Novel Orally Available Salvinorin A Analog PR-38 Inhibits Gastrointestinal Motility and Reduces Abdominal Pain in Mouse Models Mimicking Irritable Bowel Syndrome


Abstract

The opioid and cannabinoid systems play a crucial role in multiple physiological processes in the central nervous system and in the periphery. Selective opioid as well as cannabinoid (CB) receptor agonists exert a potent inhibitory action on gastrointestinal (GI) motility and pain. In this study, we examined (in vitro and in vivo) whether PR-38 (2-O-cinnamoylsalvinorin B), a novel analog of salvinorin A, can interact with both systems and demonstrate therapeutic effects. We used mouse models of hypermotility, diarrhea, and abdominal pain. We also assessed the influence of PR-38 on the central nervous system by measurement of motoric parameters and exploratory behaviors in mice. Subsequently, we investigated the pharmacokinetics of PR-38 in mouse blood samples after intraperitoneal and oral administration. PR-38 significantly inhibited mouse colonic motility in vitro and in vivo. Administration of PR-38 significantly prolonged the whole GI transit time, and this effect was mediated by μ- and κ-opioid receptors and the CB1 receptor. PR-38 reversed hypermotility and reduced pain in mouse models mimicking functional GI disorders. These data expand our understanding of the interactions between opioid and cannabinoid systems and their functions in the GI tract. We also provide a novel framework for the development of future potential treatments of functional GI disorders.

Introduction

Salvinorin A (SA) is a natural diterpenoid, isolated from the Mexican plant Salvia divinorum (Fichna et al., 2009b). Extracts from S. divinorum have been used for centuries by the Mazatec Indians for rituals and for alleviating symptoms of gastrointestinal (GI) disorders, such as abdominal pain and diarrhea (Fichna et al., 2009a). However, potential clinical application of S. divinorum derived natural compounds is strongly limited due to possible adverse effects in the central nervous system (CNS). Recently, a short-lasting but strong hallucinogenic effect of SA has been confirmed in humans (Maclean et al., 2013).

A number of in vitro and in vivo studies in animal models showed that SA undeniably plays an important role in GI motility in physiological and pathophysiological conditions. Among others, our group demonstrated in vitro that SA inhibited electrically evoked twitch contractions of mouse stomach, ileum, and colon in a concentration-dependent manner (Fichna et al., 2009a). In all tissues studied, the effect of SA was blocked by the universal opioid antagonist naloxone and a selective κ-opioid receptor (KOR) antagonist, nor-binaltorphimine (nor-BNI), which was in good agreement with classic binding assays (Capasso et al., 2006). Additionally, in the ileum and the colon, the inhibitory effect of SA was blocked by selective antagonists of cannabinoid type 1 (CB1) [N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide, AM 251] and cannabinoid type 2 (CB2) [6-iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1H-indol-3-yl][4-methoxyphenyl]methanone (AM 630)] receptors. In the in vivo studies, it has been shown that SA slowed upper...
intestinal transit, as indicated by a lower geometric center (GC) of the upper GI tract (Fichna et al., 2009a) and colonic motility (Fichna et al., 2009a), and inhibited GI motility in croton oil–induced intestinal inflammation in mice (Capasso et al., 2008). Moreover, Guida et al. (2012) have shown that SA reduces mechanical allodynia induced by peripheral formalin injection in mice. It is noteworthy that SA also produced a potent antinoceptive effect in a mouse model of abdominal pain induced by mustard oil (MO) (Fichna et al., 2012).

Opioid receptors, in particular μ-opioid receptor (MOR) and KOR, and their ligands mediate several biologic functions and are attractive molecular targets in the development of therapeutics. In the CNS, opioid agonists regulate, among others, pain perception and antinociception, mood, cognitive function, and locomotor activity (Fichna et al., 2009a; Sobczak et al., 2014). In the periphery, opioid receptor agonists inhibit intestinal propulsive contractions and GI motility, mainly by decreasing pyloric tone and increasing absorption of fluids and electrolytes into the gut lumen, which results in their antidiarrheal and constipating effect (Fichna et al., 2009a; Sobczak et al., 2014). Recently, broader attention has been given to KORs and their ligands, which may become a target in the treatment of GI disorders, such as postoperative ileus, irritable bowel syndrome (IBS), and intestinal inflammation (Fichna et al., 2009a). Clinical trials with a peripherally acting KOR agonist, asimadoline, are particularly encouraging in patients with IBS (Delgado-Aros et al., 2003; Camilleri, 2008), as they proved that asimadoline decreases pain and improves abnormal bowel function.

