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The synergistic effects of opioid and neuropeptide B/W in rat acute inflammatory and neuropathic pain models

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ABSTRACT

The use of morphine is controversial due to the incidence of rewarding behavior, respiratory depression, and tolerance, leading to increased drug dose requirements, advancing to morphine addiction. To overcome these barriers, strategies have been taken to combine morphine with other analgesics. Neuropeptide B23 and neuropeptide W23 (NPB23 and NPW23) are commonly used to relieve inflammatory pain and neuropathic pain. As NPB23 and NPW23 system shares similar anatomical basis with opioid system at least in the spinal cord we hypothesized that NPB23 or NPW23 and morphine may synergistically relieve inflammatory pain and neuropathic pain. To test this hypothesis, we demonstrated that μ opioid receptor and NPBW1 receptor (receptor of NPB23 and NPW23) are colocalized in the superficial dorsal horn of the spinal cord. Secondly, co-administration of morphine witheitherNPB23 or NPW23 synergistically attenuated inflammatory and neuropathic pain. Furthermore, either NPB23 or NPW23 significantly reduced morphine-induced conditioned place preference (CPP) and constipation. We also found that phosphorylation of extracellular-regulated protein kinase (ERK1/2) following morphine was profoundly potentiated by the application of NPB23 or NPW23. Hence, combination of morphine with either NPB23 or NPW23 reduced dose of morphine required for pain relief in inflammatory and neuropathic pain, while effectively prevented some side-effects of morphine.

1. Introduction

The clinical therapies for moderate and severe pain including cancer pain, acute and chronic inflammatory pain, and neuropathic pain, relyon opioid analgesics, such as morphine and other μ opioid receptor agonists (Portenoy and Ahmed, 2014; Stein, 2013; Zöllner and Stein, 2007). Morphine serves as the “gold standard " for severe pain management. However, caution should be taken for the utilization of morphine due to its unwanted side-effects, including drug tolerance, constipation, and physical dependence. Some of the side-effects lead to an increase in dose requirements and ultimately a severe morphine addiction (Inturrisi, 2002; Stein, 2013; Zöllner and Stein, 2007). An emerging strategy in utilizing morphine is to co-administer morphine with other analgesics that target distinct pain transmission pathways different from morphine. The combinatory therapies not only ensure a synergistic effect on pain relief, but also reduces the dose of morphine and then the incidence of its adverse side-effects. (White and Kehlet, 2010). In addition, a number of studies found that treatment by an opioid agent combined with other analgesics relieves pain better than the opioid alone (Ashish et al., 2018). For example, combining a μ opioid receptor agonist with a cannabinoid receptor agonist or ion channel antagonist produces a synergistic analgesic effect on chronic models of pain while significantly reduces unwanted opioid side-effects (Grenald et al., 2017 ; Hahm et al., 2012; Kazantzis et al., 2016). Besides, opioid compounds with dual targets interact with opioid and other systems with different pharmacokinetic and pharmacodynamic parameters are valuable for combinatory pain relief (Li et al., 2016; Schiller, 2010).

Neuropeptide B23 (NPB23) and neuropeptide W23 (NPW23), are natural ligands for the orphan G protein-coupled receptors, NPBW1 receptor (GPR7) and NPBW2 receptor (GPR8) (Shimomura et al., 2002; Fujii et al., 2002). NPBW1 receptor and NPBW2 receptor belonging to neuropeptidergic system are thought to play essential roles in regulating

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feeding behavior, energy homeostasis, neuroendocrine function, and stress responses (Mondal et al., 2003; Skrzypski et al., 2012; Takenoya et al., 2010). Evidence also suggests that NPB23 and NPW23 are considered candidates for modulating inflammatory and neuropathic pain (Yamamoto et al., 2005, 2006). Despite NPBW1 receptor and NPBW2 receptor have similar structural characteristics with opioid receptors, NPB23 and NPW23 are unable to activate opioid receptors and exert analgesic effects differently from opioid peptides (Yamamoto et al., 2005).

NPB, NPW, or NPBW1 receptor mRNA were expressed in the central nervous system (CNS), more specifically in the periaqueductal gray, amygdala, and spinal cord. These regions play important roles in pain signal transmission and pain modulation, and also express opioid receptors (Tanaka et al., 2003; Jackson et al., 2006). The expression pattern of NPBW1 receptor and μ opioid receptor suggests these receptors may localize on the same neurons of the central nervous system. Moreover, NPBW1 receptor and μ opioid receptor are all seven-transmembrane $G_{i/o}$ protein-coupled receptors sharing similar signal transduction pathways, such as inhibiting the production of 3′–5′-Cyclic adenosine monophosphate (cAMP) and triggering ERK1/2 phosphorylation (Sakurai, 2013). Owing to these similarities, we hypothesized that the NPBW1 receptor and μ opioid receptor, if activated simultaneously, may elicit synergistic (or supra-additive) antinociceptive effects.

Therefore, the primary aim of this study was to detect whether μ opioid receptor and NPBW1 receptor co-localize in the dorsal horn of the spinal cord. Secondly, we will compare the antinociceptive effects of morphine with or without NPB23 and NPW23 administration in a range of different dose in neuropathic and inflammatory pain in the rats. We want to determine whether morphine-NPB23 and morphine-NPW23 would yield synergistic effects or merely additive effects based on isobolographic analysis described by Tallarida. Thirdly, we would also test whether morphine-NPB23 or morphine-NPW23 would reduce morphine-induced constipation and conditioned place preference. Finally, to examine whether NPB23 or NPW23 could modulate the morphine-induced downstream signal, we evaluated the levels of ERK1/2 phosphorylation (well-known acute signaling via activation of NPBW1 receptor or μ opioid receptor) in NPB23, NPW23, NPB23-morphine, and NPW23-morphine, respectively.

2. Materials and methods

2.1. Animals

The experiments were performed on male Sprague–Dawley rats (180–200 g) from the Experimental Animal Center of Xuzhou Medical University (Xuzhou, Jiangsu Province, China). The rats were housed in a temperature controlled room (22 ± 1 °C) and humidity (45–75%) on a dark/light cycle of 12 h (8:00 a.m.–8:00 p.m.) with *ad libitum* access to food and water. All animal experiments were performed in accordance with a protocol approved by the Animal Care and Use Committee of Xuzhou Medical University (201909A001) in accordance with the Declaration of NIH Guide for Care and Use of Laboratory Animals (Publication No. 80–23, revised 1996).

2.2. Drugs and protocols

NPB23 (W Y K P A A G H S S Y S V G R A A G L L S G L-NH2) and NPW23 (W Y K H V A S P R Y H T V G R A A G L L M G L-NH2) were prepared by manual solid-phase synthesis using standard N-fluorenylmethoxycarbonyl (Fmoc) chemistry as described previously (Mou et al., 2011). The established peptides were then purified using semi-preparative RP-HPLC and characterized by RP-HPLC, TLC, ESI-TOF MS, and mp. Purities were determined to be 95–99% as characterized by analytical RP-HPLC. Morphine hydrochloride was purchased from Shenyang First Pharmaceutical Factory, China. All drugs were dissolved in sterilized distilled saline and stored at −20 °C.

To investigate the synergistic effects between morphine and NPB23 or NPW23, these drugs were intrathecally (i.t.) administered separately as well as co-administered and pain behavioral assays were performed subsequently. In control rats, the vehicle (saline) was administered, i. t. Each drug was examined independently, generating dose-response curves with their individual ED_{50}. Combinations of NPB23 with morphine or NPW23 with morphine were then simultaneously administered i. t. in a fixed 1:1 ED_{50} ratio of fractions (1.0, 0.5, 0.25 and 0.125) of their respective ED_{50} values. Drugs were weighed out and dissolved in the vehicle daily, prior to use.

