JPET Fast Forward. Published on October 15, 2010 as DOI: 10.1124/jpet.110.172353

Title page

A Series of DAO Inhibitors Specifically Prevents and Reverses Formalin-Induced Tonic Pain in Rats

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Running title page

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Text pages: 36

The number of figures: 10

The number of tables: 1

The number of references: 55

The number of words in the Abstract: 249

The number of words in the Introduction: 1017

The number of words in the Discussion: 1814

Total words: 9779

Non-Standard ABBREVIATIONS: DAO, D-amino acid oxidase; NMDA, N-methyl-D-aspartic acid; rsDAO, rat spinal cord-derived DAO; pkDAO, porcine kidney-derived DAO; CBIO, 5-chlorobenzo[d]isoxazol-3-ol; Compound 8 or sc-203909, 4H-Thieno[3,2-b]pyrrole-5-carboxylic acid; AS057278, 5-methylpyrazole-3-carboxylic acid; NPCA, 4-nitro-3-pyrazole carboxylic acid; E_{min} , minimum effect; E_{max}, maximum effect; ED_{50} or EC_{50}, half-effective dose or concentration.

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ABSTRACT

We have recently found that mutation of D-amino acid oxidase (DAO) diminished formalin-induced tonic pain. The present research further studied the analgesic effects of a series of DAO inhibitors in this model. CBIO, Compound 8, AS057278, sodium benzoate and NPCA inhibited rat spinal cord-derived DAO activity in a concentration-dependent manner, with maximal inhibition of 100% and potency rank of CBIO > Compound 8 > AS057278 > sodium benzoate > NPCA. In rats, intrathecal injections of CBIO, Compound 8, AS057278 and sodium benzoate but not NPCA specifically prevented formalin-induced tonic pain but not acute nociception, with the same potency order as in the DAO activity assay. The highly potent analgesia of DAO inhibitors was evidenced by CBIO, which prevented 50% pain at 0.06 μg, approximately 5-fold the potency of morphine. CBIO given post formalin challenge also reversed the established pain state to the same degree as prevention. The anti-hyperalgesic potencies of these DAO inhibitors were highly correlated to their inhibitions of spinal DAO activity. Maximum inhibition of pain by these compounds was approximately 60%, comparable to that of the NMDA receptor antagonist MK-801, suggesting that a larger portion of formalin-induced tonic pain is “DAO sensitive” while the remaining 40% of tonic pain and acute nociception are “DAO insensitive”. These findings, combined with our previous DAO gene mutation and induction results, indicate spinal DAO mediates both induction and maintenance of formalin-induced tonic pain and further validate spinal DAO as a novel and efficacious target molecule for the treatment of chronic pain.

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Introduction

D-amino acid oxidase (DAO) is a peroxisomal flavoprotein that catalyzes the oxidative deamination of neutral and polar D-amino acids, with strict stereospecificity, to α-keto acids, NH_3 and H_2O_2 (see reviews by Pollegioni *et al.*, 2007, Verrall *et al*., 2009; Williams, 2009). In the central nervous system, DAO expression is restricted to the lower brainstem, cerebellum and spinal cord, with decreasing levels in the midbrain, cortex, and hippocampus (Kapoor and Kapoor, 1997; Moreno *et al.*, 1999; Horiike *et al*., 2001; Yoshikawa *et al*., 2004). Recently we discovered that mutation of the DAO gene blocked formalin-induced tonic pain but not acute nociception by using ddY/DAO$^{+/+}$ mice *vs.* ddY/DAO$^{-/-}$ mice (Zhao *et al*., 2008), sharply contrasting an earlier finding (Wake *et al*., 2001). Systemic and intrathecal administration of the DAO inhibitor sodium benzoate also specifically attenuated L5/L6 spinal nerve tight ligation-induced neuropathic mechanical allodynia and formalin-induced tonic pain in rats and mice. In contrast, sodium benzoate administered by either route was not effective in acute pain such as the early phase flinch response in the formalin test, thermal nociceptive response in the tail flick test or hot-plate test (Zhao *et al*., 2008, 2010). Moreover, L5/L6 spinal nerve damage up-regulated spinal DAO gene expression and DAO enzymatic activity, with the same time-course as peripheral nerve damage-induced mechanical allodynia (Zhao *et al.*, 2010). Thus, we have obtained the first evidence indicating that spinal DAO specifically participates in chronic pain transmission and is a potential target molecule for the treatment of chronic pain including chronic neuropathic pain (Zhao *et al*., 2008, 2010). However, several issues need to be clarified before validation of the hypothesis of spinal DAO as an efficacious pain target molecule.

