Concurrent bullatine A enhances morphine antinociception and inhibits morphine antinociceptive tolerance by indirect activation of spinal κ-opioid receptors

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A R T I C L E   I N F O

Chemical compounds studied in this article:
Bullatine A (PubChem CID: 102040922)
dynorphin A(1−17) (PubChem CID: 91928823)
morphine (PubChem CID: 5464110)

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A B S T R A C T

Ethnopharmacological relevance: Bullatine A, a C20-diterpenoid alkaloid and one of the major effective ingredients in Aconiti brachypodi Radix (Xue-shang-yi-zhi-hao), can block pain hypersensitivity in a variety of rodent models through expression of spinal microglial dynorphin A.

Aim of the study: To assess the interaction between bullatine A and morphine on antinociception in acute nociception and pain hypersensitivity states, with the exogenous synthetic dynorphin A as a comparison

Materials and methods: Spinal nerve ligation-induced neuropathic rats and naïve mice were used for assessing the acute and chronic interactions of bullatine A/dynorphin A with morphine.

Results: Single subcutaneous injection of bullatine A or dynorphin A(1−17) did not either alter formalin- and thermally (hot-plate and water immersion tests)-induced acute nociception or potentiate morphine antinociception in naïve mice. In contrast, bullatine A dose-dependently inhibited formalin-induced tonic pain with the efficacy of 54% inhibition and the half-effective dose of 0.9 mg/kg. Concurrent bullatine A additively enhanced morphine antinociception. In neuropathic rats, the antinociceptive effects of multiple bidaily intrathecal injections of bullatine A and dynorphin A remained consistent over 13 days, whereas morphine produced progressive and complete tolerance to antinociception, which was completely inhibited by concurrent bullatine A and dynorphin A. A single intrathecal injection of bullatine A and dynorphin A immediately reversed established morphine tolerance in neuropathic rats, although the blockade was a less degree in the thermally induced mouse acute nociceptive tests. The inhibitory effects of bullatine A and dynorphin A on morphine tolerance were immediately and completely attenuated by intrathecal dynorphin A antibody and/or selective κ-opioid receptor antagonist GNTI.

Conclusion: These results suggest that bullatine A produces antinociception without induction of tolerance and inhibits morphine antinociceptive tolerance, and provide pharmacological basis for concurrent bullatine A and morphine treatment for chronic pain and morphine analgesic tolerance.

1. Introduction

Approximately 76 Aconitum species have been used for medicinal purpose in China and other Asian countries. Aconiti brachypodi Radix (Xue-shang-yi-zhi-hao), the dried root of Aconitum brachypodum diels and several other morphologically similar species (genus Aconitum, family Ranunculaceae), possesses analgesic and anti-rheumatic properties recognized by the Chinese Pharmacopoeia (China Pharmacopoeia Committee, 1977; Ren et al., 2012; Xiao et al., 2006). The bioactive extracts of Aconiti brachypodi Radix, in forms of pills, liniment, patch and injection, are widely prescribed to manage chronic pain, rheumatic arthritis, and traumatic injuries in China. Bullatine A, a C20-diterpenoid alkaloid, is one of the major effective and quality-controlled ingredients identified in Aconiti brachypodi Radix (Li et al., 2013; Teng et al., 2015). In our recent study, we demonstrated that bullatine A was effective in blockade of pain hypersensitivity in a variety of rodent pain models. Specifically, a systemic injection of bullatine A was able to effectively attenuate mechanical allodynia and/or thermal hyperalgesia induced by spinal nerve ligation, bone cancer cell inoculation, streptozotocin (STZ) diabetes, or complete Freund’s adjuvant (CFA)-induced rheumatic inflammation, with the efficacy of 45% –70% maximal possible effect, and ED50 value of 0.9–1.9 mg/kg. However,
systemic bullatine A was not effective in attenuation of acute nociceptive pain (Huang et al., 2016). This demonstrated the specific antinociceptive property of bullatine A in pain hypersensitivity, such as mechanical allodynia and thermal hyperalgesia, which is further supported by another recent study showing that bulleyaconitine A, a C19-diterpenoid alkaloid, markedly suppressed peripheral nerve injury-, bone cancer cell inoculation- and formalin-induced allodynia and hyperalgesia (Li et al., 2016a).