2.3. Immunohistochemistry assay for NPBW1 receptor and μ opioid receptor

Rats were deeply anesthetized with isoflurane and perfused through the aortic arch with 100 ml of heparin (75 U/ml of heparin in 0.9% saline) followed by 100 ml of 2% paraformaldehyde (PFA) in 0.1 M phosphate-buffered saline (PBS), pH 7.4, and then by 300 ml of 2% PFA in the same buffer at 45 ml/min. The lumbar spinal cord was removed and post fixed in 2% PFA in 0.1 M PBS for 1 h at 4 °C. Sections (30 μm thick) were cut using a cryostat and processed for NPBW1 receptor and μ opioid receptor labeling. Sections were incubated in 1% sodium borohydride for 30 min and rinsed with 0.1 M PBS and pre-incubated for 1 h at room temperature in 3% NGS in PBS. They were then incubated with antibodies against NPBW1 receptor (1:200 Abcam) and μ opioid receptor (1:200 Abcam) for 48 h at 4 °C. Sections were then rinsed twice with TBS and incubated for 1 h at room temperature with secondary antibodies both at 1: 400 (Abcam). Images were acquired using a QImaging Rolera XR digital camera on an Olympus X81 microscope, and analyzed using MetaMorph software.

2.4. Formalin-induced nociceptive behavioral test

The formalin flinch test, characterized by a biphasic response, is a well-recognized acute inflammatory pain model (Yamamoto et al., 2005). Rats were placed in a transparent acrylic observation chamber (20 × 20 × 30 cm) with a mirror positioned at a 45° angle below the floor to allow an unimpeded view of the animals' hind paw. Initially, rats were acclimatized in the test conditions for 30 min followed by injecting 50 μl 5% formalin (dissolved in 0.9% saline) solution subcutaneously under the surface of the left hind paw. Rats were observed from 0 to 60 min following formalin injection. The early nociceptive response phase (phase 1) normally peaked at 0–10 min and the late phase (phase 2) at 11–60 min after formalin injection, demonstrating the direct stimulation of nociceptors and an inflammatory nociceptive response respectively. As a control, saline was injected into the hind paws, and flinches were recorded. Rats were intrathecally (i.t.) administered NPB23 (1, 5, 7, 10 μg), NPW23 (1, 5, 7, 10 μg), morphine (1, 5, 10, 15 μg), saline or a fixed dose ratio of NPB23 and morphine or NPW23 and morphine, then received an intraplantar injection of formalin 10 min later. Following formalin injection, the rats were immediately put back in the observation chamber, and the number of flinches was counted and recordedan.

2.5. Establishment of neuropathic pain rat model

Chronic constriction injury (CCI) is a rodent model of persistent peripheral neuropathic pain (Bennett and Xie 1988). In this study, rats were anesthetized through intraperitoneal injection of40 mg/kg sodium pentobarbital. An incision was made below the left hipbone, and the sciatic nerve was exposed. Four snug ligatures with 1 mm spacing were made with 4.0 silk thread around the nerve distal to the sciatic notch, until a brief twitch in the respective hind limb was observed. As a control, Sham ratsunderwent surgery and exposure of the sciatic nerve without ligation was made. Following CCI surgery, the rats were

monitored for 6–8 days before the behavioral experiment. Rats were intrathecally administered NPB23 (1, 5, 7, 10 μg), NPW23 (1, 5, 7, 10 μg), morphine (1, 3, 5, 7 μg), saline or a fixed ratio doses of NPB23-morphine or NPW23-morphine. Following drug treatment, rats were subjected to mechanical paw withdrawal thresholds (PWT) test over a 2-h time course and compared to saline-treated animals.

2.6. Mechanical allodynia testing

Mechanical allodynia, a symptom of neuropathic pain, were measured (Maier et al., 2010). One week following surgery, rats (CCI and Sham) were administered saline, NPB23, NPW23, morphine, or a fixed ratio dose of NPB23-morphine or NPW23-morphine. Following drug treatment, rats were measured by prodding the plantar region of a hind paw with calibrated von Frey filaments (Stoelting Co., WoodDale, IL). Before the test, each rat was placed in a testing box (17 × 15 × 12 cm) with a wire-mesh grid floor, and allowed to acclimate for a minimum of 30 min. Each hair was applied for 10 s or until the rat withdrew the hind paw without ambulation, and 50% paw withdrawal threshold was calculated using the up-and-down method (Chaplan et al., 1994). Before drug administration or surgery, baseline withdrawal latencies or mechanical thresholds were measured at least three times. All experiments were performed in a blinded manner.

2.7. Condition place preference

The chamber for conditioned place preference (CPP) assay is divided into three compartments. One end compartment has black strips on the wall and a smooth floor. Another end compartment has gray walls with a textured floor. The middle transition compartment has parallel bars on the floor.

CPP assay was conducted according to previously described protocol (Largent-Milnes et al., 2013). The assay consists of pre-conditioning, conditioning, and post-conditioning sessions. On pre-conditioning session (day 1), rats were allowed to explore in CPP chamber for 15min, and the time spent in each compartment was measured. Rats that spent more than 60% of time in the same compartment were excluded from the assays (less than 5% of rats). On the conditioning session, rats were i. t. injected with saline and confined to one end compartment for 15 min. Approximately 6 h later, the rats were i. t. administered with saline, morphine (10 μg), NPB23 (2, 5, 10 μg), NPW23 (2, 5, 10 μg), or a combination of morphine-NPB23 (10 μg-2μg) or morphine-NPW23 (10 μg-2μg) and then confined to the compartment. This procedure was carried out in three consecutive days (day 2–day 4). In post-conditioning session (day 5), rats were placed in CPP chamber for 15 min, and the time they spent in each compartment was measured. CPP score was expressed as time spent in the drug-associated compartment in post-conditioning session minus that in pre-conditioning session.

2.8. Gastrointestinal transition test

The rat gastrointestinal transit test was used to evaluate the effect of drug combination on the movement of chyme in small intestine (Li et al., 2016). After being fasted for 16 h with free access to water, rats received administration of saline, low (2 μg, i. t.) or high (10 μg, i. t.) doses of morphine, NPB23 (2, 5, 10 μg, i. t.), NPW23 (2, 5, 10 μg, i. t.) or a combination treatment of morphine-NPB23 (2 μg–2 μg, 10 μg–2 μg, i. t.) or morphine-NPW23 (2 μg–2 μg, 10 μg–2 μg, i. t.). Fifteen minutes following drugs administration, a charcoal meal (an aqueous suspension of 5% charcoal and 10% gum arable) was administered orally at a volume of 0.1 ml·10 g^{-1} body weight. Thirty minutes after charcoal meal feeding, rats were sacrificed, and the small intestines from the pylorus to the caecum were carefully removed. The traveled distances of the charcoal meal in rat intestine were measured. For each rat, GIT % was calculated as the percentage of the small intestine tract that was traversed.

2.9. Establishment of HEK293 cells stably expressing MOR- NPBWR1

Human embryo kidney 293 (HEK293) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 0.1 mg/ml streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2. The eukaryotic vectors, pcDNA3.1-FLAG-μ opioid receptor and pcDNA3.1-3 × HA-NPBW1 receptor, were tranfected into HEK293 cells by Lipofectmine2000 according to the manufacturer's instruction. The day after transfection, G418 (800 μg/ml) was added to the medium for 2 weeks. Then the antibiotic-resistant clones derived from a single cell were selected and further characterized by RT-PCR and Western blotting to ensure the expression of human μ opioid receptor and NPBW1 receptor. The cellular function of the μ opioid receptor and NPBW1 receptor was confirmed by the μ-opioid receptor antagonist naloxone and NPBW1 receptor antagonist CYM50769. Briefly, HEK293-MOR-NPBWR1 cells were preincubated with or without 1 μM naloxone or 1 μM CYM50769 in DMEM medium for 30 min, then, the cells were stimulated with 1 μM morphine or 1 μM NPB23, and 10 μM forskolin to measure the cAMP accumulation level.