DAO mutation using ddY/DAO$^{-/-}$ mice entirely eliminates DAO activity (Konno *et al*., 1983; Xin *et al*., 2007, 2010; Zhao *et al*., 2008), however, compensation developed during growth and non-specific effects such as changes in motor activity might account for observed pain behaviors, and could lead to false positive or negative results in animal studies. DDY/DAO$^{-/-}$ mice were indeed reported to have

less locomotor activity than ddY/DAO$^{+/+}$ mice (Almond *et al*., 2006), thus making interpretation of our previously observed behavior changes more complex. In addition, sodium benzoate (benzoic acid) is the only DAO inhibitor used so far in animal pain studies – and without dose-response analysis. Due to its low potency and rapid excretion in urine (Kubota and Ishizaki, 1991; MacArthur *et al*., 2004; Williams and Lock, 2005; Xin *et al*., 2007), it was widely accepted to give sodium benzoate in high doses such as 100 - 1000 mg/kg for systemic administration (Moses *et al*., 1996; Williams and Lock, 2005; Wu *et al*., 2006; Xin *et al*., 2005, 2007; Zhao *et al*., 2008, 2010). It is reasonable to speculate that the analgesic action of sodium benzoate may be a result of its possible non-specific effects due to high doses. Thus, more DAO inhibitors with higher potency and chemical structures distinct from sodium benzoate are needed for further studies. A series of compounds having a carboxylic acid group or related groups have emerged recently as novel inhibitors of DAO with high potencies for the treatment of schizophrenia, including CBIO (5-chlorobenzo[*d*] isoxazol-3-ol) (Ferraris *et al*., 2008; Hashimoto *et al*., 2009; Horio *et al*., 2009), Compound 8 (sc-203909, 4H-thieno[3,2-b]pyrrole-5-carboxylic acid) from Merck (Sparey *et al*., 2008; Smith *et al*., 2009), AS057278 (5-methylpyrazole-3-carboxylic acid) (Adage *et al*., 2008), and 4-nitro-3-pyrazole carboxylic acid(NPCA)(Fang *et al*. 2005), as well as Compound 2 (3-hydroxyquinolin-2-(1H)-one) from Pfizer (Duplantier *et al*., 2009). The chemical structures of CBIO, Compound 8, AS057278, sodium benzoate, and NPCA are presented in Fig. 1 where Compound 2 is also listed for comparison.

High efficacy is critical for selection of a target molecule for establishing drug screening and development programs against chronic pain. The efficacy of spinal DAO inhibition in tonic pain is not yet established partly due to the lack of dose-response analysis of DAO inhibitors on analgesia. It is important to compare the efficacies of DAO inhibitors to a well established chronic pain drug target molecule particularly validated in humans, such as N-methyl-D-aspartic acid (NMDA) receptors. Activation of spinal NMDA receptors is believed to play an essential role

for central sensitization-mediated chronic pain (see reviews by Basbaum *et al*., 2009 and Hulsebosch *et al*. 2009). Intrathecal administration of NMDA receptor antagonists including MK-801 have been reported to suppress formalin-induced tonic pain (Nasstrom *et al*., 1992; Yamamoto and Yaksh, 1992; Chaplan *et al*., 1997).

Therefore, the aim of the present study is to systemically study a series of potent DAO inhibitors on tonic pain in the formalin test to further elucidate role of spinal DAO in central sensitization-mediated pain. Selection of the formalin test was based on the well accepted notion that subcutaneous injection of formalin produces biphasic behavioral effects in animals with the early phase reflecting an acute nociceptive state and the following tonic pain due to on going activation of sensory afferents and tissue damage as well as inflammation reflecting a state of central-sensitization, a mechanism shared by chronic or persistent pain including neuropathic pain (Cook *et al*., 1987; Coderre *et al*., 1993; Woolf *et al*., 1994; Jett *et al*., 1997; see review by Sawynok and Liu, 2003). The present study included the following protocols: 1) determination of inhibitory effects of CBIO, Compound 8, AS057278, sodium benzoate, and NPCA, compared to MK-801, on rat spinal cord derived DAO (rsDAO) and porcine kidney-derived DAO (pkDAO) enzymatic activities; 2) examination of preventive effects of CBIO, Compound 8, AS057278, NPCA and sodium benzoate administrated intrathecally by multiple doses, compared to MK-801, on formalin-induced tonic pain in rats; the blocking effect of CBIO administered post formalin challenge on the established pain state was also studied; 3) analyses of dose-response curves of DAO inhibitors on rsDAO and pkDAO enzymatic activities and formalin-induced tonic pain to yield potencies and maximum effects for further determination of the correlation between DAO inhibition and blockade of tonic pain as well as efficacy for spinal DAO pain transduction and transmission; 4) measurement of effects of intrathecally injected CBIO on carrageenan-induced inflammatory thermal hyperalgesia to further test its specificity on analgesia.

Materials and Methods

Drugs. AS057278 (5-methyl-3-pyrazole-carboxylic acid), NPCA (4-nitro-3-pyrazole carboxylic acid) , γ-carrageenan, and MK-801 were purchased from Sigma (Sigma, St Louis, MO, USA) while sodium benzoate and formalin were from Sinopharm Group Chemical Reagent Co., Ltd. (Shanghai, China). CBIO (5-chlorobenzo[*d*]isoxazol-3-ol), Compound 8 (sc-203909, 4H-thieno[3,2-b]pyrrole-5-carboxylic acid) and porcine kidney DAO (5 unit/mg) were from Maybridge PLC (Cornwall, U.K.), Santa Cruz Biotechnology Inc. (Santa Cruz, California, USA) and SERVA (Heidelberg, New York, USA), respectively. All drugs and reagents were freshly dissolved in sterile normal saline solution (Sinopharm Group Chemical Reagent Co., Ltd.) with the pH adjusted by 1 M NaOH solution as needed, or 50% DMSO in normal saline in one case as indicated below.

