Interactions of the potent D-amino acid oxidase inhibitor CBIO with morphine in pain and tolerance to analgesia

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ABSTRACT

A series of experiments using technologies of gene mutation and silencing as well as chemical biology have demonstrated that spinal D-amino acid oxidase (DAAO) contributes to the development of central sensitization-mediated chronic pain and might be a potential molecular target for the treatment of chronic pain. DAAO inhibitors are now under clinical investigations for the management of chronic neuropathic pain. This study examined the interactions between morphine and the DAAO inhibitor CBIO (5-chloro-benzo[d]isoxazol-3-ol) in pain and analgesia tolerance mainly in the formalin test. Given subcutaneously CBIO acutely interacted with morphine in analgesia in an additive manner both in the acute nociception settings (the formalin acute phase nociception, hot-plate test and tail immersion test) and in formalin-induced tonic pain. Bi-daily exposure of CBIO given subcutaneously for 7 days did not produce self-tolerance to analgesia or cross-tolerance to morphine whereas 7-day subcutaneous morphine induced self-tolerance to analgesia but not cross-tolerance to CBIO. More importantly, subcutaneous co-administrations or even single dose of CBIO completely prevented or reversed morphine tolerance to analgesia (exhibited by a single dose or a dose–response curve of morphine) in both formalin-induced acute phase nociception and tonic phase pain. These results, for the first time, identified DAAO as an efficacious molecule mediating morphine tolerance, in addition to clarifying the complex interactions between morphine and DAAO inhibitors probed by CBIO, and provided a pharmacological basis for DAAO inhibitors in combination with morphine to clinically manage pain.

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1. Introduction

We have recently demonstrated that spinal D-amino acid oxidase (DAAO) contributes to the development of central sensitization-mediated chronic pain and is a potential target molecule for the treatment of chronic pain (Chen et al., 2012; Gong et al., 2011a; Zhao et al., 2008, 2010). Mutation of DAAO gene (Zhao et al., 2008) and knock-down of spinal DAAO gene by the siRNA oligonucleotide against DAAO delivered by intrathecal poly-etherimide complexation and adenoviral vector (Chen et al., 2012; Huang et al., unpublished data, 2012) efficaciously relieved formalin-induced tonic phase pain or bone cancer pain. Intrathecal

injection of a series of DAAO inhibitors including CBIO (5-chloro-benzo[d]isoxazol-3-ol, IC_{50} value: 150 nM), "Compound 8" (4H-thieno[3,2-b]pyrrole-5-carboxylic acid, IC_{50} value: 520 nM), AS057278 (5-methylpyrazole-3-carboxylic acid, IC_{50} value: 14.6 μM), and sodium benzoate (IC_{50} value: 44.6 μM) all specifically prevented and reversed formalin-induced tonic phase pain in a dose-dependent manner by approximately 60% but not acute phase nociception, with a significant positive correlation between the potencies of analgesia and the inhibition of spinal DAAO enzymatic activity (Gong et al., 2011a). Given systemically and intrathecally, DAAO inhibitors produced analgesia in chronic pain including neuropathic pain (Zhao et al., 2010; Fang and Jones, 2005) and bone cancer pain (Huang et al., 2010), but not in acute pain such as thermally-induced paw licking and tail-flicking responses in the hot-plate test and tail immersion test (Gong et al., 2011a; Zhao et al., 2008, 2010). Moreover, DAAO inhibitors produced analgesia in the formalin test via specific blockade of the increase in spinal hydrogen peroxide rather than raising the D-serine level (Lu et al., 2012). DAAO inhibitors are now under early clinical

investigation for the management of chronic neuropathic pain (http://www.sumitomo-chem.co.jp), although there have been studies reporting the opposite role of DAAO in pain transmission (Wake et al., 2001; Ying et al., 2006; Miraucourt et al., 2011).