2.10. Detection of MAPK phosphorylation

ERK phosphorylation was measured by immunoblotting as described (Belcheva et al., 2005; Korzh et al., 2008). HEK293 cells stably expressing the μ opioid receptor and NPBW1 receptor were seeded in 12-well plates. Sixteen h before the addition of ligands, the culture medium was removed and replaced by a fresh serum-free medium. For rapid ERK1/2 phosphorylation assay, the cells were treated with morphine, NPB23, NPW23, and their combination (1:1 ratio) at different concentrations and incubated at 37 °C for 10 min. Cell monolayers were rinsed with ice-cold PBS, and whole lysates were prepared by the addition of RIPA lysis buffer containing 10 μM PMSF and phosphatase inhibitor cocktails (P5726, Sigma-Aldrich) for 5 min. Soluble proteins were separated by centrifugation at 12,000*g* for 10 min. Protein concentration was determined by using BCA protein assay kit (Pierce, Thermo Scientific, U.S.). A total amount of 30 μg protein from each sample was prepared for 10% SDS−polyacrylamide gel electrophoresis and electroblotted onto polyvinylidene difluoride membranes. Membranes were probed with primary antibody against phosphorylated-ERK1/2 or ERK1/2 (1:1000 dilution in blocking solution, Cell Signaling Technology Inc.). Immunoreactive proteins were visualized using a horseradish peroxidase sensitive ECL chemiluminescent Western blotting kit (Pierce, Thermo Scientific, U.S.).

2.11. Analysis

GraphPad Prism 5.0 software (Graph Pad Inc., San Diego, CA) was used to analyze and plot data. Responses to time and drug treatments in formalin flinch and CCI assays were analyzed using two-way repeated measures ANOVAs. The extent and duration of analgesia were estimated by area under the curve (AUC) values. The AUC depicting total number of flinches or paw withdrawal threshold versus time was computed by trapezoidal approximation. The AUC date calculated from 0 to 60 min of formalin flinch and 0–120 min of CCI assay in the dose- and time-response curves of individual drug (Jens et al., 2003). Levene's test were used to evaluate whether data from different groups are equal in variances. Data obtained from GIT and CPP assay test were shown as means ± S.E.M. and further analyzed with one-way ANOVA followed by Dunnett's post-hoc test. P values less than 0.05 ($P < 0.05$) were considered statistically significant. Data from formalin assay were expressed as a percentages of maximal possible effect (%MPE) calculated as:

%MPE = 100x (test value-vehicle control value)/ (cutoff value-vehicle control value). Data from mechanical PWT was calculated as %MPE = 100 × (test

value - baseline value)/ (cutoff value - baseline value), and a cutoff value was set at 300 g to minimize tissue damage

Isobolographic analyses were used to examine the synergism of the antinociceptive effects of morphine alone or morphine-NPB23 or morphine-NPW23 on formalin flinch and neuropathic pain (Tallarida, 2006 ; Tallarida and Raffa, 2010). Dose-response relationships were obtained to provide the magnitude of the effect of drug combination. Following the creation of dose-response curves for morphine, NPB23 and NPW23, the median effective dose (ED_{50}) for each drug was determined. The ED_{50} was plotted on the X- and Y-axes to obtain the additive line. To determine whether the combination had a synergistic effect, a theoretical additive ED_{50} (ED_{50}add) was calculated (Pinardi et al., 2001; Miranda et al., 2002) using the following equation:

$$ED_{50\ add} = ED_{50\ NPB23/NPW23}/(P_1 + R \times P_2)$$

where R is the potency ratio of NPB or NPW alone to morphine alone, P_1 is the proportion of NPB23 or NPW23 and P_2 is the proportion of morphine in the total mixture.

To evaluate the statistical significance of the synergistic effect, a Student's-test was used to compare the theoretical ED_{50} values calculated as described above with the ED_{50} values experimentally obtained for the drug mixture. For this comparison, the variance of the predicted additive effect was calculated from the fraction (FR) each dose of interest using:

$$Var\ ED_{50\ add} = Var\ ED_{50\ NPB23/NPW23} \times (FR_{NPB23/NPW23})^2 + Var\ ED_{50\ morphine} \times (FR_{morphine})^2$$

The interaction index was calculated as the ratio of experimental ED_{50}/theoretical ED_{50} (Miranda et al., 2008). Values lower than 1 indicate synergistic interactions.

3. Results

3.1. μ opioid receptor and NPBW1 receptor co-localize in the superficial dorsal horn of the spinal cord

First of all, we examined the cellular distribution of μ opioid receptor and NPBW1 receptor in rat spinal cord using immunofluorescent histochemistry. μ opioid receptor (MOR) (Fig. 1A) (red) immunoreactivity was detected in laminae I and II of the dorsal horn. The immunoreaction was particularly dense in the inner part of lamina II (IIi). NPBW1 receptor (NPBWR1) (Fig. 1B) (green) immunoreactive fibers were also observed in laminae I and II. Laminae I was more strongly labeled by NPBW1 receptor staining. In the superficial dorsal horn of the spinal cord a considerably high population of μ opioid receptor positive nerve fibers were co-stained with the NPBW1 receptor (Fig. 1C). Moreover, 71.3% of μ opioid receptor-labeled cells contained detectable NPBW1 receptor (Figs. 1D), and 74% of the NPBW1 receptor -positive cells also expressed μ opioid receptor (Fig. 1E). These results suggested that these two receptors of morphine and NPB23/NPW23 are proximal, therefore, a combination of selective agonists for each receptor might possibly elicit synergistic antinociceptive effects.

3.2. Intrathecal (i.t.) co-administration of morphine-NPB23 or morphine-NPW23 synergistically attenuates acute inflammatory nociception

To determine whether combination of morphine withNPB23 or morphine withNPW23 confers synergistic effects on relieving formalin-induced acute inflammatory pain, rats were intrathecally administered with saline, morphine, NPB23, NPW23, morphine-NPB23 or morphine-NPW23 [1: 1 fixed ratio (ED_{50} of agonist A: ED_{50} of agonist B)), respectively, following intraplantar injection of formalin. Formalin injection caused apparent flinching behaviors i. Phase 1 flinching occurs within 10 min after injection and is characterized by an acute and phasic peak in neuronal firing. Phase 2 is more prolonged and tonic, and occurs between 11 and 60 min after injection. Phase 2 is thought to be associated with an inflammatory response Hunskaar et al. (1985).