In search of novel potential drugs for the treatment of IBS, the aforementioned reports on the action of SA in the GI tract encouraged us to develop a series of novel SA derivatives with potentially preserved therapeutic properties, improved kinetics, and that were deprived of any side effects, particularly in the CNS. One of the novel analogs, PR-38 (2-O-cinnamoylsalvinorin B; for structure, please see Supplemental Fig. 1), which has been synthesized from SA and MOR with Kᵢ values of 9.6 and 52 nM, respectively. Moreover, in the guanosine 5’–3’-(thio)triphosphate functional test, PR-38 was proved to be a potent mixed KOR and MOR agonist (unpublished results) and has therefore been selected for further studies.

In this study, we characterized the effect of PR-38 on mouse intestinal contractility in vitro and GI motility in animal models. Furthermore, we investigated the antidiarrheal and antinociceptive effect of PR-38. We also performed several tests that allowed us to investigate the action of PR-38 in the CNS to rule out its potential hallucinogenic, anxiogenic, or anxiolytic action. Moreover, by using specific opioid and CB antagonists, we examined the mechanism of action of PR-38. This characterization concludes into a preclinical study that clarifies the potential of PR-38 as a drug in the treatment of functional disorders of the human intestine.

Materials and Methods

Animals. Experimentally naive male albino Balb/c mice were obtained from the Animal House of the University of Lodz, Poland. All animals used in experiments weighed 22–30 g. The animals were housed at a constant temperature (22°C) and maintained under a 12-hour light/dark cycle (lights on 6:00 AM) in sawdust-lined plastic cages with access to chow pellets and tap water ad libitum. All animal protocols were in accordance with the European Communities Council Directive of November 24, 1986 (86/609/EEC) and Polish legislation acts concerning animal experimentation. The experimental protocol was approved by the Local Ethics Committee at the Medical University of Lodz (animal use approval no. 590/2012). All efforts were made to minimize animal suffering and to reduce the number of animals used.

In Vitro Organ Bath Studies. In vitro organ bath experiments were conducted according to the methodology described previously (Fichna et al., 2014). Mice were killed by cervical dislocation. Full-thickness segments (1 cm) of ileum and distal colon were removed and kept in ice-cold oxygenated Krebs-Ringer solution (115 mM NaCl, 8.0 mM KCl, 2.0 mM KH₂PO₄, 25 mM NaHCO₃, 2.4 mM MgCl₂, 1.3 mM CaCl₂, and 10 mM glucose). Luminal contents were gently flushed. All experiments lasted less than 3 hours, and each preparation was used for a single experiment only. Each segment was mounted between two platinum electrodes in organ baths containing Krebs (25 ml) equilibrated with 95% O₂ and 5% CO₂ at 37°C. One end of each preparation was attached to the bottom of the organ bath using a silk thread, whereas the other end was connected to an FT03 force displacement transducer (Grass Technologies, West Warwick, RI). A tension of 0.5 g was applied, and the preparations were allowed to equilibrate for 30 minutes. Changes in tension were amplified by a P11T amplifier (Grass Technologies) and recorded on a personal computer using the POLYVIEW software (Polybytes Inc., Cedar Rapids, IA). Tissue strips were subjected to electrical field stimulation applied by an S88X stimulator (electrical field stimulation (EFS)), 1.0 Hz, 60 V, pulse duration 0.5 ms, train duration 10 seconds; Grass Technologies), and delivered through electrodes placed around the tissue. EFS of isolated smooth muscle strips caused twitch contractions, which were virtually abolished by the muscarinic receptor antagonist atropine (10⁻⁶ M) or the neural blocker tetrodotoxin (10⁻⁶ M).

The contractile responses were characterized in the presence of increasing cumulative concentrations of SA and PR-38 (both 10⁻¹⁰ to 10⁻⁶ M), with a contact time for each concentration of 10 minutes. Before the addition of drugs, the mean amplitude of four successive twitch contractions was used as an internal control. Changes in contractions were reported as the percentage of the internal control. In control experiments, the effect of the vehicle was tested. To examine the involvement of opioid and CB receptors, specific receptor antagonists were added into the organ baths 15 minutes prior to tested compounds: β-funaltrexamine (β-FNA, 10⁻⁶ M, to block MOR), nor-BNI (10⁻⁶ M, to block KOR), and AM 251 (10⁻⁷ M, to block CB1 receptors).