Animals. Male Wistar rats (180-250 g) were obtained from Shanghai Experimental Animal Institute for Biological Sciences (Shanghai, China). The animals were housed in a temperature and humidity controlled environment on a 12 h light/dark cycle (lights on at 6:00 am). Food and water were freely available. The research protocol was approved by the Animal Care & Welfare Committee of Shanghai Jiao Tong University School of Pharmacy and followed the animal care guidelines of the National Institutes of Health. Animals were acclimated to the laboratory environment for 3 - 5 days before entering the study. Experimental study groups were assigned in random and the researcher was blind for behavior testing.

Measurement of rsDAO and pkDAO Enzymatic Activities. The enzymatic activities of rsDAO and pkDAO were determined according to the “keto acid method” (D’Aniello *et al*., 1993). The rats were killed by decapitation and the spinal cords were quickly removed on ice and weighed. The tissue was homogenized (10,000 rpm for 15 seconds) with a homogenizer (Fluko Equipment Co., Shanghai, China) in 0.1 M Tris-HC1 (pH 8.2) (1 g/3 ml) and centrifuged (4,000 rpm for 10 minutes) at 4 ºC. The protein content was measured by the method of Coomassie Brilliant Blue

Staining. 50 μl D-alanine (0.015 M dissolved in 0.1 M Tris-HC1 buffer, pH 8.2) was added to 50 μl of supernatants with final concentration of 6 mM D-alanine and incubated (700 rpm) at 37 ºC for 60 minutes. Preliminary results showed the Km value of D-alanine in this assay was 5.8 mM. 25% Trichloroacetic acid (50 μl) was then added to the assay mixture, mixed, and centrifuged (14,000 rpm, 5 min). The supernatant (50 μl) was mixed with 50 μl 1 mM 2,4-dinitrophenylhydrazine (in 1 M HCl) and incubated (700 rpm) at 37 ºC for 10 minutes. Finally, 100 μl of 1.5 M sodium hydroxide was added, mixed, and incubated (700 rpm) at 37 ºC for 10 minutes. The absorbance was read at 450 nm on an ELx800 Universal Microplate Reader (Bio-Tek Instruments, Inc., USA) against a blank sample consisting of the same homogenates without D-alanine. The activity of DAO in the homogenates was quantified against the standard curve of pyruvic acid (from 100 - 800 μM, $R^2 > 0.99$). The specific enzymatic activity was expressed as pyruvate production per mg protein per minute. The measurement of enzymatic activity of pkDAO was the same as in the spinal DAO assay except that the spinal cord supernatant solution was replaced by pure pkDAO solution (final concentration: 0.2 unit/0.04 mg/ml), final D-alanine concentration was 1 mM (Km value in this assay) instead of 6 mM and incubation time was 5 minutes instead of 60 minutes.

Intrathecal Catheterization. A 24-cm polyethylene catheter (PE-10: 0.28 mm i.d. and 0.61 mm o.d., Clay Adams, Parsippany, NJ, USA) with volume of approximately 13 μl was inserted into the rat lumbar level of the spinal cord under intraperitoneal injection of pentobarbital (50 mg/kg) anesthesia as described elsewhere (Wei *et al*., 2007). Following recovery from anesthesia, the placing of the catheter in the spinal cord was verified by administering 4% lidocaine (10 μl followed by 15 μl of normal saline for flushing) with a 50-μl micro injector (Shanghai Anting Micro-injector Factory, Shanghai, China). The lidocaine test was performed 5 - 7 days prior to the start of the drug testing sessions. Only those rats that had no motor impairment before lidocaine injection but had bilateral paralysis of hindlimbs following intrathecal administration of lidocaine were selected for the study, but the exclusion rate in our laboratory was zero. For intrathecal administration of control and test articles, the

drugs were microinjected with a 50 μl micro injector in a volume of 10 μl (or 30 μl in one case due to low drug solubility as indicated below) followed by a normal saline flush in a volume of 15 μl.

The Rat Formalin Test of Acute Nociceptive Pain and Tonic pain. Rats were acclimated individually to the observation cage for 30 minutes prior to testing. The formalin test was performed as previously described (Wang *et al*., 2000) with slight modifications, by injecting 50 μl of 5% (or 1% in some experiments as indicated) formalin in 0.9% saline subcutaneously on the dorsal side of the left hindpaw and the rat was immediately placed in a 23 cm × 35 cm × 19 cm transparent polycarbonate box. Nociceptive behavior was manually quantified by counting the number of the formalin-injected paw flinches supplemented by licking duration (considered as 1 count per second matching the duration of the typical flinching behavior) in 1-min epochs. Measurements were taken at 12-min intervals beginning immediately after formalin injection and ending 96 minutes later.

The Rat Carrageenan Model of Acute Inflammatory Pain. In order to induce a local inflammation, right hindpaws of rats received subcutaneously intraplantar surface injection of 100 μl of 2% carrageenan in normal saline. Hyperalgesia was assessed by placing the hindpaw above a radiant heat source (setting to a low intensity of 45) and measuring the paw-withdrawal latency with a noxious heat stimulus, using 390 G Plantar Test (IITC Life Science Instruments, Woodland Hills, CA, USA) as described by Hargreaves *et al*. (1988). Paw-withdrawal latencies were determined both in carrageenan-injured and contralateral (uninjured) hindpaws. The cut-off latency was 30 seconds to avoid tissue damage. The paw-withdrawal latencies were evaluated in no less than 30 minutes at different time points before and after control and test article administration. Each test was calculated as a mean of three repeated measurements.