In contrast to saline group, morphine (1, 5, 10, 15 μg, i. t., Fig. 2A, *P*

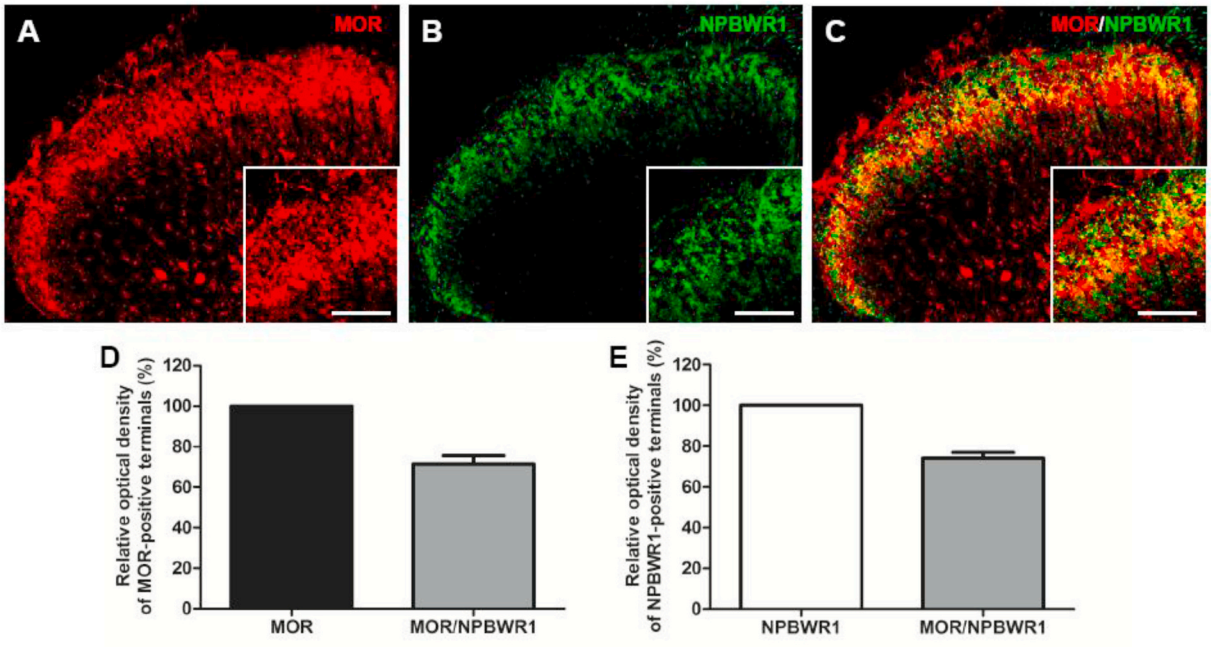

Fig. 1. MOR and NPBWR1 colocalize within rat spinal cord. Double-immunofluorescence staining of MOR (A, red fluorescence) and NPBWR1 (B, green fluorescence) in the superficial dorsal horn of the spinal cord. Dual colocalization of MOR and NPBWR1 (C, yellow) can be observed. Statistical analysis of the relative optical density (ROD) of MOR-positive terminals (D) and NPBWR1-potive terminals (E) in rat spinal cord. The bar = 100 μm n = 5.

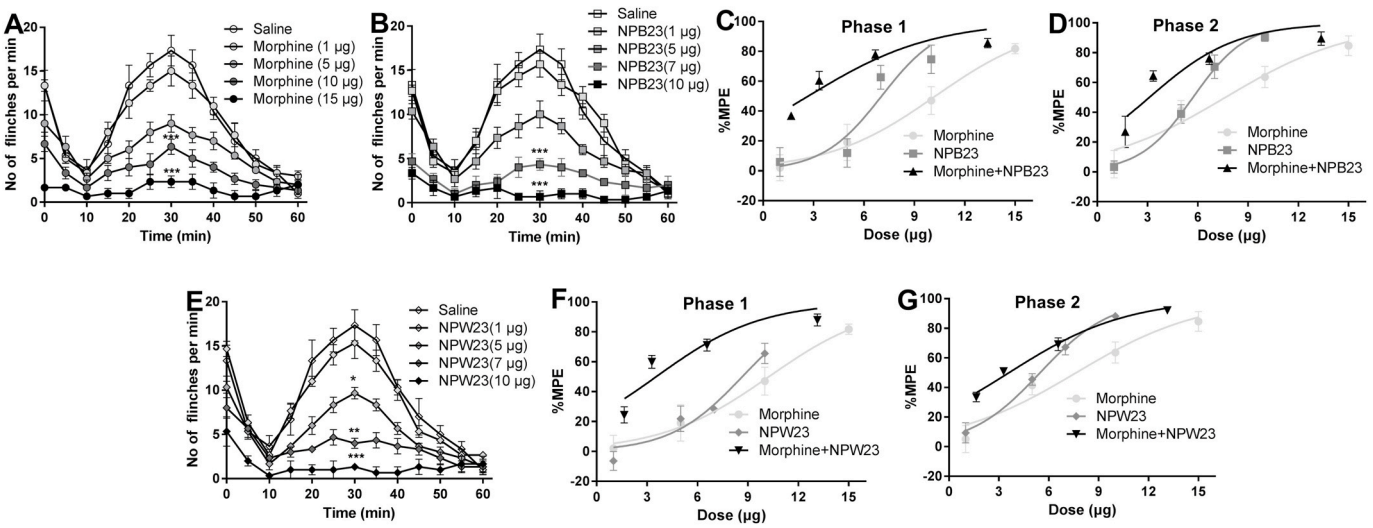

Fig. 2. Combined morphine and NPB23 or NPW23 result in a synergistic inhibition of formalin-induced hind paw flinching. Time- and dose-response curve for the antinociception induced by i. t. injection of (1–15 μg; A) morphine, (1–10 μg; B) NPB23 and (1–10 μg; E) NPW23. The flinches observed after the 5% formalin injection into the plantar surface of the left rat hind-paw. Dose-response analyses illustrate left-ward shifts at 1:1 morphine: NPB23 (C, D) and 1: 1 morphine: NPW23 (F, G) dose ratio in phase 1 and phase 2. (AUC calculated during 0–60 min from these data were statistically analyzed and are presented in the text. $^{*}P < 0.05$ $^{**}P < 0.01$, $^{***}P < 0.001$ versus saline according to repeated measures ANOVAs; n = 6–7).

< 0.05), NPB23 (1, 5, 7, 10 μg, i. t., Fig. 2B, $P < 0.05$) and NPW23 (1, 5, 7, 10 μg, i. t., Fig. 2E, $P < 0.05$) caused a dose-dependent decrease in number of flinches in the phase 1. A significant reduction in the number of flinches was also observed in the inflammatory phase 2 in response to morphine (Time $F_{9,250} = 603.2$, $P < 0.05$; Dose $F_{4,250} = 354.7$ $P < 0.05$; Dose × Time Interaction, $F_{36,250} = 14.4$ $P < 0.05$), NPB23 (Time $F_{9,250} = 553.8$, $P < 0.05$; Dose $F_{4,250} = 346.4$ $P < 0.05$; Dose × Time Interaction, $F_{36,250} = 14.7$ $P < 0.05$) and NPW23 (Time $F_{9,300} = 586.2$, $P < 0.05$; Dose $F_{4,300} = 362.5$ $P < 0.05$; Dose × Time Interaction, $F_{36,300} = 18.4$ $P < 0.05$). The antinociception of NPB23 and NPW23 was inhibited by NPBW1 receptor specific antagonist CYM 50796 (Fig. S4A). Administration of morphine-NPB23 or morphine-NPW23 also reversed the formalin-induced increase in the number of flinches in phase 1, with an ED_{50} of 2.6 μg (Fig. 2C, Table 1) or 3.5 μg (Fig. 2F, Table 1). Likewise, morphine-NPB23 or morphine-NPW23 reversed the number of flinches in phase 2, with an ED_{50} of 3.1 μg (Fig. 2D, Table 1) or 3.6 μg (Fig. 2G, Table 1), respectively. Dose-response relationships of morphine-NPB23 and morphine-NPW23 in phases 1 and 2 exhibited significant leftward shifts relative to those of r morphine, NPB23 and NPW23. Furthermore, the experimentally obtained antinociceptive effects of morphine-NPW23 (Fig. 3A and B and Table 1) and morphine-NPB23 (Fig. 3C and D and Table 1) were much greater than those predicted by their additive effects. Collectively, the administration of morphine-NPB23 and morphine-NPW23 yielded synergistic effects on attenuating acute inflammatory nociception in rats.