Whole Gastrointestinal Transit. The whole gastrointestinal transit was conducted as described previously (Fichna et al., 2014). The whole gut transit test evaluates the time of passage of the nonabsorbable colored marker (150 μl of glutinous liquid, consisting of 5% Evans blue and 5% gum Arabic) through the GI tract. Vehicle, SA, or PR-38 [intraperitoneally and orally] was injected 15 minutes before marker administration. If the antagonist (β-FNA, nor-BNI, or AM 251) was used in the experiment, it was injected intraperitoneally 15 minutes before the administration of SA or PR-38. Immediately after the intragastric administration of the marker, mice were returned to individual cages, which were placed on a white sheet to facilitate recognition of colored boluses. Time elapsed between intragastric administration of the marker and excretion of the first colored fecal bolus was considered as the whole-gut transit time.

Colonic Bead Expulsion Test. Distal colonic expulsion was measured as reported recently (Sibaev et al., 2009). In brief, animals were fasted overnight; PR-38 or vehicle was administered (150 μl p.o. or 100 μl i.p.), and 15 minutes later, a prewarmed (37°C) glass bead (2 mm) was inserted into the distal colon (2 cm depth) using a silicone pusher. After insertion of the bead, mice were moved to individual cages, and the time to bead expulsion was measured. Mice that did not expel the bead within 30 minutes were killed to confirm the presence of the bead in the lumen of the colon.
Gastric Emptying and Geometric Center of Upper Gastrointestinal Transit. Gastric emptying (GE) and GC experiments were performed according to techniques described earlier (Smits and Lefeber, 1996; Capasso et al., 2005; Sibaev et al., 2009). In brief, after an overnight fasting period with free access to tap water, animals received a gavage of 200 μl of a marker solution (50 mg of phenol red in 100 ml of 1.5% methylcellulose, constantly stirred and held at 37°C). Twenty minutes after administration of the meal, mice were killed, and the stomach and the small intestine were carefully removed. The stomach was opened and its contents transferred to a tube containing 4 ml of distilled water. After 20 minutes of sedimentation, 1 ml of supernatant was transferred to another tube containing 1 ml of 1 M NaOH to intensify its color. The solutions were colorimetrically assayed with a spectrophotometer [iMark Microplate Reader; Bio-Rad Laboratories, Hercules, CA] at 560 nm. Gastric emptying (percentage) was calculated according to the following formula:

\[ GE = \frac{100 \times (1 - \frac{\text{amount of phenol red after 20 min}}{\text{amount of phenol red after 0 min}})}{\text{segment number}} \]

In the GE experiments, 20 minutes after gavage of a marker solution, the entire small intestine with its contents was isolated and divided into 10 segments of equal length. The intestinal motility of each bowel segment was vortexed with 2 ml of distilled water. After 20 minutes of sedimentation, 1 ml of supernatant was transferred to another tube containing 1 ml of 1 M NaOH to develop the maximum intensity of the color. The solutions were colorimetrically assayed with a spectrophotometer (iMark Microplate Reader; Bio-Rad Laboratories) at 560 nm. GC of small intestinal transit was calculated according to the following formula:

\[ GC = \sum \left(\frac{\% \text{ per segment} \times \text{segment number}}{\text{amount of phenol red after 20 min}}\right) \]

GC ranged from 1 (minimal motility) to 10 (maximal motility). In all GE and GC experiments, PR-38 or vehicle (intraperitoneally) was injected 15 minutes before the start of the experiment.

Upper Gastrointestinal Transit Test. The upper gastrointestinal tract was performed according to techniques described earlier (Kamysh et al., 2013). The upper GI transit was assessed using the following protocol. After an overnight fasting period with free access to tap water, the mice received 150 μl of charcoal marker (10% activated charcoal and 5% gum Arabic), gavaged into the stomach using an 18-gauge animal feeding tube. After 20 minutes, the mice were killed, and the small intestine was immediately removed. The distance from the pyloric sphincter to the front of the marker was measured and expressed as a percentage of the total length of the small intestine. PR-38 (or vehicle) was injected intraperitoneally 15 minutes prior to the gavage of the marker.