Bullatine A treatment could specifically stimulate primary cultures of microglia (but not astrocytes or neurons) to express dynorphin A but not proinflammatory cytokines. Intrathecal injection of bullatine A markedly suppressed spinal nerve ligation-induced mechanical allodynia and specifically simulated expression of dynorphin A but not proinflammatory cytokines in spinal homogenates of rats. The stimulatory effects of bullatine A on dynorphin A expression and secretion were totally attenuated by the microglial inhibitor minocycline (Bhandare et al., 2016, 2015). More directly, the double immunofluorescence staining showed that bullatine A-stimulated dynorphin A in the spinal dorsal horn was specifically expressed in microglia but not in neurons or astrocytes. Given intrathecally, bullatine A antinociception in neuropathic rats was completely abolished by intrathecal injection of minocycline, the dynorphin A antibody and 5′-guanidinonaltrindole (GNTI), a highly selective κ-opioid receptor antagonist (Huang et al., 2016). These results indicated that bullatine A produced anti-hyperalgesia activity by specific stimulation of dynorphin A expression in spinal microglia. The same mechanism for aconitines to produce antinociception is expanded by recent studies of aconite and bullataconitine A (Li et al., 2016a, 2016b, 2017).

To date, morphine is recognized as the gold standard treatment for severe pain, although morphine can induce serious side-effects, such as constipation, nausea/vomiting, somnolence, and respiratory depression, significantly decreasing the quality of life of patients (Gong et al., 2012; Resine and Pasternak, 1996). Besides, repeated morphine administration can also cause morphine analgesic tolerance, which is characterized by a reduced sensitivity to its antinociceptive effects and requires a higher dose to achieve the desired analgesic effect (Bekhit, 2010). To sustain its clinical analgesic effect, repeated administrations and increased doses of morphine (to as high as 2000 mg as reported) (Gong et al., 2012; Schneider et al., 2009) are often required and thus bring unavoidable risks and suffering to patients. Therefore, analgesic agents are often used concurrently with morphine to reduce the dosage of morphine and attenuate its unwanted adverse effects, and potentially attenuate morphine analgesic tolerance. Thus far, many target molecules have been studied to implicate their roles in morphine analgesic tolerance, including N-methyl-D-aspartate (NMDA) receptors (Liu et al., 2015; Mendez and Trujillo, 2008; Shu et al., 2008), κ-opioid receptors (Shu et al., 2008; Takahashi et al., 1991; Tokuyama et al., 2007), α2-adrenoceptors (Nakagawa et al., 2012; Ozdogn et al., 2004), and n-amino acid oxidase (Gong et al., 2014b, 2012; Ma et al., 2015).

Given that morphine and the Aconitum extracts as well as their purified ingredients are widely used in China and other Eastern Asian countries, it is very likely that these two classes of drugs are used concurrently in the clinic. This study aimed to elucidate their interaction in respect to antinociception and tolerance to antinociception. Moreover, κ-opioid receptors are known to interact with various µ-opioid (e.g., morphine)-mediated biological functions, including analgesia, antinociceptive tolerance, and reward, and may be a potential target molecule for attenuation of morphine antinociceptive tolerance (Pan, 1998; Tokuyama et al., 2007). It would provide useful neurobiological information to assess the blockade effect of bullatine A on morphine antinociceptive tolerance and explore its underlying mechanism, as bullatine A-induced antinociception is known to be entirely mediated by spinal microglial dynorphin A expression (Huang et al., 2016). The following research protocols were included in this study: i). Acute (single injection) interaction between subcutaneous administration of bullatine A and morphine in nociception in naïve mice and neuropathic rats; ii). Chronic (up to 13-d treatments) interaction between systemic or intrathecal morphine and bullatine A in antinociception and tolerance to antinociception in naïve mice and neuropathic rats; iii) The comparison with the synthetic dynorphin A throughout the study; and iv). The mechanism underlying bullatine A blockade of morphine antinociceptive tolerance. We expected to provide pharmacological and translational medicinal basis for concurrent uses of morphine and aconitines in chronic pain.

2. Material and methods

2.1. Drugs and reagents

Bullatine A was purchased from the National Institute for the Food and Drug Control (Beijing, China) with purity not less than 98%, determined by the manufacturer with high performance liquid chromatography (HPLC). Its molecular weight was verified in house by a high-resolution mass spectrum (Waters Corporation, Milford, MA, USA). Dynorphin A(1-17) with peptide sequences of YGGFLRIRPRKLKDQNG was synthesized by Dan Gang Peptides Co. (Hangzhou, China) with purity not less than 98%. Morphine hydrochloride, lidocaine hydrochloride, and minocycline were obtained from the Northeast Pharmaceuticals Group (Shenyang, China), the First Chengdu Pharmaceuticals Group (Chengdu, China) and Yuanye Biotech (Shanghai, China), respectively. 5′-Guainidinonaltrindole (GNTI) was from Sigma-Aldrich (St. Louis, MO, USA). The rabbit polyclonal antibodies neutralizing dynorphin A were purchased from Phoenix Pharmaceuticals (Burlingame, CA, USA), with specificity to dynorphin A (100%), but not to dynorphin B (0%), β-endorphin (0%), α-neo-endorphin (0%), or leu-enkephalin (0%) according to the manufacturer’s descriptions. Its specificity was also validated by the antigen absorption test from other laboratories (Wakabayashi et al., 2010; Yamada et al., 2013). All the drugs and reagents were dissolved or diluted in 0.9% normal saline.