3.3. Synergistic effects of morphine-NPB23 or morphine-NPW23 on neuropathic pain

We further assessed the effects of morphine-NPB23 or morphine-NPW23 on neuropathic pain. Neuropathic pain was induced by chronic constriction injury (CCI) of the sciatic nerve. Dose- and time-dependent antinociceptive effects of drug administration indicated in von Frey assays were investigated and shown in Fig. 4. On day 7, following CCI, a baseline mechanical withdrawal latency was assessed. The rats bearing CCI exhibited a significant decrease in mechanical paw withdrawal thresholds (PWT) as compared to non-injured baselines. Rats given intrathecal injection of morphine (1, 3, 5, 7 μg, i. t., Fig. 4A, Time $F_{5,150} = 249.4$, $P < 0.05$; Dose $F_{4,150} = 149.5$ $P < 0.05$; Dose × Time Interaction, $F_{20,150} = 18.2$ $P < 0.05$), NPB23 (1, 5, 7, 10 μg, i. t., Fig. 4B, Time $F_{5,180} = 264.4$, $P < 0.05$; Dose $F_{4,180} = 165.7$ $P < 0.05$; Dose × Time Interaction, $F_{20,180} = 20.4$ $P < 0.05$) or NPW23 (1, 5, 7, 10 μg, i. t., Fig. 4E, Time $F_{5,150} = 242.4$, $P < 0.05$; Dose $F_{4,150} = 175.3$ $P < 0.05$; Dose × Time Interaction, $F_{20,150} = 17.6$, $P < 0.05$) significantly reversed the CCI-induced reduction in mechanical thresholds as compared to sham-operated and saline-treated controls in a dose-dependent manner. The elevated PWT induced by NPB23 and NPW23 was inhibited with NPBW1 receptor specific antagonist CYM 50796 (Fig. S4A). Dose-response curves were plotted, used to determine ED_{50} values and isobolographic analysis from data collected at 30 min time point. In these animals, morphine, NPB23 and NPW23 produced an increase in mechanical PWT with an ED_{50} of 3.8 μg, 4.8 μg, and 5.8 μg, respectively. Theoretical additive ED_{50} of combinations were calculated, with ED_{50} of 4.3 μg and 4.8 μg. Compared with theoretical ED_{50}, morphine-NPB23 (Fig. 4C and Table 1, ED_{50} = 2.5 μg) and morphine-

Table 1
Relative potencies of morphine, NPB23, NPW23 and their combination observed in inflammatory and neuropathic pain models.

	Morphine	NPB23/NPW23	Morphine + NPB23/NPW23 (1:1)	Theoretical additive	Drug interaction
5% formalin flinch					
ED_{50} (μg)	7.7 (0.8)	5.7 (0.8)/5.5 (0.6)	3.1 (0.5)/3.6 (0.4)	6.7 (0.8)/6.5(1.1)	Synergistic/Synergistic
E_{max} (%)	85 (6)	90 (4)/93 (5)	103 (5)/98 (7)	N.D.	
Mechanical PWT (CCI)					
ED_{50} (μg)	3.8 (0.7)	4.8 (0.5)/5.8 (1.2)	2.5 (0.3)/2.2 (0.5)	4.3 (0.9)/4.8 (1.0)	Synergistic/Synergistic
E_{max} (%)	83 (4)	92 (5)/94 (6)	102 (7)/100 (8)	N.D	

Parameters, including ED_{50} (μg), Emax (%) is the maximal possible effect of that agonist. Values are shown as the mean (SEM or rang).

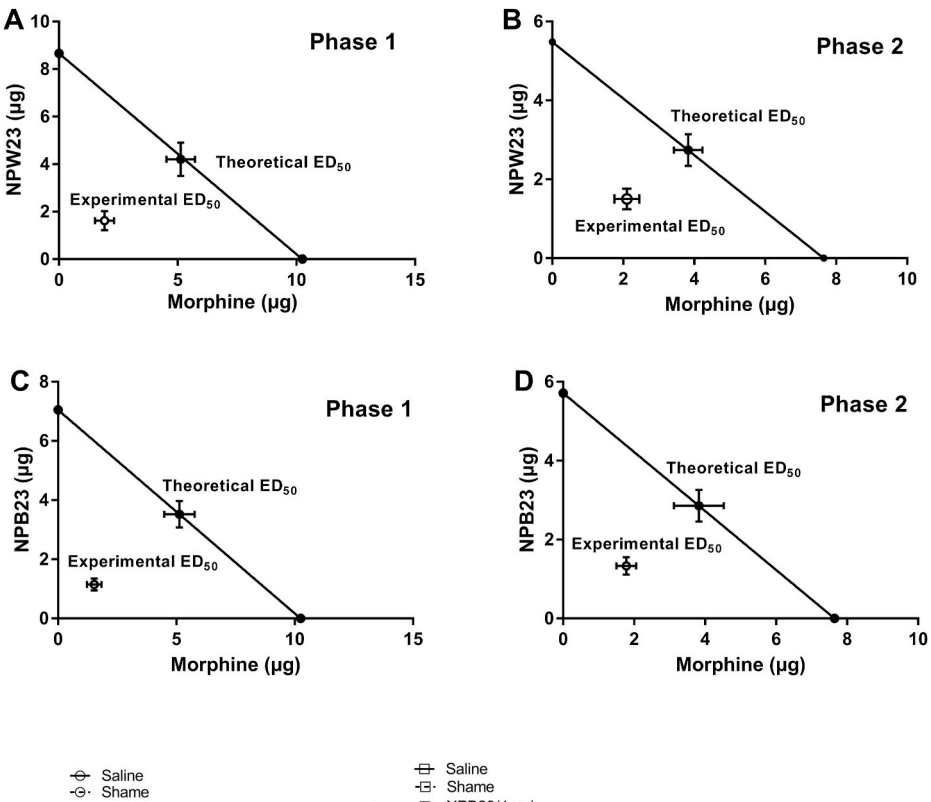

Fig. 3. Isoboles for combined morphine plus NPB23 or NPW23 at a range of effect levels (in 1:1 fixed ratio of ED_{50}). Isobologram for combined between (A, B) morphine-NPB23, (C, D) morphine-NPW23 during phase 1 and phase 2 in the formalin assay. The ED_{50} values for each drug are plotted on the x- and y-axes, respectively. Symbols represent theoretical additive and experimental ED_{50} values, with their associated 95% confidence intervals. The experimental ED_{50} point was significantly different than the theoretical ED_{50} point, indicating a synergistic interaction.