Mouse Model of Castor Oil-Induced Diarrhea. The model was used according to the methodology described earlier (Fichna et al., 2014). To induce diarrhea, animals (fasted for 12 hours before experiment) were gavaged with 200 μl of castor oil. The animals were then put into individual cages, which were placed over clean paper. Time elapsed between administration of castor oil and the appearance of symptoms of diarrhea (excretion of liquid feces) was measured and compared between groups. PR-38/SAL/OP (loperamide) (or vehicle) was administered intraperitoneally 15 minutes prior to the gavage of the castor oil.

Mouse Models of Hypermotility. The fecal pellet output was examined in nonfasted animals. Fifteen minutes after drug administration (PR-38 or vehicle), mice were placed on a metal grid, and the number of fecal pellets excreted over a 60-minute period was counted in 15-minute intervals (Fichna et al., 2014).

Behavioral Pain Responses. Behavioral responses to i.a. administration of MO (allyl isothiocyanate) were determined as described previously (Laird et al., 2001; Eijkenkamp et al., 2007). In brief, Vaseline Petroleum Jelly was applied to the perianal area to rule out the stimulation of somatic areas, and then 50 μl of MO (1% in 70% ethanol) was administered i.c. under isoflurane anesthesia. After 5 minutes of recovery, spontaneous behaviors were recorded on a videotape for 20 minutes for later analysis by an observer blinded to experimental conditions. Pain-related behaviors were defined as 1) licking of the abdomen, 2) squashing of the lower abdomen against the floor, 3) stretching the abdomen, and 4) abdominal retractions, and were each counted as 1.

PR-38 was administered 10 minutes (i.c.) and 15 minutes (intraperitoneally) before the MO instillation. Selective MOR (μ-FNA, 1 mg/kg i.p.), KOR (nor-BNI, 10 mg/kg i.p.), and CB1 (AM 251, 1 mg/kg i.p.) receptor antagonists were administered 10 minutes before PR-38. All drugs were dissolved in 5% dimethylsulfoxide in saline, which was used as vehicle in control experiments.

The writhing test was performed as described earlier (Gach et al., 2010). Fifteen minutes after the administration of vehicle or PR-38, mice were injected intraperitoneally with acetic acid solution (10 ml/kg of 0.5% vol/vol, in 0.9% NaCl). After the injection, mice were placed in individual cages, and the total number of writhes was counted 5 minutes later, during three periods of 5 minutes each. The writhing response, regarded as a nociceptive behavior, was characterized by elongation of the body and the development of tension in the abdominal muscles and hind paws.

Qualitative Determination of PR-38 in Mouse Plasma by Mass Spectrometry. Proteins from mouse plasma were precipitated by the addition of 2 volumes of acetonitrile. Two hundred microliters of acetonitrile was added to 100 μl of plasma with gentle vortexing. The mixtures were then left at room temperature for 30 minutes. The supernatants were then centrifuged at 13,000g for 15 minutes and lyophilized. Samples then were acidified with 2% trifluoroacetic acid prior to desalting with ZipTipC18 pipette tips (Merck Millipore, Darmstadt, Germany). In brief, disposable SPE (solid-phase extraction) pipette tips packed with C18 material were conditioned with two washes of methanol, 75% ACN (acetonitrile)0.1% trifluoroacetic acid. Coelutions were performed directly onto the matrix-assisted laser desorption/ionization–time of flight mass spectrometry (MALDI-TOF MS) target (spectra were obtained in reflector positive mode within a mass range of 200–1200 Da, a focus mass of 500 Da). The experiments were repeated at least twice.

The values of the molecular ion were as expected. The strong signal and low background allowed for easy identification of compound by MALDI-TOF MS.

Measurement of Locomotor Activity. Locomotor activity was assessed according to the method introduced by Fichna et al. (2007). In brief, measurement was made automatically in a Digiscan actimeter (Omnitech Electronics Inc., Columbus, OH), which monitored horizontal displacements and vertical movements. The animals were placed individually in 20 × 20 × 30-cm compartments, in a dimly illuminated and quiet room. The responses were expressed as the number of crossed infrared beams by a mouse during four consecutive 15-minute periods.

Elevated O-Maze Test. To identify differences in exploratory behavior and anxiety, the elevated O-maze was used, as described earlier (Fuss et al., 2010). The maze consisted of a gray plastic annular runway (width 6 cm; outer diameter 46 cm; 50 cm above ground level) covered with black cardboard paper to prevent mice from slipping off. The maze is composed of two opposing open and two opposing closed sectors protected by inner and outer walls of gray polyvinyl (height 10 cm). The maze was illuminated with 25 lux. Animals were placed in one of the protected sectors and observed for 5 minutes. The following parameters were analyzed: total time spent in the open and closed compartments, and total distance traveled in open and closed compartments.