2.2. Experimental animals

Male adult Swiss mice (18–20 g body weight) and Wistar rats (160–170 g body weight) were purchased from the Shanghai Experimental Animal Institute for Biological Sciences (Shanghai, China). The animals were caged 4–6 in the Shanghai Jiao Tong University Experimental Animal Center (Shanghai, China) at standard room temperature (22 ± 2 °C), under conditions of a 12/12-hr reversed light-dark cycle (7:00 a.m.–7:00 p.m.), and received food and water ad libitum. They were accustomed to the laboratory environment for 3 days before the experiments. Experimental study groups (n = 6–8 in each group) were assigned randomly, and the researcher was blinded for the behavior testing. The research protocols were approved by the Animal Care and Welfare Committee of Shanghai Jiao Tong University and carried out in accordance with the animal care guidelines of the National Institutes of Health.

2.3. Mouse formalin test

Animals were acclimated individually to the observation cage for 30 min prior to test. The formalin test was performed, as described previously (Gong et al., 2014a) to subcutaneously inject 10 μL of 5% formalin in 0.9% saline to the dorsal side of the left hindpaw. The animal was immediately placed in a transparent polycarbonate box to monitor their behaviors. The duration of nociceptive behaviors (licking/biting) was manually quantified in the pooled durations at 0–5 and 20–40 min that were considered as the acute phase nociception and tonic phase pain, respectively.
2.4. Mouse hot-plate and tail-immersion test

Pain reflex in response to thermal stimulus in the hot plate was measured using the YLS-6B Intelligence Hot-Plate Analgesia Meter (Shandong Academy of Medical Sciences Device Station, Shandong, China). The surface of the hot plate was heated to a constant temperature at 55 ± 0.1 °C, as measured by a built-in digital thermometer with an accuracy of 0.1 °C and verified by a surface thermometer. During the test, mice were placed on the hot plate, which was surrounded by a clear acrylic cage, and the start/stop button on the timer was activated. The latency to respond with either hindpaw lick or jump (whichever came first) was measured to the nearest 0.1 s by deactivating the timer when the response was recorded. The mouse was then immediately removed from the hot plate and returned to its housing cage. Trials were terminated if the animals did not respond within 40 s to avoid tissue damage.

2.5. Mouse intrathecal injection

The intrathecal injection followed the previously described procedure (Zhu et al., 2014). In brief, a 10-μL microsyringe with a tube to deliver test drugs was inserted into the skin and through the L5 to L6 intervertebral space directly into the subarachnoid space. A tail flick provided a reliable indicator that the needle had penetrated the spinal arachnoid mater. The vehicle and test drugs in 5 μL, sufficiently enough to consistently spread to the caudal thoracic vertebrae, where there is the lumbar enlargement of the spinal cord, were injected into the subarachnoid space. The injection success rate, confirmed by 2% lidocaine injection, was consistently over 95% in our laboratory.

2.6. Rat intrathecal catheterization and injection

The rats were under inhaled isoflurane anesthesia (i.e., 4% for induction and 1% for maintenance) run by an anesthesimeter (Ugo Basile Gas Anesthesia System, Comerio, Italy). A 18-cm polyethylene catheter (PE-10: 0.28 mm inner diameter and 0.61 mm outer diameter; Clay Adams, Parsippany, NJ, USA), in a volume of 13 μL, was inserted into the rat lumbar level of the spinal cord, with approximately 6-cm long tubing inside the subarachnoid space and the rest tubing placed subcutaneously and externally for the skin fixation and injection (Storkson et al., 1996). Two days after recovery from anesthesia, the right installation of the catheter in the spinal cord was verified by administering 4% lidocaine (10 μL followed by 15 μL of saline flushing) through the catheter. The intrathecal cannulation was considered successful when the rat did not show motor impairment following insertion of the intrathecal catheter and developed immediate bilateral paralysis of the hindlimbs following lidocaine. For the intrathecal delivery, 10 μL of the drug solution was administered in a 50-μL microinjector (Shanghai Anting Micro-Injector Factory, Shanghai, China) followed by a 15-μL saline flush.

2.7. Rat model of neuropathic pain and behavioral assessment of mechanical allodynia

To induce neuropathic pain, rats were subjected to spinal nerve ligation as described previously (Chung et al., 2004; Kim and Chung, 1992; Zhang et al., 2013). Spinal nerve ligation was used to induce peripheral neuropathy. Under inhaled isoflurane anesthesia (4% for induction and 1% for maintenance) delivered by an anesthesimeter (Ugo Basile Gas Anesthesia System), the left L5 and L6 spinal nerves were isolated and tightly ligated with 6-0 silk thread. After ligation, the wound was sutured and the rats were allowed to recover. After that, only those animals with marked unilateral allodynia to mechanical stimulation (hind limb withdrawal thresholds in the operated side < 8 g) and no major impairment were included in the experiment. Since intrathecal injection was needed in neuropathic rats in this study, the intrathecal catheterization was performed at the same time just before spinal nerve ligation. Drug testing started on 2–4 weeks after surgery of spinal nerve ligation.