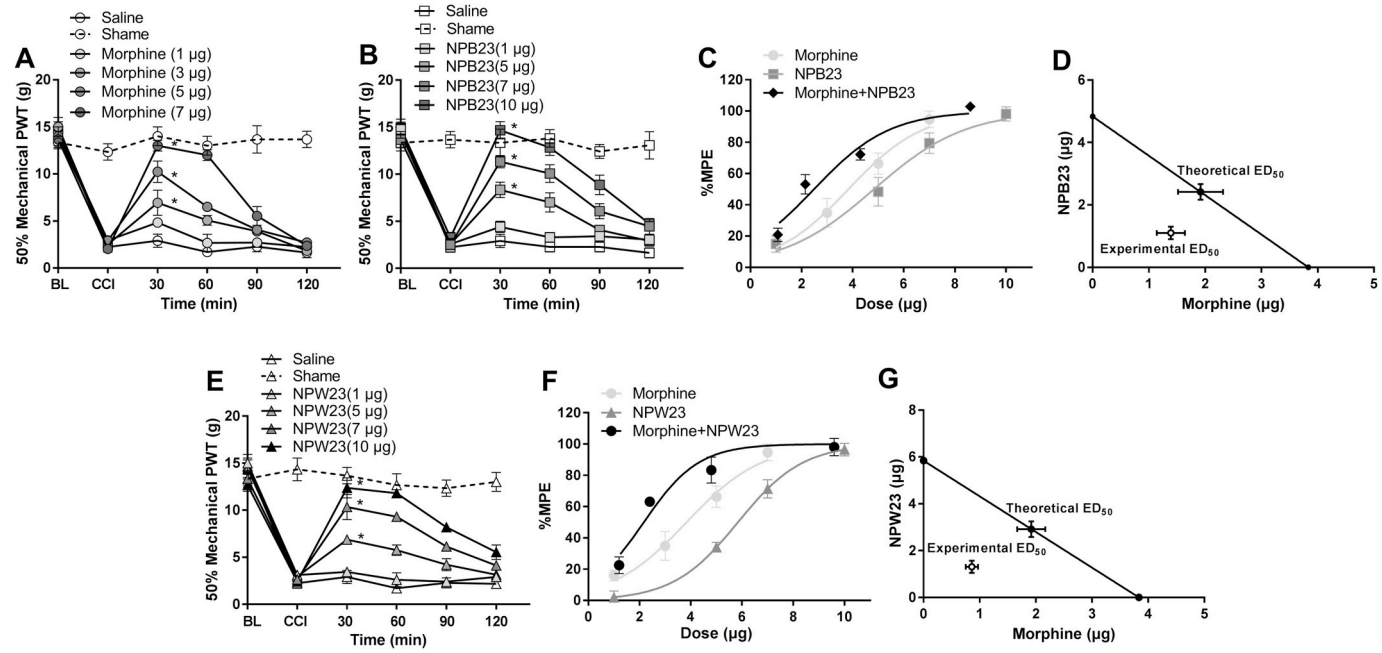

Fig. 4. Synergistic drug interaction between morphine and NPB23 or NPW23 in neuropathic pain. Time- and dose-dependent antinociceptive effects of single i. t. administration of (1–7 μg; A) morphine, (1–10 μg; B) NPB23 and (1–10 μg; E) NPW23 on mechanical PWT. Combination therapy using 1:1 ED_{50} fixed ratio resulted in a significant leftward shift in the dose-response curve for both (C, D) morphine-NPB23 and (F, G) morphine-NPW23, indicative of synergy. Animals received a i. t. injection of morphine, NPB23 or NPW23 at time 0 min, 6–8 days after CCI surgery. BL = baseline. (AUC calculated during 1–120 min from these data were statistically analyzed and are presented in the text. *$P < 0.05$ versus saline according to repeated measures ANOVAs; n = 6).

NPW23 (Fig. 4F and Table 1, ED_{50} = 2.2 μg) conferred a stronger effect on mechanical PWT. Under 1:1 fixed ED_{50} ratios, morphine-NPB23 (Fig. 4D and Table 1) or morphine-NPW23 (Fig. 4G and Table 1) displayed an overt synergistic in the von Frey assay.

3.4. Co-administration of morphine with NPB23/NPW23 attenuates the side-effects of morphine

Morphine carries reward liability and abuse potential. To confirm whether combining morphine with either NPB23 or NPW23 would attenuate rewarding effect of morphine, conditioned place preference (CPP) paradigm was performed. The saline group did not show place preference change, indicating that i. t. injection *per se* did not induce rewarding or aversive behaviors in an unbiased CPP paradigm. Rats treated with NPB23 (Fig. 5A) or NPW23 (Fig. 5B) dose-dependently exerted CPP as morphine, since rats with either NPB23 or NPW23 spent longer time ($P < 0.001$) in the administration chamber as rats with morphine (10 μg, i. t.) compared to saline group. The time spent in the

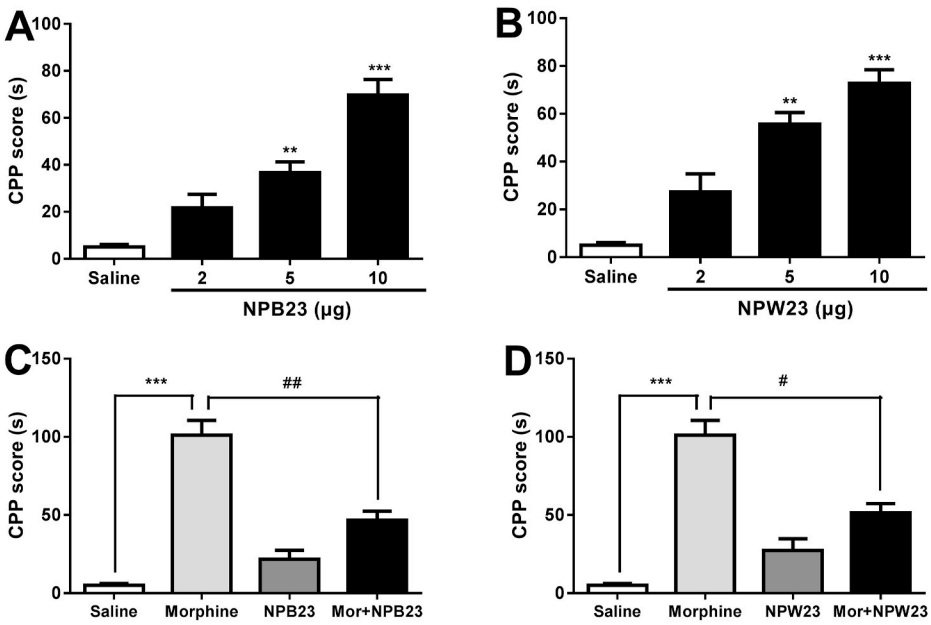

Fig. 5. NPB23 and NPW23 attenuates morphine-induced condition place preference.

chamber at low dose NPB23 (2 μg, i. t.) or NPW23 (2 μg, i. t.) was not significantly increased compared to the saline group. Moreover, rats with morphine-NPB23 (10 μg-2μg, $P < 0.01$) or morphine-NPW23 (10 μg-2μg, $P < 0.05$) showed decreased time in the administration chamber compared with morphine, indicative of overall effects of NPB23 (Fig. 5C) and NPW23 (Fig. 5D) on preventing morphine-induced CPP. In