Opioids are widely used for the clinical management of chronic pain. Among them, morphine is considered as the benchmark of analgesics used to relieve severe pain and suffering. However, clinical usefulness of opiates in the treatment of chronic pain is hampered by their side effects such as constipation, respiratory depression, physical and psychological dependence, and tolerance to analgesia. Morphine tolerance to analgesia develops quickly and is a common clinical phenomenon that decreases antinociceptive effect with repeated administrations and increases the doses of morphine (to as high as 2000 mg) to sustain its clinical analgesic effect as a consequence. Moreover, morphine induces cross-tolerance to other opioids and many other analgesic agents. As other analgesics are often used in combination with morphine to reduce the doses of morphine, several criteria should be met for an ideal fixed ratio combination of morphine and other analgesics. For example, synergistic or at least additive interaction rather than antagonism between morphine and other analgesics is expected; cross-tolerance to each other's analgesia should be avoided; and ideally the combined analgesics should be able to block morphine self-tolerance to analgesia.

This study aimed to elucidate possible interactions between morphine and the DAAO inhibitor with respect to nociception and tolerance to analgesia. CBIO is one of the most potent inhibitor of DAAO available (Ferraris et al., 2008; Gong et al., 2011a). Therefore we employed CBIO as a DAAO probe inhibitor in this study to investigate the interactions between morphine (opiates) and DAAO inhibition. The specific protocols were to determine (1) the acute interaction between systemic administration of CBIO and morphine in analgesia; (2) the subacute (7-day) interaction between subcutaneous morphine and CBIO in tolerance to analgesia; (3) whether subcutaneous CBIO treatment prevented or reversed morphine tolerance to analgesia exhibited by a single dose and a dose–response curve of morphine. Our preliminary findings were presented at the 11th National Conference of Pharmacology of China in an abstract (Gong et al., 2011b).

2. Materials and methods

2.1. Drugs

MK801 was obtained from Sigma-Aldrich (St. Louis MO, USA) while CBIO (5-chloro-benzo[d]isoxazol-3-ol) was purchased from Maybridge PLC (Cornwall, U.K.). Formalin and morphine hydrochloride injection were purchased from Sinopharm Group Chemical Reagent Co., Ltd. (Shanghai, China) and Shenyang First Pharmaceutical Co., Ltd. (Shenyang, Liaoning, China), respectively. All test drugs were freshly dissolved in sterile normal saline solution (Sinopharm Group Chemical Reagent Co., Ltd.) with the pH adjusted to 7.3–7.5 by 1 N NaOH solution as needed.

2.2. Animals

Weighing 20–30 g adult male Swiss mice were purchased from Shanghai Laboratory Animal Center (Shanghai, China). Following shipment, mice were housed in a temperature (22–24 °C)- and humidity (60%)-controlled environment on a 12-h light/dark cycle (lights on at 7:00 AM) for 3–7 days allowing acclimatization prior to experimental studies. The animals were given free access to food and water ad libitum unless otherwise noted. All procedures were approved by the Laboratory Animal Use Committee of Shanghai Jiao Tong University School of Pharmacy. All efforts were made to reduce the number of animals used, to minimize their sufferings, and to utilize alternatives to *in vivo* techniques, if available. Experimental study groups were assigned randomly, and the researcher was blind for behavior tests.

2.3. The mouse formalin test

Animals were acclimated individually to the observation cage for 30 min prior to testing. The mouse formalin test was performed, as previously described (Hunskaar

et al., 1985; Lu et al., 2012) by injecting 10 μl of 5% formalin in 0.9% saline subcutaneously on the dorsal side of the right hindpaw and the animal was immediately placed in a transparent polycarbonate box. The duration of nociceptive behaviors (licking/biting) was manually quantified in the pooled durations at 0–5 min and 20–40 min which were considered as the acute phase nociception and tonic phase pain, respectively.