To evaluate mechanical allodynia, the animals were acclimatized for at least 30 min to the test environment, namely a plexiglass box on a metal grid. The hind paw withdrawal threshold was measured by a 2391 CE Electronic Von Frey hair (ITTC Life Science Inc, Woodland Hill, CA, USA). The monofilament (with forces ranging between 0.1 and 90 g) was applied to the footpad with increasing force until the rats suddenly withdrew their hindlimbs. The lowest force producing a withdrawal response was considered as the threshold. Three repeated measurements were made at intervals of approximately 3 min each, and the mean of the three threshold values was averaged for each hind paw at each time-point.

2.8. Data calculation and statistical analysis

For the dose-response curve analysis, the parameters, minimum or maximum effect (E_{max}), half-effective dose (ED_{50}), and Hill coefficient (n), were calculated by fitting nonlinear least-squares curves to the relation Y = a + bx, where x = [D]^{n}/(ED_{50}^{n} + [D]^{n}). The value of ED_{50} and b (E_{max}) was projected by yielding a minimum residual sum of squares of deviations from the theoretical curve according to previous studies (Wang and Pang, 1993; Gong et al., 2012; Zhang et al., 2013). For the drug interaction analysis, value of the theoretic additive ED_{50} was calculated according to the previously described method (Tallarida et al., 1989, 1997) and the drug interactions were presented in the conventional isobolography (Gong et al., 2012; Tallarida et al., 1989; Zhang et al., 2005).

The data were summarized and expressed as means ± SEM or with 95% confidence limits. The statistical significance was evaluated by one-way or two-way repeated-measures ANOVA using Prism (version 5.01) (GraphPad Software, San Diego, CA, USA). The post-hoc Student–Newman–Keuls test was conducted when the effect of the drug (dose) (for one-way ANOVA, the factor was drug [dose]; for two-way ANOVA, the factors were drug [dose], time and their interaction) was observed to be statistically significant. The probability values were two-tailed and the statistical significance was set to be 0.05.

3. Results

3.1. Single subcutaneous injection of bullatine A additively enhanced morphine antinociception in mice

To examine the interaction between bullatine A and morphine in formalin-induced acute nociception and tonic pain, the antinociceptive effects of bullatine A, morphine, and their combination were studied in a total of twenty groups of naïve mice. The first seven groups of mice received subcutaneous injection of 10 mL/kg saline or bullatine A (0.1, 0.3, 1, 3, 10, or 30 mg/kg). The second seven groups of mice received subcutaneous injection of 10 mL/kg saline or morphine (0.03, 0.1, 0.3, 1, 3, or 10 mg/kg). The last six groups of mice received subcutaneous injection of 10 mL/kg saline or five doses of the combination of bullatine A and morphine in a fixed dose ratio of 3:1, i.e., 0.1 mg/kg bullatine A +0.03 mg/kg morphine, 0.3 mg/kg bullatine A +0.1 mg/kg morphine, 1 mg/kg bullatine A +0.3 mg/kg morphine, 3 mg/kg bullatine A +1 mg/kg morphine, and 10 mg/kg bullatine A +3 mg/kg morphine. All mice were treated with the test articles 30 min before...
formalin challenge.

Our data showed that subcutaneous injection of bullatine A up to 30 mg/kg did not significantly inhibit formalin-induced acute biting/licking response, whereas subcutaneous morphine markedly attenuated acute nociception in a dose-dependent manner, with the ED\textsubscript{50} value of 1.2 mg/kg (95% confidence intervals: 0.8–1.8 mg/kg) (Fig. 1A). Furthermore, in the presence of bullatine A, subcutaneous morphine produced similar dose-dependent inhibition of acute phase nociception, with the ED\textsubscript{50} value of 0.7 mg/kg (95% confidence intervals: 0.5–1.1 mg/kg), which was not significantly different from that of morphine alone (Fig. 1B). In contrast, unlike giving bullatine A alone, subcutaneous bullatine A in the presence of morphine exhibited dose-dependent inhibition of acute nociception, with the ED\textsubscript{50} value of 2.3 mg/kg (95% confidence intervals: 1.5–3.5 mg/kg), which was not significantly different from that of morphine alone (Fig. 1C).