Catwalk Quantitative Gait Analysis Test. The quantitative analysis of gait was performed as described recently by Vandeputte et al. (2010). In brief, the Catwalk is a video-based analysis system used to assess gait in voluntarily walking mice (Noldus, Wageningen, the Netherlands). The system measures various aspects of footfalls in

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a dynamic manner. When the animal crosses the walkway, the areas of contact are illuminated. In this way, the different paw contacts are visualized, and multiple parameters are calculated. Here, to examine the possible influence of PR-38 on the CNS, several selected parameters were analyzed, including run duration, intensity of the paws (signal depends on the degree of contact between a paw and the glass plate and increases with increasing pressure), print width (width of the complete paw print), print area (surface area of the complete print), swing (duration of no contact with the glass plate in a step cycle), and swing speed (speed of the paw during swing). For data collection, three runs per animal and time point were performed by placing the animal in front of the start zone of the catwalk runway. Analysis was performed on a minimum of four normal step sequence patterns in each uninterrupted run.

Drugs. All drugs and reagents, unless otherwise stated, were purchased from Sigma-Aldrich (Poznan, Poland). SA (purity: 99% by high-performance liquid chromatography) was isolated from *S. divinorum* leaves, purchased from the Sage Wisdom Salvia Shop (Malibu, CA) by one of the authors (J.K.Z.). PR-38 was synthesized from SA at the Department of Pharmacognosy, University of Mississippi (University, MS), as described earlier (unpublished data). Nor-binaltorphimine dihydrochloride, β-funaltrexamine hydrochloride, loperamide, and AM 251 were purchased from Tocris Bioscience (Ellisville, MO). In the in vitro experiments (isolated smooth muscle strips), all drugs were dissolved in dimethylsulfoxide. In the in vivo tests, drugs were dissolved in 5% dimethylsulfoxide in saline, which was used as vehicle in control experiments. The vehicles in the used concentrations had no effects on the observed parameters.

Statistics. In the in vitro experiments, *n* denotes the number of individual tissues from at least three different animals. Statistical analyses were performed using PRISM 5.0 (GraphPad Software Inc., La Jolla, CA). The data are expressed as means ± S.E.M. Student’s *t* test was used to compare single treatment means with control means. Analysis of variance followed by Newman-Keuls post-hoc test was used for analysis of multiple treatment means. *P* values ≤0.05 were considered statistically significant.

### Results

**PR-38 Inhibits Smooth Muscle Contractility In Vitro.**

We first investigated the effect of SA and PR-38 on isolated mouse colon contractility to predict the potential effect on gastrointestinal motility in vivo and to estimate the optimal dose for in vivo experiments. Both compounds (10⁻¹⁰ to 10⁻⁶ M) inhibited the EFS-induced twitch contraction in a concentration-dependent manner, but PR-38 was less potent than the parent compound (IC₅₀ value of 533 ± 46 and 59.5 ± 3.8 nM for PR-38 and SA, respectively; Fig. 1A). The maximum inhibitory effect of both compounds was approximately 40%.

As shown in Fig. 1B, the effect of SA was blocked by the KOR antagonist nor-BNI (10⁻⁶ M), but not by MOR or CB1 antagonists. In contrast, the inhibitory effect of PR-38 was reversed by the MOR antagonist β-FNA (10⁻⁶ M) and significantly decreased by KOR antagonist nor-BNI (10⁻⁶ M) (Fig. 1C), suggesting the involvement of MOR and KOR in the action of PR-38. The effect of PR-38 was not blocked by CB1 receptor antagonist AM 251 (10⁻⁷ M). These results encouraged us to investigate the effect of PR-38 on mouse gastrointestinal motility in vivo.

**PR-38 Prolongs the Time of the Whole Gastrointestinal Transit In Vivo.**

To test the effect of PR-38 on mouse GI motility in vivo, several animal models were used. As shown in Fig. 2A, PR-38 administered intraperitoneally produced a potent, dose-dependent inhibitory effect on whole GI motility. A dose of 10 mg/kg for PR-38 was required to produce the inhibitory action equipotent to that observed after the intraperitoneal; injection of SA at a dose of 3 mg/kg.