The Rotarod Motor Coordination Test and Motor Activity Test. Motor coordination performance was assessed by means of a YLS-4C Rota Rod with automatic timers and falling sensors (Yiyan Scientific Ltd., Shandong, China). The rats were trained and tested using an accelerated speed from 5 rpm to 25 rpm within 1

minute followed by 25 rpm for two more minutes. The accumulated time (seconds/3 min) for animals to spend on the rotarod was recorded during the 3-min observation period after the animals were trained once a day each for 9 minutes for three days. The accumulated time spent on the rod had to be at least 120 seconds allowing inclusion in the study. Motor activity was also measured using a YLS-1c electromagnetic activity monitor (YLS-1C Motor) with a printer (Yiyan Scientific Ltd., Shandong, China) located in a quiet environment. Animals were each placed into an individual testing cage identical to their home cages with free access to diet and water. Three hours later automatic counting of accumulated movements (counts/15 min) were continuously recorded and printed out in a 15-min interval for the scheduled period.

Statistical Analysis. For dose (concentration)-response curve analysis of DAO inhibitors on DAO enzymatic activity or formalin-induced tonic pain, the parameters, *i.e.*, minimum effect (E_{min}), maximum effect (E_{max}), half-effective dose or concentration (ED_{50} or EC_{50}) and Hill coefficient **(*n*),** were calculated from individual dose-response curves. To determine the parameters of dose-response or concentration-response curves, values of response (*Y*) were fitted by nonlinear least-squares curves to the relation $Y = a + bx$, where $x = [D]^n/(ED_{50}^n + [D]^n)$ or $x = [C]^n/(EC_{50}^n + [C]^n)$, to give the value of ED_{50} or EC_{50} and *b* (E_{max}) yielding a minimum residual sum of squares of deviations from the theoretical curve (Wang *et al*., 1993). For correlationship between potencies of DAO inhibitors on DAO enzymatic activity and formalin-induced tonic pain, lineal correlation coefficient was calculated.

The results are expressed as mean ± SEM and statistical significance was evaluated by a two-way repeated measures analysis of variance (ANOVA) followed by post-hoc two-tailed Student's t-tests, or by an unpaired and two-tailed Student 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).

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Results

Inhibitory Effects of DAO Inhibitors on rsDAO and pkDAO Enzymatic Activities. Five DAO inhibitors, CBIO (5-chlorobenzo[*d*]isoxazol-3-ol), Compound 8 (sc-203909, 4H-Thieno[3,2-b]pyrrole-5-carboxylic acid), AS057278 (5-methylpyrazole-3-carboxylic acid), sodium benzoate, and NPCA (4-nitro-3-pyrazole carboxylic acid), were assayed (triplicates) for effects on DAO enzymatic activity in homogenates from rat spinal cords. In the control homogenates incubated for 60 minutes, the α-keto acid produced by D-alanine was determined by measurement of pyruvic acid and the specific DAO activity was about 1.5 nmol/mg protein/min. CBIO (10^{-8} - $3*10^{-6}$ M), Compound 8 (10^{-8} - 10^{-5} M), AS057278 ($3*10^{-7}$ - 10^{-4} M), sodium benzoate ($3*10^{-6}$ - 10^{-3} M), and NPCA (10^{-4} - $3*10^{-2}$ M) all inhibited DAO enzymatic activity in a concentration-dependent manner (Fig. 2A), with maximum inhibition (E_{max}) values of approximately 100%, and half-inhibitory concentration (IC_{50}) values of 0.09 μM, 0.17 μM, 15.4 μM, 75.4 μM and 20 mM, respectively. In addition, NMDA receptor antagonist MK-801 was tested and found not to inhibit DAO enzymatic activity up to 0.8 mM (data not shown).

The inhibitory effects of these compounds were also tested in the pkDAO enzymatic activity assay (triplicates) where the specific DAO activity was about 1 μmol/mg protein/min after 5 minutes of incubation. Five DAO compounds inhibited pkDAO enzymatic activity in a concentration-dependent manner (Fig. 2B), with E_{max} values of approximately 100%. The IC_{50} values for these compounds were 0.15 μM, 0.52 μM, 14.6 μM, 44.6 μM and 5.9 mM, respectively, with the same potency order as in the rsDAO assay, i.e., BIO > Compound 8 > AS057278 > sodium benzoate > NPCA. Our results with CBIO, Compound 8, AS057278, and sodium benzoate were consistent with previous reports where their IC_{50} or Ki values were 145 or 245 nM (Sparey *et al*., 2008; Smith *et al*., 2009), 188 nM (Ferraris *et al*., 2008), 0.91 μM (Adage *et al*., 2008), and 16 μM (Ki, Vanconi *et al*., 1997), respectively. However, NPCA less potently inhibited rsDAO and pkDAO DAO with IC_{50} values of 20 mM and 5.9 mM, respectively, in contrast to a granted patent where the IC_{50} value of

NPCA was less than 10 μM (Fang *et al*. 2005).