In the tonic phase, subcutaneous injection of bullatine A and morphine produced a dose-dependent inhibition of formalin-induced tonic biting/licking responses, with the ED\textsubscript{50} value of 0.9 mg/kg (95% confidence intervals: 0.4–1.9 mg/kg) and 0.6 mg (95% confidence intervals: 0.4–0.8 mg/kg), and \( E_{\max} \) values of 54% and 100% inhibition, respectively. Subcutaneous injection of their combination in a fixed dose ratio of 3:1 also produced dose-dependent inhibition of tonic pain (Fig. 1D). The analgesic ED\textsubscript{50} value of morphine in the presence of bullatine A was 0.2 mg/kg (95% confidence intervals: 0.1–0.4 mg/kg), and the ED\textsubscript{50} value of bullatine A in the presence of morphine was 0.4 mg/kg (95% confidence intervals: 0.3–0.8 mg/kg). The analgesic \( E_{\max} \) value of the combination of bullatine A and morphine was 100% inhibition (Figs. 1E and 1F).

Moreover, the theoretic additive ED\textsubscript{50} values of morphine in the presence of bullatine A and bullatine A in the presence of morphine were 0.3 mg/kg (95% confidence intervals, 0.2–0.4 mg/kg) and 0.4 mg/kg (95% confidence intervals, 0.4–0.6 mg/kg), respectively. The conventional isobolographical analysis showed that the experimental mixture ED\textsubscript{50} values were within the 95% confident limits of the theoretic additive ED\textsubscript{50}s (Fig. 1G), indicating that bullatine A and morphine produced antinociception in an additive rather than synergistic or antagonistic manner.

To further confirm the interaction between morphine and bullatine A or dynorphin A in thermally-induced acute reflex nociception, the antinoceptive effects of subcutaneous morphine, bullatine A or dynorphin A, and the mixture of morphine and bullatine A or morphine and dynorphin A were assessed in mice using hot-plate and tail-
immersion tests. Six groups of mice received subcutaneous injection of saline (10 mL/kg), bullatine A (10 mg/kg), dynorphin A (1 mg/kg), morphine (10 mg/kg), bullatine A (10 mg/kg) + morphine (10 mg/kg), or dynorphin A (1 mg/kg) + morphine (10 mg/kg). The paw lick/jump and tail flick latencies were consecutively (with 10-min interval) measured before, and 0.5, 1 and 2 h after injection in the hot-plate and hot-water bath. As shown in Fig. 2A and B, subcutaneous morphine injection markedly alleviated thermally evoked licking or jumping responses of the hot-plate test by 81%, with the peak effect of 30 min after administration. Subcutaneous morphine also inhibited thermally evoked tail flicking responses by 71% using the tail-immersion test (P < 0.05 by two-way ANOVA followed by the post-hoc Student–Newman–Keuls test). In contrast, subcutaneous bullatine A or dynorphin A did not significantly either reduce thermally induced nociceptive responses or potentiate morphine antinociception using both hot-plate and tail-immersion tests.

3.2. Multiple bidaily intrathecal bullatine A and dynorphin A inhibited morphine antinociceptive tolerance in neuropathic rats

To test whether chronic intrathecal injection of bullatine A or dynorphin A can inhibit morphine-induced antinociceptive tolerance in pain hypersensitivity, we performed bidaily injections of bullatine A, dynorphin A, and morphine into the rat model of neuropathy induced by spinal nerve ligation. Six groups of neuropathic rats received bidaily intrathecal injections (at 12-hr intervals) for 7 days as the following regimen: saline (10 μL), bullatine A (10 μg), dynorphin A (1 μg), morphine (20 μg), bullatine A (10 μg) + morphine (20 μg), or dynorphin A (1 μg) + morphine (20 μg). The paw withdrawal thresholds were measured 1 h after each morning injection. The testing time point of 1 h was determined based on the previous study showing that the intrathecal injection of bullatine A or dynorphin A time-dependently alleviated mechanical allodynia with the peak effect at 1 h after injection (Huang et al., 2016). As shown in Fig. 3A, spinal nerve ligation induced marked mechanical allodynia. Intrathecal injection of saline, bullatine A, dynorphin A, and morphine, and the mixture of bullatine A + morphine and dynorphin A + morphine did not significantly change the withdrawal thresholds to mechanical stimuli in the contralateral paws. In contrast, intrathecal bullatine A, dynorphin A, and morphine produced marked antiallodynic effect at 1 h after injection in the ipsilateral paws. In addition, the combination of morphine with bullatine A or dynorphin A produced apparent additive antiallodynia. Over the 7-d bidaily treatments, the mechanical allodynia remained unchanged in the saline-treated rats; however, intrathecal morphine injections induced progressive and complete tolerance to antiallodynia. In contrast, the antiallodynic effects of intrathecal bullatine A and dynorphin A remained consistent during the 7-d treatment. Co-injections with both bullatine A and dynorphin A completely inhibited morphine antinociceptive tolerance (P < 0.05 by one-way ANOVA followed by the post-hoc Student–Newman–Keuls test on Day 7).