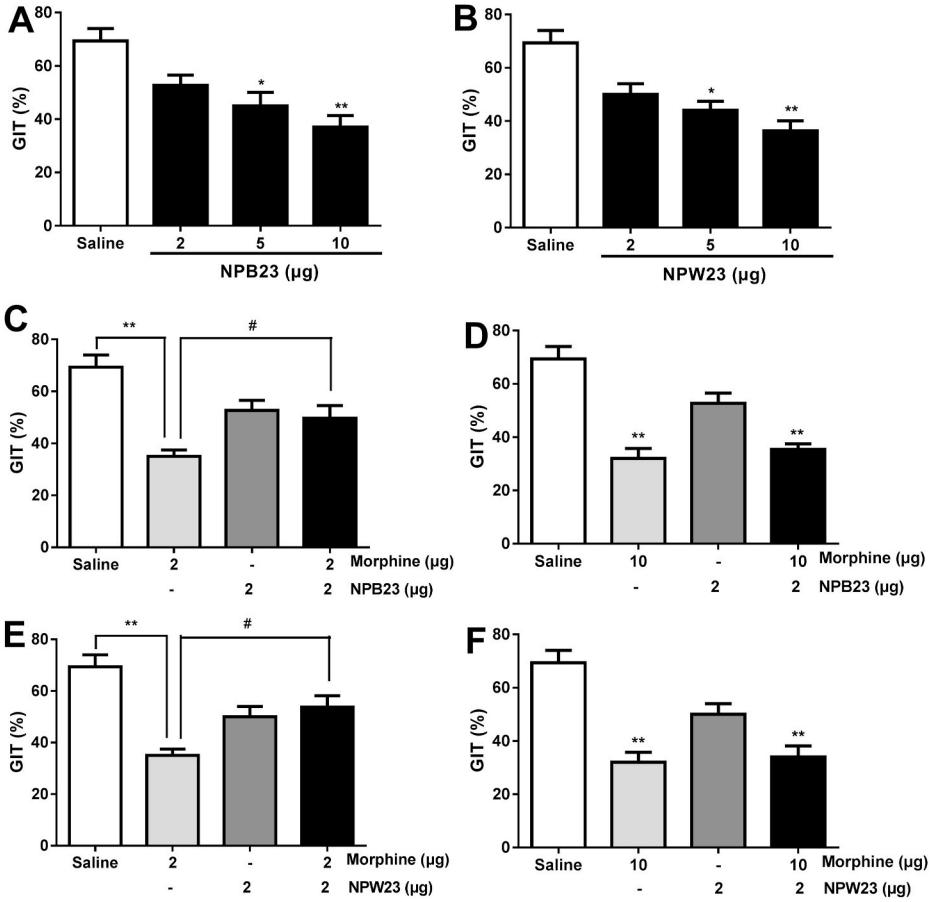

Fig. 6. The effects of NPB23 and NPW23 attenuate morphine-induced constipation. Gastrointestinal transit was evaluated in rats treated with (A) NPB23, (B) NPW23 or their combination. (C, D) NPB23 or (E, F) NPW23, at low dose, had no effect alone and reinstated gastric transit to saline-treated levels with low, but not high, doses of morphine. (*$P < 0.05$, **$P < 0.01$ versus saline and #$P < 0.05$ versus morphine according to one-way ANOVA followed by the Tukey HSD test; n = 5–6).

conclusion, combining NPB23 or NPW23 with morphine had overtly greater effects on antinociception but reduced CPP than morphine alone.

Opioid-induced constipation is a common problem associated with chronic use of opioids. To further investigate whether co-administration of morphine-NPB23 or morphine-NPW23 would alter morphine-induced constipation. We examined the effect of morphine, NPB23, NPW23 and their combination on gastrointestinal transit (GIT) in rats. Low dose NPB23 (2 μg, i. t., 52.7%) and NPW23 (2 μg, i. t., 50.0%) did not cause a profound decrease in GIT compared with the saline group (69.3%) (Fig. 6A and B). We also examined whether administration of morphine (low dose at 2 μg and high dose at 10 μg) with or without NPB23 or NPW23 led to constipation. The results showed that both low dose (35.0%) and high dose (32%) morphine significantly ($P < 0.01$) caused a marked decrease in GIT compared with the saline-treated group (69.3%). Interestingly, low dose morphine-induced constipation was attenuated by co-administration of NPB23 (49.7%, $P < 0.05$, Fig. 6C) or NPW23 (53.7%, $P < 0.05$, Fig. 6E). While either NPB23 (35.3%) or NPW23 (34.2%) did not restore GIT affected by high dose morphine.

3.5. NPBW1 receptor might enhance the sensitivity of μ opioid receptor

It had been reported that phosphorylation of ERK1/2 and reduction of cAMP are results of activation μ opioid receptor or NPBW1 receptor activation (Sakurai, 2013; Belcheva, 2001). To examine whether NPB23 or NPW23 could modulate the morphine-induced downstream signaling cascade, we evaluated the alteration of ERK1/2 phosphorylation in HEK293 cells co-expressing μ opioid receptor andNPBW1 receptor. Cells were treated with 10^{-11}-10^{-7} M of either NPB23, NPW23, or morphine alone or in combination [morphine + NPB23 (1:1) or morphine + NPW23 (1:1)] for 10 min followed by lysis and Western blot analysis (Fig. 7). Morphine, NPB23 andNPW23 significantly stimulated ERK1/2 phosphorylation in a concentration-dependent manner, respectively. At lower concentration (10 pM), either drug caused ERK1/2 phosphorylation, whereas combining morphine with either NPB23 or NPW23 induced much stronger phosphorylation at the same concentration (Fig. 7). Similarly, morphine and NPB23/NPW23 have a synergistic effect on cAMP production (Fig. S5). The results implied that NPBW1 receptor might enhance the sensitivity of the μ opioid receptor, therefore, the μ opioid receptor exhibited an active state with low dose morphine.

4. Discussion

The ultimate goal of a drug combination is to potentiate therapeutic effects but to attenuate adverse side-effects of individual drugs. Evidence indicates that drug combination has therapeutic potential as antinociception with reduced side-effects. (Yibo et al., 2014; Cun et al., 2018). In the present study, we examined antinociceptive efficacy of a combination of morphine-NPB23 or morphine-NPW23 in the treatment of inflammatory pain and neuropathic pain. We also quantitatively evaluated the interaction between NPB23/NPW23 and morphine in terms of antinociception and some side-effects. We found that μ opioid receptor and NPBW1 receptor were co-localized in the superficial dorsal horn of the spinal cord, where it is known to be highly implicated in antinociception (Jackson et al., 2006). The co-expression suggests that agonist of μ opioid receptor and NPBW1 receptor may simultaneously regulate neurons in the superficial dorsal horn of the spinal cord. Indeed, we showed for the first time that μ opioid receptor agonist, morphine, and NPBW1 receptor agonists, NPB23 and NPW23, synergistically relieved inflammatory pain and neuropathic pain, while preventing the induction of morphine-induced rewarding behaviors and constipation.

NPB23 and NPW23 dose-dependently relieved acute inflammatory painand mechanical allodynia induced by CCI, and these are in line with the previous studies. (Yamamoto et al., 2005, 2006). Moreover, the NPB23 and NPW23 produced antinociception through NPBW1 receptor but not opioid receptor (Paola F. et al., 2005; Yamamoto et al., 2005). NPB23 and NPW23 showed dose-dependent side-effects, including rewarding effect and constipation. However, at a low dose (2 μg), they displayed no potency to induce these side-effects. Note that NPB23 and NPW23 caused the side-effects and antinociception with different dose-relationships. NPB23 and NPW23 exhibited a higher potency in reducing CCI-induced mechanical allodynia and acute inflammatory pain than in inducing side effects.

Like NPB23 and NPW23, morphine exhibited attenuation in the acute inflammatory nociception and mechanical allodynia induced by CCI with high efficacy, consistent with previous studies (Bian et al., 1995). Morphine also produced rewarding effect and constipation. Unlike NPB23 and NPW23, the doses of morphine caused antinociception and side-effects were largely overlapped. We observed that morphine induced acute constipation at dose as low as 2 μg (i.t.), indicating that morphine produced antinociception simultaneously with constipation.

Recently, drugs with multimodal targets have been pursued to confer profound analgesic effects, but to suppress unwanted side-effects. Considering that μ opioid receptor and NPBW1 receptor are co-localized in the spinal cord and share similar signal transduction pathways, their agonists may elicit synergistic antinociceptive actions. We revealed that combinatory activation of μ opioid receptor and NPBW1 receptor was beneficial compared to activating μ opioid receptor alone.