To determine the potential mechanism of action of PR-38, antagonists of opioid and CB receptors were also used. The effect of PR-38 (10 mg/kg) was blocked by MOR antagonist β-FNA (1 mg/kg), KOR antagonist nor-BNI (10⁻⁶ M), and CB1 antagonist AM 251 (10⁻⁷ M) (Fig. 2A).

We also characterized the effect of PR-38 on the GI tract motility after oral administration at a dose of 20 mg/kg and...
observed a significant prolongation of the whole GI transit time (Fig. 2B). SA did not produce any effect after oral administration (data not shown). We next aimed at investigating the effect of PR-38 within different segments of the GI tract.

**PR-38 Inhibits Colonic Motility In Vivo.** The action of PR-38 was first evaluated in the colon. PR-38 (10 mg/kg i.p.) produced a time-dependent inhibitory effect on colonic expulsion up to 45 minutes after administration (Fig. 3A). To determine the bioavailability of PR-38, we also examined its action after p.o. administration. At a dose of 20 mg/kg, PR-38 significantly prolonged the time to bead expulsion 15 minutes after injection (Fig. 3B). This effect was no longer observed 45 minutes after administration of the drug (Fig. 3B).

**PR-38 Does Not Affect Gastric Emptying, but Lowers Geometric Center and Inhibits Upper Gastrointestinal Transit In Vivo.** We have shown previously that SA inhibits small intestinal transit by lowering the GC, but it does not affect the GE (Fichna et al., 2009a), and here we have observed a similar effect for PR-38. The intraperitoneal administration of PR-38 at a dose of 10 mg/ kg had no effect on GE (Fig. 4A) and significantly slowed the upper intestinal transit, as indicated by a lower GC (Fig. 4B). Additionally, we observed that PR-38, at a dose of 10 mg/kg i.p. significantly slowed the motility of the gut in the mouse model of the upper GI transit (Fig. 4C).

**Antidiarrheal Action of PR-38 in Mouse Models of Diarrhea and Hypermotility.** Encouraged by the results showing that PR-38 inhibits motility of both the small and large intestine, we investigated the antidiarrheal activity of PR-38 in a mouse model of castor oil–induced diarrhea. Intragastric administration of castor oil caused an accumulation of water and electrolytes in the mouse intestine, which resulted in acute diarrhea in the control animals (Fig. 5A). PR-38 (10 mg/kg i.p.), SA (3 mg/kg i.p.), and the reference drug...
loperamide (1 mg/kg i.p.) significantly delayed the emergence of liquid feces (Fig. 5A). The effect of PR-38 after oral administration was not statistically significant (data not shown).

To further characterize the influence of PR-38 on the GI tract in pathophysiological conditions, two mouse models of GI hypermotility were used in the study. In the stress-induced mouse model of hypermotility, the number of fecal pellets excreted by animals was measured for 60 minutes. Placing animals in a novel environment significantly increased fecal output compared with nonstressed control animals (Fig. 5B). PR-38 administered at a dose of 10 mg/kg i.p. inhibited GI hypermotility in both control and stressed mice nearly to the same extent as loperamide, which was used as a reference drug at a dose of 1 mg/kg i.p. (Fig. 5B). PR-38 was not active in this model after p.o. administration (data not shown).

**PR-38 Is a Potent Analgesic Agent in Mouse Models of Abdominal Pain.** Abdominal pain is one of the major symptoms of IBS. To assess the analgesic activity of PR-38, two different mouse models of abdominal pain were used. In the model elicited by the i.c. administration of MO, the systemic injection of PR-38 (10 mg/kg i.p.) and SA (3 mg/kg) resulted in a significant decrease of pain-related behaviors, and this effect was blocked by MOR antagonist β-FNA (1 mg/kg i.p.), but not by KOR antagonist nor-BNI or CB1 antagonist AM 251 (Fig. 6A).

The antinociceptive effect of PR-38 after oral administration was not tested in this model, as our preliminary experiments with well established analgesics produced conflicting results for this route of administration and require further studies. However, PR-38 produced statistically significant analgesia after local (i.c.) administration at a dose of 10 mg/kg (Fig. 6B). Increase in the dose to 20 mg/kg did not strengthen the effect of PR-38 (data not shown).

In the writhing test, the administration of PR-38 at a dose of 10 mg/kg i.p. resulted in a significant reduction of the number of writhes (Fig. 6C). Furthermore, PR-38 at a dose of 20 mg/kg produced a statistically significant analgesic effect after oral administration (Fig. 6D).