To study the possible cross-antinociceptive tolerance between morphine and bullatine A or dynorphin A, the above-named six groups of rats on Day 8 received consequent intrathecal injection of bullatine A (10 μg) (for the saline, morphine, bullatine A, and the mixture of bullatine A + morphine groups) or dynorphin A (1 μg) (for the dynorphin A and the mixture of dynorphin A + morphine groups) and morphine (20 μg) 4 h later. The paw withdrawal thresholds were measured hourly after injection. Injections of bullatine A, dynorphin A and morphine in these 7-d saline-treated rats produced marked and reversible antiallodynia, and bullatine A produced the same antiallodynic effect in bullatine A-, morphine- and the mixture of bullatine A + morphine-treated rats. However, intrathecal morphine was still not antinociceptive in morphine tolerant rats of 7-d treatment after a single dose of bullatine A given intrathecally 4 h earlier (P < 0.05 by two-way ANOVA followed by the post-hoc Student–Newman–Keuls test) (Fig. 3B).

In order to determine whether the inhibitory effect of bullatine A or dynorphin A on morphine tolerance was also associated with activation of κ-opioid receptors, the above-named six groups of rats continuously received bidaily injections with the same regimen for four more days as Day 1–7. On Day 13, all rats received a single intrathecal injection of the highly selective κ-opioid receptor antagonist GNTI (50 μg) (Jones and Portoghese, 2000) 30 min before the respective treatment. The paw withdrawal thresholds were measured 1 h thereafter. Intrathecal GNTI did not significantly affect baseline mechanical allodynia in saline-treated rats (Huang et al., 2016) (data not shown), but immediately and completely diminished bullatine A and dynorphin A antiallodynia (Fig. 3C). GNTI also immediately and entirely reversed bullatine A- and dynorphin A-induced blockade of morphine antinociceptive tolerance. The blocking effects of GNTI on bullatine A and dynorphin A antinociception disappeared two days after injection, parallel to those with bullatine A- and dynorphin A-induced blockade...
of morphine antinociceptive tolerance.

3.3. Multiple bidaily subcutaneous bullatine A and dynorphin A inhibited morphine-induced antinociceptive tolerance in naïve mice

To assess whether chronic treatment of bullatine A or dynorphin A is able to inhibit morphine-induced antinociceptive tolerance in acute pain where there would be no confounding effects due to bullatine A antinociception, we injected bidaily injections of bullatine A, dynorphin A, and morphine into naïve mice using the hot-plate and tail-immersion test. Six groups of mice received subcutaneous injections of saline (10 mL/kg), bullatine A (10 mg/kg), dynorphin A (1 mg/kg), morphine (10 mg/kg), bullatine A (10 mg/kg) + morphine (10 mg/kg), or dynorphin A (1 mg/kg) + morphine (10 mg/kg), twice daily at 12-hr intervals for 7 days. The paw lick and tail flick latencies were consequently (with 10-min interval) measured 30 min after every other morning injection.

As shown in Fig. 4A and B, the paw lick and tail flick latencies remained unchanged during the 7-d injections of saline, whereas bidially injections of morphine over 7 days produced progressive and nearly complete tolerance to antinociception. In contrast, bidially injections of bullatine A and dynorphin A produced no antinociceptive throughout the observation period of time. However, co-injections with both bullatine A and dynorphin A significantly inhibited morphine antinociceptive tolerance by 44% and 39% (in the hot-plate test), 60% and 57% (in the tail-immersion test), respectively (P < 0.05 by one-way ANOVA followed by the post-hoc Student–Newman–Keuls test on Day 7).

To determine whether the inhibitory effect of bullatine A or dynorphin A on morphine tolerance is associated with spinal κ-opioid receptor activation, the above-named six groups of mice received a single intrathecal injection of GNTI (5 μg) on Day 8 30 min before the routine daily treatment. The paw lick and tail flick latencies were consequently (with 10-min interval) measured 30 min thereafter. As shown in Figs. 4C and 4D, intrathecal GNTI did not significantly affect the paw lick or tail flick latency in either saline-, bullatine A-, or dynorphin A-treated mice (data not shown), but it immediately and completely blocked bullatine A- and dynorphin A-attenuated morphine antinociceptive tolerance. The blockade effect of GNTI was reversible and disappeared two days after injection.

The mice continuously received bidaily injections of the regimens (as day 1–7 treatment) for two more days until Day 11. To determine whether the inhibitory effect of bullatine A on morphine tolerance was also associated with spinal dynorphin A release, all mice received a single intrathecal injection of dynorphin A antibody (at a 1:50 dilution in 5 μL) 30 min before the routine daily treatments (Li et al., 2016a) on Day 11 and the paw lick and tail flick latencies were consequently measured 30 min thereafter. Intrathecal injection of the dynorphin A antibody did not significantly affect the paw lick or tail flick latency either in saline-, bullatine A-, or dynorphin A-treated mice (data not shown), but it immediately and completely blocked bullatine A- and dynorphin A-attenuated morphine antinociceptive tolerance. The blockade effect of the dynorphin A antibody was reversible and disappeared two days after injection (Figs. 4C and 4D).