2.4. The mouse NMDA (N-methyl-D-aspartate) test and intrathecal injection

After acclimation, mice were intrathecally injected with 5 μl of NMDA (0.4 nmol) in 0.9% saline and immediately placed in an observation chamber and the duration of the nociceptive behaviors (licking, scratching and biting directed toward the hind limb, gluteal region and base of the tail) were recorded for 10 min immediately after NMDA injection (Sakurada et al., 1990; Tsukamoto et al., 2010).

Mouse intrathecal injection followed the previously described procedures (Mestre et al., 1994). Briefly, a 10 μL micro-syringe with a tube for delivering testing drugs was inserted into the skin and through the L5-L6 intervertebral space directly into the subarachnoid space. A flick of the mouse's tail provided a reliable indicator that the needle had penetrated the spinal arachnoid mater. The test article in 5 μL was subsequently injected into the subarachnoid space.

2.5. The mouse tail-flick and hot-plate tests

The model SSY-H digital display thermostatic water-bath (Shanghai Sanshen Medical Instrument Co., Shanghai, China) was used to maintain a constant water temperature of 50 ± 0.5 °C. While the mice were placed in a tubular restrainer, their tails were immersed 3.5 cm in the water-bath as described previously (Zhao et al., 2008). The nociceptive latency was defined as the time required to elicit a flick of the tail. The cut-off time was 30 s for tail-flick measurements to minimize tissue injury.

Pain reflexes in response to thermal stimulus in the hot-plate test were measured using YLS-6B Intelligence Hot-Plate Analgesia Meter (Shandong Academy of Medical Sciences Device Station, Shandong, China) (Suaudeau et al., 2005). 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. 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 a hindpaw lick, hindpaw flick, biting, or jump (whichever came first) was measured to the nearest 0.1 s by deactivating the timer when the response was observed. The mouse was immediately removed from the hot-plate and returned to its home cage. Trials were terminated if the animals did not respond within 40 s to avoid tissue damage.

2.6. Statistical analysis

For dose–response curve analysis of CBIO or morphine on formalin-induced tonic phase pain, the parameters, *i.e.*, minimum effect (E_{min}), maximum effect (E_{max}), half-effective dose (ED_{50}) and Hill coefficient (n), were calculated from dose–response curves. To determine the parameters of dose–response curves, values of response (Y) were fitted by nonlinear least-squares curves to the relation $Y = a + bx$, where $x = [D]^n/(ED_{50}^n + [D]^n)$, to give the value of ED_{50} and b (E_{max}) yielding a minimum residual sum of squares of deviations from the theoretical curve (Wang and Pang, 1993). Theoretic additive ED_{50}s were calculated according to the method (Tallarida et al., 1989, 1997) and the drug interactions were also presented in the conventional isobolography (Tallarida et al., 1989; Wang et al., 2000; Zhang et al., 2005).

The results are expressed as mean ± SEM and statistical significance was evaluated by a one-way analysis of variance (ANOVA) or two-way repeated measure ANOVA followed by post-hoc Student-Newman–Keuls test, or unpaired and two-tailed Student's *t*-test. The statistical significance criterion *P* value was 0.05. All data calculations and statistics analysis were done by using the Version 5.01 GraphPad Prism Program (GraphPad Software Inc., San Diego, CA, USA).

3. Results

3.1. Given systemically CBIO is analgesic on formalin-induced tonic phase pain but not NMDA-induced pain or acute pain

In our recent paper (Lu et al., 2012), we demonstrated that subcutaneous injection of CBIO blocked formalin-induced tonic phase pain but not acute phase nociception in mice, with the ED_{50} value of 0.9 mg/kg (95% confidence limits: 0.3–2.7 mg/kg) and maximum inhibition of 61.5%. Here we further tested the analgesic effect of CBIO by the oral route. Six groups of fasted mice (*n* = 6–7 in each group) received gavage of saline (10 ml/kg) or CBIO (1, 3, 10, 30 or 100 mg/kg) 30 min before formalin challenge. Formalin