Differentially Inhibitory Effects of Intrathecal Injections of DAO Inhibitors and the NMDA Receptor Antagonist on Formalin-Induced Tonic pain. The analgesic effects of DAO inhibitors (CBIO, Compound 8, AS057278, sodium benzoate) as well as NPCA, compared to that of the NMDA receptor antagonist MK-801, on formalin-induced nociceptive pain and tonic pain were studied via direct spinal cord administration in rats chronically implanted with intrathecal cannulas. Seven groups of rats (n = 5 in each group) received intrathecal bolus injection of normal saline (10 μl) or CBIO (0.01, 0.03, 0.1, 0.3, 1, or 3 μg) 30 minutes before formalin injection. Subcutaneous injection of formalin in control rats receiving intrathecal injection of normal saline produced a characteristic bi-phasic flinching response supplemented by licking response consisting of an initial, rapidly decaying acute phase (within 12 minutes after formalin injection) followed by a slowly rising and long-lived (12 - 96 minutes) tonic phase as shown in Fig. 3A. Although formalin-induced flinching and licking may be independent behaviors (Abbott et al., 1995) and may have different underlying mechanisms (Wheeler-Aceto and Cowan, 1993; Sawynok and Reid, 2002), both reflect the established pain state. Our preliminary experiment demonstrated CBIO blocked 1% or 5% formalin-induced flinching and licking behaviors in the tonic phase to a similar degree, thus both behaviors were combined for overall measurement. Compared with the normal saline control, CBIO up to 3 μg did not prevent formalin-induced flinch response (acute nociception) in the acute phase ($P > 0.05$ by ANOVA). However, CBIO prevented formalin-induced tonic pain in the tonic phase in a dose-dependent manner (Fig. 3A). The areas under the flinching response curve from 12 to 96 min ($AUC_{12\text{-}96\ min}$s) were calculated. Dose-response analysis of CBIO by best fit showed that maximum inhibition (E_{max}) (at 1 μg or 6.2 nmol) of formalin-induced tonic pain was 67.3% and half-effective dose (ED_{50}) was 0.06 μg (0.39 nmol) (Fig. 3B). For a comparison, 1.5 (2 nmol) and 10 μg (13.2 nmol) of morphine sulfate administered intrathecally abolished formalin-induced tonic pain by 50% and 100% respectively (Wang *et al*., 2000; Zhao *et al*., 2010). As the tonic phase flinches were measured subsequently

after the acute phase, the differential effects of CBIO on the acute and tonic phases may be due to its variable tissue concentrations/pharmacokinetics changes at different time points. Therefore, two groups of intrathecally cannulated rats (n = 4 in each group) received intrathecal bolus injection of saline (10 μl) or CBIO (3 μg) 90 minutes before 5% formalin challenge (matching the time to the peak of the tonic phase in the previous experiment). As shown in Fig. 4A, CBIO given 90 minutes before did not significantly prevent formalin-induced nociceptive flinches in the acute phase ($P > 0.05$, unpaired and two-tailed Student t-test), the same as CBIO given 30 minutes before formalin injection as described above; but CBIO still significantly inhibited tonic pain measured as $AUC_{12\text{-}96\ min}$ by 52.0%, which is a moderately smaller effect than when CBIO was given 30 minutes before formalin injection presumably due to its longer disposition in the spinal cord. In addition, because the ineffectiveness of CBIO on the acute nociception induced by 5% formalin might also be due to its ceiling effect, CBIO was examined by employing 1% formalin. Two groups of rats (n = 4 in each group) received intrathecal bolus injection of saline (10 μl) or CBIO (3 μg) 30 minutes before formalin challenge. Compared to 5% formalin, 1% formalin produced similar acute nociception but less and shorter-lived tonic pain. 3 μg CBIO blocked 1% formalin-induced tonic pain measured as $AUC_{12\text{-}96\ min}$ by 72.7%, but not acute nociception ($P > 0.05$ by unpaired and two-tailed Student t-test) (Fig. 4B).

In order to test whether CBIO injected post-formalin challenge still effectively reversed the established pain state, two groups of intrathecally cannulated rats (n = 4 in each group) received intrathecal bolus injection of 10 μl normal saline or 1 μg CBIO 24 minutes after formalin administration. CBIO injected after formalin challenge markedly reversed the established pain state in the tonic phase, with the inhibitory rate of 60.3% measured by $AUC_{36\text{-}96\ min}$ (Fig. 4C), the same as the preventive effect of 66.6% by CBIO administered before formalin challenge (ref. Fig. 3A).

Since it was reported that DAO mutant mice had less locomotor activity (Almond *et al.*, 2006), which might account for our observed pain behaviors, the rotarod motor

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coordination test and motor activity test were conducted, respectively, to examine the locomotor functions of CBIO, although CBIO did not exhibit apparent motor side effects during the observation period of all above experiments. Two groups of rats (n = 4 in each group) received intrathecal bolus injection of normal saline (10 μl) or CBIO (3 μg), and the accumulated time for rats to spend on the rotarod (at a rate of 5 rpm to 25 rpm within one minute followed by 25 rpm for two more minutes) was recorded before (151.8 ± 28.3 seconds/3 min for saline *vs.* 143.8 ± 23.8 seconds/3 min for CBIO), 30 minutes (90.8 ± 30.6 seconds/3 min *vs.* 95.8 ± 29.2 seconds/3 min) and 90 minutes (116.3 ± 37.9 seconds/3 min *vs.* 141.8 ± 34.7 second/3 min) after administration of control and test articles. Both groups of rats did not show significant difference at any time points measured over the observation period ($P > 0.05$ by two-way repeated measures ANOVA). In addition, both groups of rats (n = 4 in each group) receiving intrathecal injection of normal saline (10 μl) or CBIO (3 μg) showed the same motor activity at all time points during observation of at least 2 hours after injection (2 hours' total counts: 173.2 ± 84.7 for saline *vs.* 172.1 ± 45.1 for CBIO, $P > 0.05$, by unpaired and two-tailed Student t-test). Thus the analgesia of CBIO is not falsely positive due to locomotor behaviors.