4. Discussion

In this study, we demonstrated that i) A single injection of bullatine A and dynorphin A acutely interacted with morphine in nociception in an additive manner; ii) Chronic exposure (up to 13-d treatments) of bullatine A and dynorphin A did not produce self-tolerance to antinociception or cross-tolerance to morphine antinociception; iii) Co-administration of bullatine A and dynorphin A significantly inhibited morphine antinociceptive tolerance; iv) The inhibitory effects of bullatine A and dynorphin A on morphine tolerance were immediately and entirely blocked by intrathecal the dynorphin A antibody and/or the selective κ-opioid receptor antagonist GNTI. Our current data provided pharmacological and translational medicinal basis for concurrent use of morphine and aconitines for control of chronic pain. Future clinical study will assess the usefulness of their combination for treatment of chronic pain in patients.

In our previous study, we demonstrated that systemic and intrathecal bullatine A markedly attenuated mechanical allodynia and thermal hyperalgesia after spinal nerve ligation, CFA challenge, chemically-induced diabetes, or bone cancer cell inoculation (Huang et al., 2016). In the current study, we extended our finding to that a single subcutaneous injection of bullatine A dose-dependently inhibited
formalin-induced tonic pain, with maximal inhibition of 54% and the half-effective dose of 0.9 mg/kg. Moreover, the combination of single dose of bullatine A and morphine in a fixed dose ratio of 3 to 1 produced more potent antinociception than bullatine A or morphine alone. The conventional isobolographical analysis indicated that the acute interaction of bullatine A and morphine was in an additive manner because the real ED50 value was not statistically significantly different from the theoretical ED50 value. The acute antinociception to morphine was also confirmed in neuropathy by that a single intrathecal injection of bullatine A produced apparently additive antiallodynia to morphine. However, bullatine A did not have an intrinsic activity on acute reflex nociception, which, consistent with the previous data (Huang et al., 2016), included formalin-induced acute nociception, thermally-induced nociception (the hot-plate and tail-immersion tests) in naive mice and mechanical thresholds in the contralateral paws in neuropathic rats. Systemic bullatine A at a dose up to 30 mg/kg did not potentiate or reduce morphine antinociception in all acute nociceptive models tested in this study. Thus, the additive antinociception of bullatine A to morphine was also considered in acute nociception in a sense of mathematical calculation.

Furthermore, our current study also showed that there were more complicated interactions after chronic treatments. As expected, multiply bidaily subcutaneous and intrathecal injections of morphine over 13 days led mice and rats to develop progressive and complete antinociceptive tolerance in acute reflexive nociception (using the hot-plate and tail-immersion test) and neuropathic pain (induced by spinal nerve ligation). In contrast, bullatine A, given by bidially intrathecal injections, induced no antinociceptive tolerance in neuropathic rats during the total observation period of 13 days. On the other hand, concurrent administrations of bullatine A completely inhibited morphine antinociceptive tolerance in neuropathy. In addition, concurrent treatment of acute nociception with bullatine A also showed significant, although with lesser degree by approximately 44% –60%, inhibition (delay) of morphine antinociceptive tolerance, which excluded the possibility that bullatine A suppressed morphine antinociceptive tolerance by potentiating morphine antinociception. The mechanism of the difference is not known, but may be related to the mode action of dynorphin A, as the exogenous dynorphin A also demonstrated to differentially (completely vs. approximately 39% –57% inhibition) block morphine tolerance in neuropathic pain and acute nociception. Bullatine A is known to produce antinociception in neuropathic pain via specific spinal microglial dynorphin A expression and release (Huang et al., 2016). Our current study confirmed that intrathecal dynorphin A antibody and/or the highly selective κ-opioid receptor antagonist GNTI completely blocked bullatine A antinociception in neuropathy, and further demonstrated that they also immediately and completely reversed systemic and intrathecal bullatine A-induced blockade of morphine tolerance in both acute and chronic pain states. These results are consistent with previous data in which the blockade effects of the Aconitum extracts and their effective ingredients bulleyaconitine A and mesaconitine (although notaconitine or hypaconitine to our surprise) on morphine antinociceptive tolerance were reduced by the dynorphin A antibody and/or κ-opioid receptor antagonist nor-BNI (Li et al., 2016a; Shu et al., 2006a, 2007, 2008). To confirm the involvement of dynorphin A in the inhibitory effect of bullatine A, we also investigated the blockade effect of the synthetic dynorphin A on morphine tolerance to antinociception, which was also totally attenuated by the dynorphin A antibody and GNTI, in parallel to
both acute nociception and neuropathic pain. All these results suggest a cross-talk between microglia and neurons within the spinal cord, in which aconitines including bullatine A inhibit morphine antinociceptive tolerance by stimulating microglia to express and secrete dynorphin A, which subsequently activates κ-opioid receptors presumably located on post-synaptic neurons.