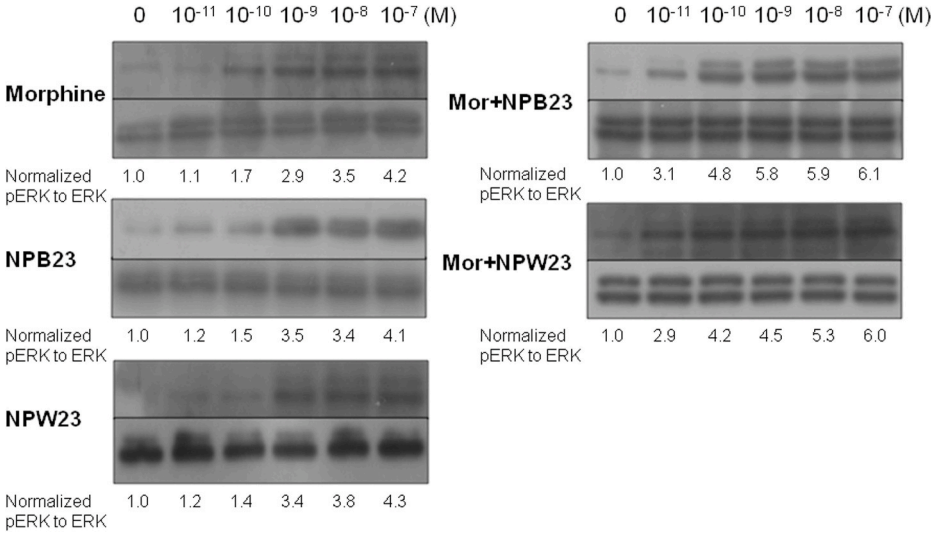

Fig. 7. Morphine, NPB23, NPW23 or their combination stimulated ERK1/2 phosphorylation in HEK293 cells expressing human MOR and NPBWR1. HEK293 cells stably expressing MOR and NPBWR1 were treated with morphine, NPB23, NPW23, morphine + NPB23 (1:1), morphine + NPW23 (1:1) for the indicated concentrations (mole). Compared with administration of morphine, NPB23, NPW23 alone, their combination exhibited higher potencies for ERK1/2 phosphorylation. Results are representative of at least three independent experiments.

The isobolographic analysis was used to confirm if morphine andNPB23 or NPW23 interact synergically. In our analysis, only a single fixed ratio (1:1 ED_{50} dose) for combination was used. It remains to be determined whether this is the optimal dose ratio for reversing acute inflammatory pain and neuropathic pain (Grenald et al., 2017). In our study, the combination of μ opioid receptor agonist + NPBW1 receptor agonist synergistically reduced both acute inflammatory pain and mechanical allodynia in a neuropathic pain. Furthermore, combinatory therapy resulted in a significant increase in the maximal antinociceptive efficacy compared with administration of either drug. Thus, a combination of morphine andNPB23 orNPW23 may be an advancement of therapeutic strategy to treat both inflammatory pain and neuropathic pain.

Opioid analgesics have propensity to induce undesirable rewarding effects and constipation. In this study, we showed that NPBW1 receptor agonists, NPB23 and NPW23, produced dose-related CPP and GIT impairmentsimilar to morphine. The combination of morphine with NPB23 or NPW23 had a synergistic rewarding effect and caused constipation. But, the synergistic analgesic action of morphine-NPB23 or morphine-NPW23 could be an offset of these side effects (Stone et al., 2014). The present results showed that NPB23, NPW23 alone had no significant effect on CPP and GIT at low dose (2 μg, i. t.). Interestingly, the intrathecal administration of low dose NPB23 or NPW23 profoundly reduced the low dose morphine-induced rewarding effect. In GIT assays, NPB23 and NPW23, blocked slowness of GIT induced by low dose morphine (2 μg), but not that induced by high dose morphine (10 μg). Because μ opioid receptor agonist with NPBW1 receptor agonists had a synergistic interaction in relief of inflammatory pain and neuropathic pain, it can explain our observation that coadministration of morphine with NPB23 or NPW23 significantly reduced the doses of opioids required for antinociception, which may limit rewarding effects and GIT impairment.

The common mechanisms underlying the synergistic antinociception by opioid agents and other analgesics include the co-expression of their receptors in pain transmission circuits and the similarity of signal transduction of these receptors (Tham et al., 2005; Schoffelmeer et al., 2006; Rodrigue et al., 2001). For instance, Co-administration of μ opioid receptor/cannabinoid receptor agonists synergistically inhibits preclinical inflammatory pain, post-operative pain and neuropathic pain because μ opioid receptor is co-localized with cannabinoid receptor on neurons, and μ opioid receptor and cannabinoid receptor share similar downstream signaling cascades (Salio et al., 2001; Seely et al., 2012). The assumption is that both receptors coexist on neurons and therefore share a common pool of G proteins. The coupling of these receptors to a common G protein family may lead to inter-receptor signaling whereby activation of one receptor causes the redistribution of its G proteins, which increase the sensitivity of the other receptor (Djellas et al., 2000; Ashish et al., 2018). There were some differences in the distribution of the μ opioid receptor and NPBW1 receptor in the dorsal horn. An interaction between morphine and NPB23/NPW23 at this level would imply an indirect mechanism.

Recent studies reported that ERK1/2 pathway had an influence on neuropathic pain and formalin-induced pain (Xinqiang et al., 2020; Ghallab et al., 2019). Moreover, studies have shown that ERK1/2 signaling played important roles in the synergistic effects of two drugs (Yibo et al., 2014; Cun et al., 2018). Hence, we investigated the role of the ERK1/2 pathway in the synergistic effects betweenNPB23/NPW23 and morphine. We found that NPB23 and NPW23 effectively potentiated the phosphorylation of ERK1/2 induced by morphine. Our results revealed that ERK1/2 phosphorylation is a crucial event to mediate synergistic antinociception caused by morphine and NPB23/NPW23.

In summary, our data indicate that intrathecal co-administration of morphine and NPB23 or morphine andNPW23 significantly inhibited acute inflammatory pain and CCI-induced mechanical allodynia in a synergistic manner. In addition, the combination of a μ opioid receptor agonist and NPBW1 receptor agonists at optimal ratios significantly decreased dose of individual drugs required for antinociception, and potentially attenuated rewarding effects of morphine and morphine-induced GIT,. The synergistic effect also appeared in the phosphorylation of ERK1/2. Together, the combination therapies of morphine and NPB23or NPW23 provides a new avenue to treat inflammatory and neuropathic pain with less risk of adverse effects of morphine including rewarding effects and constipation.

CRediT authorship contribution statement

Yanhong Xing: performed the research, designed the research study, analyzed the data, Writing – original draft. **Yao Liu:** performed the research. **Mengqiu Deng:** performed the research. **Hui-Ping Wang:** contributed essential tools. **Zhe Zhang:** designed the research study, Writing – original draft. **Jun-Li Cao:** designed the research study, analyzed the data, Writing – original draft.

Declaration of competing interest

The authors state no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejphar.2021.173979.

The effects of i. t. administration of (A) NPB23 or (B) NPW23 alone on place conditioning in rats. Low dose (C)NPB23 (2 μg, i. t.) or (D) NPW23 (2 μg, i. t.) alone did not result in a positive CPP and when co-administered with morphine resulted in a significant attenuation of morphine-induced CPP. CPP score was expressed as time spent in the drug-associated compartment on the post-conditioning day minus time spent in the drug-associated compartment during a period of 15 min on the pre-conditioning day. ($^{**}P < 0.01$, $^{***}P < 0.001$ versus saline and $\#P < 0.05$, $\#\#P < 0.01$ versus morphine according to one-way ANOVA followed by the Tukey HSD test; n = 5–6).

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