of overexpressed GRK2 to amplify the arrestin response.

In the present study, PZM21 did produce antinociception in the hot plate assay, but contrary to the previous report on this drug (Manglik et al., 2016), we also observed that PZM21 produced prolonged respiratory depression. PZM21 is therefore very similar to the prototypic opioid morphine, although in vivo it is less potent. While the current dogma is that, for μ receptor agonists, respiratory depression results from activation of arrestin signalling (see Chan et al., 2017; Madariaga-Mazón et al., 2017; Roth et al., 2017; Smith et al., 2018), there is evidence that signalling through G\textsubscript{i}/G\textsubscript{o} is also involved in mediating this behaviour. μ Receptor coupling through G\textsubscript{i}/G\textsubscript{o} proteins results in activation of G protein-activated inwardly rectifying potassium channels (GIRKs) and inhibition of voltage-gated calcium channels (North and Williams, 1985; Seward et al., 1991), and it has been reported that the respiratory depression induced by either DAMGO,
applied locally in the ventrolateral medulla, or fentanyl, administered systemically, is attenuated in GIRK2 subunit knockout mice (Montandon et al., 2016). The effect of DAMGO on respiration was also reduced in wild-type mice by local administration of Tertiapin-Q, a blocker of GIRK channels. This would imply that respiratory depression involves \( \mu \) receptors coupling to GIRK channels through activation of \( G_\text{i}/G_\text{o} \) proteins. Furthermore, the role of \( G_\text{i}/G_\text{o} \)-coupling to voltage-dependent calcium channels in respiratory depression has not been evaluated. On the other hand, morphine-induced respiratory depression was reported to be reduced in arrestin-3 knockout mice (Raehal et al., 2005), while Schmid et al. (2017) have recently described several novel \( G \) protein-biased \( \mu \) receptor ligands that appear to induce less respiratory depression than morphine at equi-antinociceptive doses. Nevertheless, our present results with PZM21 question the assumption that arrestin-dependent signalling mediates the respiratory depression observed with \( \mu \) receptor agonists.

We observed that on repeated administration, tolerance developed to the antinociceptive effect of PZM21, as has previously been widely reported with morphine (see references in Williams et al., 2013). This finding suggests that the evasion of tolerance to the analgesic effects, a suggested benefit of \( G \) protein-prefering \( \mu \) receptor agonists (Smith et al., 2018), is not achieved and that instead such agonists remain able to trigger signalling mechanisms that lead to profound tolerance. On the other hand, with twice daily injections tolerance did not develop to the respiratory depressant effect of PZM21. We have previously reported similar differential tolerance development to morphine (Hill et al., 2016). With morphine, more sustained drug delivery using subcutaneous pellet or osmotic mini-pump implantation did result in the development of tolerance to respiratory depression but at a slower rate than tolerance to antinociception (Hill et al., 2016; Withey et al., 2017). Unfortunately, we had insufficient supplies of PZM21 to perform further experiments with osmotic mini-pump delivery. The molecular mechanisms underlying the development of tolerance to \( \mu \) receptor agonists, particularly lower efficacy agonists that have lower propensity for arrestin recruitment, are complex and contentious (see Williams et al., 2013). Future studies should address the mechanism(s) underlying tolerance induced by prolonged PZM21 treatment.

Conclusions

In conclusion, our data on respiratory depression are in direct contrast to the previously published observation by Manglik et al. (2016) that PZM21 did not cause respiratory depression in mice. Given that PZM21 did induce some arrestin-3 translocation as well as \( G_i \) activation, we are unable to conclude definitively that, for \( \mu \) receptor agonists, respiratory depression is mediated by \( G \) protein or arrestin signalling. The future identification of higher efficacy \( \mu \) receptor agonists showing greater bias for \( G \) protein over arrestin signalling remains a promising avenue for the development of novel opioid agonists and should answer this important question.

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Author contributions

R.H. performed the in vivo respiration and antinociception experiments, analysed the data and prepared the figures. A.D. and S.H. synthesized PZM21. A.C. and K.S. performed the in vitro BRET experiments and analysed the data. W.D., C.B., E.K. and G.H. conceived the study and wrote the paper. All authors revised 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 recommended by funding agencies, publishers and other organisations engaged with supporting research.

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