Two groups of intrathecally cannulated rats received intrathecal bolus injection of 30 μl of 50% DMSO in normal saline (n = 4) or 750 μg NPCA (n = 5) 30 minutes before formalin injection. Formalin produced the characteristic bi-phasic flinch responses. Compared with the normal saline control, NPCA was not effective in prevention of either formalin-induced tonic pain in the late phase or the flinch response in the early phase (Fig. 4D). No apparent motor side effects of NPCA were observed during the study period.

The inhibitory effects of Compound 8 (0.1, 1, 3, or 10 μg), AS057278 (0.3, 1, 3, or 10 μg), and sodium benzoate (3, 10, 30, 100, or 300 μg) on the formalin test were also tested in 16 groups of intrathecally cannulated rats (n = 3 - 6 in each group). Compared with the normal saline control, Compound 8 (Fig. 5A), AS057278 (Fig. 6A), and sodium benzoate (Fig. 7A) all prevented formalin-induced tonic pain measured by $AUC_{12\text{-}96\ min}$, in a dose-dependent fashion, but not the flinch response in

the acute phase. Dose-response analysis by best fit showed that E_{max} values for Compound 8, AS047278 and sodium benzoate to prevent tonic pain were 67.0%, 55.6%, and 57.6%, while their ED_{50} values were 0.17 μg (1.0 nmol), 1.4 μg (11.1 nmol) and 10.2 μg (70.8 nmol), respectively (Fig. 5B, 6B, 7B). No apparent motor side effects were observed for these compounds during the study period.

Seven groups of intrathecally cannulated rats (n = 4 in each group) received intrathecal bolus injection of normal saline (10 μl) or MK-801 (0.03, 0.1, 0.3, 1, 3, or 10 μg) 30 minutes before formalin challenge. Formalin produced the same characteristic bi-phasic flinch responses. Compared with the normal saline control, MK-801 produced a dose-dependent prevention of formalin-induced tonic pain in the late phase measured by $AUC_{12\text{-}96\ min}$ in a dose-dependent manner, but not the nociceptive response in the acute phase (Fig. 8A). Dose-response analysis by best fit showed that E_{max} and ED_{50} for MK-801 to block tonic pain were 50.9% and 0.3 μg (0.9 nmol), respectively (Fig. 8B). The potency (Yamamoto and Yaksh, 1992) and efficacy (Chaplan *et al*., 1997) of MK-801 were in agreement with previous reports.

Correlation between inhibition of DAO enzymatic activity and blockade of formalin-induced tonic pain. Correlation analysis among the above DAO inhibitors between their inhibition of DAO enzymatic activity and prevention of formalin-induced tonic pain was carried out. As NPCA was not effective in the blockade of tonic pain up to a maximally allowable dose (750 μg) and thus no ED_{50} value was obtained, the maximal allowable dose of NPCA was then plotted instead (Fig 8). Correlation analysis showed that blockade potencies (ED_{50} values) of formalin-induced tonic pain by CBIO, Compound 8, AS057278, sodium benzoate, and NPCA were highly correlated to their IC_{50} values on enzymatic activities of both rsDAO (Fig. 9A, r^2 = 0.9906, P = 0.0004) and pkDAO (Fig. 9B, r^2 = 0.9925, P = 0.0003).

Ineffectiveness of Intrathecal Injection of CBIO on Carrageenan-induced Acute Heat Hyperalgesia. Two groups of intrathecally cannulated rats (n = 4 in each group) received intraplantar injection of 100 μl of 2% carrageenan in normal saline and two and half hours later received intrathecal injection of normal saline (10 μl) or

JPET Fast Forward. Published on October 15, 2010 as DOI: 10.1124/jpet.110.172353
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CBIO (1 μg). Normal and inflamed paw withdrawal latency to radiant heat stimuli were measured 2.5 hours before subcutaneous carrageenan injection, 0.5 hour before and 0.5, 1, 2, and 3 hours after intrathecal administration of control and test articles. As shown in Fig. 10, carrageenan produced inflammation observed by swelling and redness, and hyperalgesia reflected by reduction of inflamed paw withdrawal latency compared to normal paws ($P < 0.05$ by two-way repeated measures ANOVA). Intrathecal injection of CBIO did not produce any analgesic or anti-hyperalgesic effects on either the normal paw or inflamed paw. ($P > 0.05$ by two-way repeated measures ANOVA).