It is a challenge to find effective therapeutics in clinical prevention and reverse of morphine tolerance to analgesia and morphine hyperalgesia. As we know, morphine tolerance is characterized by central sensitization and highly dependent upon activation of neuronal NMDA receptors (DuPen et al., 2007; Zhou et al., 2011). The NMDA receptor antagonists, such as MK-801 (Mendez and Trujillo, 2008; Trujillo, 2000; Trujillo and Akil, 1991) and ketamine (Bell, 1999; Lilius et al., 2015; Raith and Hochhaus, 2004) are probably the most intensively investigated drugs for treatment of morphine tolerance in both basic and clinical setting. MK-801 effectively prevented morphine tolerance in preclinical studies. However, it did not significantly reduce established morphine tolerance to antinociception (Ma et al., 2015; Shu et al., 2008). In our current study, we demonstrated that a single intrathecal injection of bullatine A immediately reversed already-established morphine tolerance in neuropathy, while the 13-d concurrent injections inhibited the development of morphine tolerance. Moreover, the failure of bullatine A given 4 h earlier to block morphine tolerance and the immediate reversion of GNTI and the dynorphin A antibody on bullatine A-attenuated morphine tolerance also suggest that bullatine A could inhibit expression of morphine tolerance, rather than prevent the cellular and molecular development underlying morphine tolerance. The concurrent inhibitory and reversing effects of dynorphin A were also in parallel demonstrated in naive mice. Thus, bullatine A not only inhibited the development of morphine antinociceptive tolerance but also reversed its establishment, which appears to make aconitines and direct κ-opioid receptor agonists superior to NMDA receptor antagonists with respect to managing morphine analgesic tolerance. It is because they may have the ability to restore the analgesic potency of morphine in morphine-tolerant patients at any therapeutic stage.

A variety of preclinical studies have demonstrated that κ-opioid receptor agonists, such as U-50, 488 H, and dynorphins (including dynorphin A), blocked morphine antinociceptive tolerance (Shu et al., 2008; Souvoravong et al., 2004; Takemori et al., 1993). However, direct κ-opioid receptor agonists, particularly the synthetic nonpeptidic agonists, frequently produced serious dysphoric and psychotomimetic adverse effects in humans (Pfeiffer et al., 1986; Preston et al., 2004; Walsh et al., 2001). This may be one of the reasons why these agonists so far failed to treat morphine tolerance in clinic. In contrast, bullatine A, as an endogenous stimulator of spinal microglia, may induce much smaller quantity of dynorphin A expression and secretion in relative to the exogenous synthetic agonists applied, although the small quantity is still high enough to produce antinociception and attenuate morphine tolerance. We did not measure bullatine A-stimulated spinal level of dynorphin A in this study, a similar study may provide comparative information. Shanzhiside methylster, a principle effective iridoid glycoside from the analgesic herb Lamiophlomis rotata, reduced neuropathic pain by stimulating spinal microgialβ-endorphin expression (Zhu et al., 2014). With the similar antinociceptive efficacy, shanzhiside-increased spinal levels of β-endorphin were only approximately 1/250 fold of those by intrathecal exogenous β-endorphin (Fan et al., 2016; Gong et al., 2014c). Indeed, the Aconitum extracts were reported to be devoid of such direct agonists-mediated adverse effects in humans (Shu et al., 2006a, 2006b). Therefore, targeting spinal microgial expression and secretion of dynorphin A is probably a better approach than directly applying exogenous κ-opioid receptor agonists, and provides an attractive and promising strategy for treatment of chronic pain and morphine analgesic tolerance. It is noted that bullatine A, as a C20-diterpenoid alkaloid, exhibits significant lower toxicity, compared to the C18- and C19-diterpenoid alkaloids, such as aconitine and bulleyaconitine A (Singhuber et al., 2009; Xiao et al., 2006), and the Aconitii brachypodii extracts have been widely used to treat chronic pain patients for years without significant adverse effects.

5. Conclusion

Concurrent administration with bullatine A not only additively enhanced morphine antinociception, but also effectively inhibited and reversed morphine tolerance to antinociception. The antinociceptive and blockade effects of bullatine A on morphine antinociceptive tolerance were through stimulation of spinal microglia to express and secrete dynorphin A, which subsequently activates probably post-synaptic neuronal κ-opioid receptors. Its unique property on spinal dynorphin A expression appears to make bullatine A application a novel approach for treatment of chronic pain and morphine analgesic tolerance as adjuvants to morphine or other opioids, and thus, warrants clinical investigation.

Author contributions

Conceived and designed the experiments: YXW, QH; performed the experiments: QH, MLS, YC, XYL; analyzed the data: QH, YXW; and preparation of the manuscript: YXW, QH.

Conflict of interest

The authors declare that there is no competing financial interest in this work.

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