**PR-38 Is Present in Mouse Blood after Oral and Intraperitoneal Injection.** Encouraged by the effect observed in mice after oral administration of PR-38, we aimed at
determining whether PR-38 penetrates from the GI tract into the blood stream in mouse plasma samples using MALDI-TOF MS analysis.

We observed peaks proving the presence of diterpene skeleton in the plasma collected from mice administered both by oral and intraperitoneal route (20 and 10 mg/kg, respectively). There was no signal indicating diterpene presence in plasma from control animals (see Supplemental Material for details).

**PR-38 Affects Neither Motor Functions nor Exploratory Behavior or Anxiety Levels in Mice.** SA, which is a parent compound of PR-38, is known for its CNS-related actions, e.g., hallucinogenic. Here, we investigated the effect of PR-38 in the CNS using relevant mouse models. The influence of intraperitoneal administration of PR-38 on mouse locomotor activity was measured over six consecutive periods of 10 minutes each. The drug administered at a dose of 10 mg/kg did not modify horizontal or vertical locomotor activity in any of the time periods of the test (Fig. 7, A and B).

To identify potential differences in exploratory behavior and changes in axiogenic or anxiolytic activity after PR-38 administration versus control mice, an elevated O-maze test was used. No significant differences were found for measures of anxiety, such as the percentage of time spent in open and closed sectors after intraperitoneal administration of PR-38 at a dose of 10 mg/kg (Fig. 7C).

We also analyzed the gait parameters of mice after administration of PR-38 at a dose of 10 mg/kg i.p. None of more than 50 parameters has changed after administration of PR-38 in comparison with control animals, including intensity of the paw prints (Fig. 7D), print width (Fig. 7E), print area (Fig. 7F), maximal area of print (Fig. 7G), swing (Fig. 7H), swing speed (Fig. 7I), and run duration (data not shown).
Discussion

Extracts from plants producing opioids are well known for their actions in the GI tract. SA, the active component of *S. divinorum*, has recently been demonstrated to exhibit significant effects on smooth muscle relaxation, neuroprotection, and analgesia (Fichna et al., 2009a) and, similar to other KOR agonists, has a great potential to become a drug used in the clinical treatment of GI disorders dominated by diarrhea and abdominal pain, such as diarrhea-predominant IBS. However, in the case of SA, this potential is strongly hampered by its adverse effects, since it rapidly crosses the blood-brain barrier and causes short-lived hallucinations (Ranganathan et al., 2012). Therefore, attempts to develop new analogs with similar activity are undertaken.

In this study, we evidenced that one of the newly synthesized SA analogs, PR-38, is a strong regulator of intestinal motility and pain signaling in physiological and pathophysiological conditions. Furthermore, it should be noted that PR-38 is orally available. These findings may open up opportunities for the design of novel therapeutics for functional GI ailments, including diarrhea-predominant IBS.

Originally, SA was identified as a potent KOR agonist, but recently it has also been shown to possess, unusual for plant-derived opioids, the ability to activate CB receptors in in vitro and in vivo assays (Roth et al., 2002; Capasso et al., 2008; Fichna et al., 2009a, 2012; Aviello et al., 2011). Here, we observed that the inhibitory effect of PR-38 on twitch contractions in mouse colon in vitro and on the GI motility in vivo was blocked by MOR and KOR antagonists. Furthermore, PR-38 preserved the ability of SA to interact with CB receptor–dependent pathways in vivo. The fact that PR-38 can additionally activate MOR-dependent pathways widens its spectrum of potential pharmacological actions and clearly shows that PR-38 is of even higher therapeutic potential than SA because of the number of pharmacological targets it can interact with.