Discussion

Sodium benzoate (benzoic acid) is a prototype competitive DAO inhibitor (Vanconi *et al*., 1997) and the crystal structure of DAO complexed with benzoic acid showed that critical hydrogen bonds were formed between the carboxylate group of benzoic acid and Arg283 and Tyr228 residues of the enzyme (Mattevi *et al*., 1996). A series of compounds that share the same functional groups such as the carboxylate group or isoxazole group have emerged recently as novel inhibitors of DAO with high potencies, including carboxylate grouped compounds, i.e., AS057278 (5-methylpyrazole-3-carboxylic acid) (Adage *et al*., 2008), Compound 8 (4H-thieno[3,2-b]pyrrole-5-carboxylic acid) (Sparey *et al*., 2008; Smith *et al*., 2009), and NPCA (4-nitro-3-pyrazole carboxylic acid) (Fang *et al*. 2005), as well as isoxazole grouped compound CBIO (5-chlorobenzo[d] isoxazol-3-ol) (Ferraris *et al*., 2008) and hydroxyquinolin grouped Compound 2 (3-hydroxyquinolin-2-(1H)-one) (Duplantier *et al*., 2009). In the present study, we systemically studied inhibitory effects of these DAO inhibitors on rsDAO and pkDAO enzymatic activities. Our results confirmed that CBIO, Compound 8, AS057278, and sodium benzoate all inhibited both spinal cord derived DAO and porcine kidney DAO enzymatic activity in a concentration-dependent manner with maximal inhibition of 100%, and potency rank of CBIO (0.09 μM for rsDAO *vs.* 0.15 μM for pkDAO) > Compound 8 (0.17 μM *vs.* 0.52 μM) > AS057278 (15.4 μM *vs.* 14.6 μM) > sodium benzoate (75.4 μM *vs.* 44.6 μM) > NPCA (20 mM and 5.9 mM). consistent with previous results (Vanconi *et al*., 1997; Ferraris *et al*., 2008; Adage *et al*., 2008; Smith *et al*., 2009). However, NPCA less potently inhibited DAO enzymatic activity with IC_{50} values of 20 mM or 5.9 mM, in contrast to a patent where the IC_{50} value of NPCA was less than 10 μM (Fang *et al*., 2005). It is believed that AS057278, CBIO, Compound 8, and Compound 2 exhibit DAO inhibition via the same mechanism as benzoic acid through formation of hydrogen bonds with Arg283 and Tyr228 residues of the enzyme (Mattevi *et al*., 1996; Ferraris *et al*., 2008；Sparey *et al*., 2008; Duplantier *et al*., 2009; Smith *et al*.,

2009). NPCA is a 4-nitro-substituted 3-pyrazole-carboxylic acid analog and thus its low potency may be due to its nitro group-induced electron withdrawing effect, in contrast to AS57278, which is a 5-methyl-substituted 3-pyrazole-carboxylic acid analog and provides electrons to assist the formation of hydrogen bonds.

The current study has extended the previous results where systemic and intrathecal administrations of sodium benzoate prevented formalin-induced tonic pain (Zhao *et al*., 2008, 2010), by demonstrating that the more potent DAO inhibitors CBIO, Compound 8 and AS057278 administered by the intrathecal route all powerfully prevented formalin-induced tonic pain in a dose-dependent manner. The potency order determined was CBIO > Compound 8 > AS057278 > sodium benzoate > NPCA (ineffective). Highly potent analgesic actions of DAO inhibitors were evidenced by the fact that as little as 0.06 μg (0.39 nmol) CBIO blocked pain by 50%, approximately 5- and 2-fold more potent than the μ-opioid receptor agonist morphine and the NMDA receptor antagonist MK-801, respectively. In contrast, NPCA was ineffective up to 750 μg. Taken together, preventive effects of DAO inhibitors on formalin-induced tonic pain were correlated very well to their inhibitions of spinal cord-derived DAO (as well as porcine kidney-derived DAO) enzymatic activity. It is important to point out that sodium benzoate, Compound 8 and CBIO are structurally unrelated to the pyrazole-carboxylic acids (i.e., AS057278 and NPCA), which rules out the possibility that the analgesic effects are due to their structure-related but non-DAO specific effects. This is also emphasized by the finding that AS057278 is effective but NPCA is ineffective although both are 3-pyrazole-carboxylic acid analogs. Furthermore, we show that CBIO, whether given before or post formalin challenge, markedly prevented or reversed formalin-induced tonic pain to the same degree, suggesting that spinal DAO involves both induction and maintenance of tonic pain. In comparison, previous studies indicated that the NMDA receptor antagonist MK-801 prevented formalin-induced tonic pain but was not effective in reversion of the established pain state (Yamamoto and Yaksh, 1992; Vaccarino *et al*., 1993). These findings along with previous DAO gene mutation results (Zhao *et al*., 2008), indicate that spinal DAO mediates both induction and

maintenance of formalin-induced tonic pain and further validates spinal DAO as a target molecule for the treatment of chronic pain.