The interaction of PR-38 with opioid and CB receptors suggests that the potential sites of its action in the periphery...
are KOR, MOR, and CB receptor–expressing cells, i.e., acetylcholine- and neuropeptide-releasing motor neurons of the enteric nervous system. Opioid and CB receptor agonists are well known inhibitors of cholinergic and noncholinergic excitatory pathways in these cells (Chamouard et al., 1993; Sobczak et al., 2014). Of interest, in the in vitro experiments, we did not observe any potentiation of the antagonist effect of opioid and CB blockers when combined. Recently, we demonstrated that the coapplication of CB and KOR antagonists did not exhibit any synergistic effect on the inhibitory action of SA on the smooth muscle contraction induced by EFS in vitro in mouse colon, indicating that KOR and CB signaling could be channeled into a common final pathway in neuronal cells (Fichna et al., 2009a). It is likely that, at the cellular level, SA and PR-38 activate opioid and CB receptors, which may transmit a signal through a common G protein or form functional heterodimers. Recently, Hojo et al. (2008) have shown that MOR and CB1 receptors can form such dimers, which is in line with our experimental data. Moreover, it has been shown that KOR and CB1 ligands are able to activate G protein–coupled inwardly rectifying K+ channels (Henry et al., 1995; McAllister et al., 1999). G protein–coupled inwardly rectifying K+ channels might therefore represent a common link for opioid and CB receptor–dependent pathways.

Our earlier experimental data suggest that the antimotility action of SA is stronger in pathophysiological conditions than in healthy mice (Capasso et al., 2006, 2008), and thus in this study, we used models of diarrhea and hypermotility to evaluate the antidiarrheal properties of PR-38. The administration of PR-38 significantly prolonged the time that elapsed from administration of croton oil to emergence of liquid pellets and significantly altered defecation pattern in stressed mice. Although the effect of PR-38 in both models was weaker than that of the well known peripherally active opioid agonist loperamide, it was comparable to the antidiarrheal action of SA (Fichna et al., 2009a). The fact that PR-38 can be synthesized directly from an easily accessible and cheap plant material may become an unquestionable advantage over currently available synthetic opioids, such as loperamide.

To fully characterize the pharmacological effect of PR-38 in the GI tract, we also examined its potential antinociceptive activity in two well established mouse models of abdominal pain. In both tests, we observed a potent analgesic effect after local (i.c.) and systemic (intraperitoneal and oral) administration, which was mediated by MOR, but not KOR or CB1. This suggests that, among all the receptors targeted by PR-38, MOR is mostly responsible for its analgesic activity in abdominal pain models, and implicates the lack of the synergistic effect
upon activation of opioid and CB receptors for the induction of analgesia. We have shown previously that the analgesic effect produced by SA in the same pain model was mediated by KOR and CB1 (Fichna et al., 2012). Our observations demonstrate that the modification of the SA molecule, which resulted in the increased affinity for MOR, changed the mechanism of the action of the compound and shifted the equilibrium toward production of the analgesic effect, which is crucial for further structure-activity relationship studies.

One of our most prominent goals was to find a novel SA analog deprived of the CNS-related side effects typical for SA. It is widely known that SA is a strong, short-acting psychoactive compound, and its actions in humans range from impaired motor activity, visual effects, memory impairment, and responsiveness to feelings of anxiety/fear, distance from usual daily reality, and paranoia (Ranganathan et al., 2012; Maclean et al., 2013). To determine the potential side effects of PR-38, we focused our attention on behavioral parameters that undergo changes after administration of SA in rodents and mimic the effects of the compound in humans (Braida et al., 2009; Walentiny et al., 2010). We observed that none of the parameters measured changed after administration of PR-38. The lack of changes in spontaneous locomotor activity and, of particular importance, several gait parameters indicates that PR-38 does not affect the CNS. Moreover, results obtained from the O-maze test suggest no anxiogenic and/or anxiety-like activity of PR-38. The pharmacological profile of PR-38, which is a mixed agonist of MOR, KOR, and CB receptors, would suggest SA-like actions in the CNS. Our observations indicate the opposite and suggest that the modifications made in the SA molecule to obtain PR-38 prevent it from crossing the blood-brain barrier, increasing the therapeutic safety profile. Further studies are warranted to confirm this observation.

Conclusion

Modification of the chemical structure of SA produced a novel orally available analog, PR-38, with great potential for clinical use in functional GI disorders associated with pain and diarrhea. It seems likely that PR-38 will be further developed for its significant impact on the lower GI tract combined with good bioavailability and the lack of psychotomimetic side effects.

Authorship Contributions

Participated in research design: Fichna.
Conducted experiments: Salaga, Fichna, Sobczak, Grzywacz, Storr, Sibae, Do Rego.
Contributed new reagents or analytic tools: Zjawiony, Polepally.
Performed data analysis: Salaga, Sobczak, Storr, Sibae, Grzywacz, Kamyzd, Do Rego, Fichna.
Wrote or contributed to the writing of the manuscript: Salaga, Fichna.

References