Dose-response analysis is a powerful tool for pharmacodynamic studies, which calculates and projects several parameters such as maximum effect (E_{max}) and half-effective doses (or concentrations) (ED_{50} or EC_{50}) (Wang *et al*., 1993). ED_{50} values are used to determine the potencies of individual DAO inhibitors and correlate to their spinal DAO inhibitory effects while the values of E_{max} are used to calculate the efficacy of individual DAO inhibitor and putting E_{max} values together to assess the efficacy of spinal DAO for pain transmission and transduction. Intrathecal injection of CBIO, Compound 8, AS078278, and sodium benzoate blocked chronic pain response in rats by maximum inhibition of 67%, 67%, 56% and 58%, respectively, while these compounds inhibited rat spinal cord-derived DAO enzymatic activity by 100%. By the systemic route, DAO inhibitors also exhibited the same degree of inhibition. Subcutaneous injection of CBIO blocked formalin-induced tonic pain in a dose-dependent manner with maximum effects of 60%, and 65%, respectively, in rats and Swiss mice (Gong *et al*., unpublished data, 2010). Moreover, intravenous administration of a single dose of sodium benzoate at 400 mg/kg, which was sufficient to entirely block in vivo DAO activity (Xin *et al*., 2005, 2007, 2010), prevented formalin-induced tonic pain by 63% and 68%, respectively, in Swiss mice (Zhao *et al*., 2008) and Balb/c mice (Gong *et al*., unpublished data, 2010). In addition, mutation of the DAO gene, leading to entire loss of DAO enzymatic activity, also blocked formalin-induced tonic pain by 60% in ddy/$DAO^{-/-}$mice compared to ddy/$DAO^{+/+}$ (Zhao *et al*., 2008). All these results summarized in Table 1 indicate that DAO is largely (approximately 60%) involved in formalin-induced tonic pain. Because both DAO inhibitors and DAO mutation did not alter formalin-induced nociception in the acute phase, formalin-induced pain state could be divided into two portions: i.e, a “DAO-sensitive” (60% tonic pain) component and a “DAO-insensitive” (40% tonic pain and 100% nociception) component.

Although the NMDA receptor in the spinal cord dorsal horn is silent under normal conditions, increased release of neurotransmitters such as glutamate and substance P

from nociceptors after injuries depolarizes postsynaptic neurons to activate quiescent NMDA receptors, which in return exacerbates responses to noxious stimuli to generate tonic pain. Therefore, activation of NMDA receptors is well known to be essential for central sensitization-mediated chronic pain (see reviews by Basbaum *et al*., 2009 and Hulsebosch *et al*., 2009). Intrathecal administration of NMDA receptor antagonists has been reported to suppress formalin-induced tonic pain (Nasstrom *et al*., 1992; Yamamoto and Yaksh, 1992; Chaplan *et al*., 1997). For example, MK-801 attenuated tonic pain in rats by 67 ± 16% (Chaplan *et al*., 1997), consistent with our MK-801 observation. It is important to note that the analgesia efficacy of DAO inhibitors was the same as that of MK-801, which is a potent, selective, and non-competitive antagonist of NMDA receptors (Wong *et al*., 1986) without inhibiting DAO activity (from the current study), and is commonly used as a prototype in electrophysiological and behavioral pharmacology of NMDA receptor functions. Since high efficacy is critical for selection of an ideal target molecule for treating chronic pain, the comparability of DAO inhibitors to NMDA receptor antagonists strengthens the significant role of the spinal DAO system in pain transmission and transduction. Indeed, SEP-227900, a DAO inhibitor of unknown structure, was reportedly in early stage clinical investigation for the treatment of neuropathic pain (Williams, 2009). The similarity (e.g., efficacy and specificity) and differences (e.g., pre *vs.* post formalin) of DAO inhibitors and NMDA receptor antagonists also make it difficult to link the two pain signal pathways. It has been proposed that activation of NMDA receptors leads to the up-regulation of DAO expression (Yoshikawaa *et al*., 2004).

Sodium benzoate's analgesic effects are chronic pain-specific as it blocked formalin-induced tonic pain and peripheral nerve damage-induced neuropathic mechanical allodynia but not acute nociception in the formalin test, hot-tail immersion test, or hot-plate test (Zhao *et al*., 2008, 2010). As both formalin-induced tonic pain and peripheral nerve injury-provoked neuropathic pain are mediated by on going activation of sensory afferents leading to central sensitization, i.e., increased responsiveness of higher order spinal neurons to peripheral input (Cook *et al*., 1987;

Coderre *et al*., 1993; Woolf *et al*., 1994; Jett *et al*., 1997), the analgesic action of sodium benzoate is suggested to be central sensitization-specific (Zhao *et al*., 2008, 2010). This study extended the specificity of DAO inhibitors by confirming that CBIO, Compound 8 and AS057278 are also not effective in acute nociception in the formalin test, and by further demonstrating that CBIO is ineffective in carrageenan-induced acute thermal hyperalgesia. Serving as an extensively employed model of inflammation and inflammatory pain (as the formalin test), carrageenan produced peripheral inflammation and heat hyperalgesia and mechanical allodynia via the mechanism of peripheral sensitization in the early phase (< 3-4 hrs after carrageenan challenge) (Kocher *et al*., 1987；Dirig *et al*., 1998; Cui *et al*., 1999) without generating overt firing of sensory afferents (see review by Sawynok and Liu, 2003). Thus the ineffectiveness of DAO inhibitors on carrageenan-induced thermal hyperalgesia may further suggest that DAO is specifically involved in continuous activation of sensory afferents and central sensitization-mediated persistent pain. However, beyond this peripheral role for inflammatory mediators in carrageenan-mediated hyperalgesia, a component of spinal sensitization, at least in the late phase (> 3-4 hrs after carrageenan challenge), may also participate in the hyperalgesia (Dirig *et al*., 1998; Cui *et al*., 1999；Hedo *et al*., 1999; Tao *et al*., 2003). The exact mechanism for the differential effects of DAO inhibitors on formalin-induced analgesia and carrageenan-induced hyperalgesia warrants further investigations.

In conclusion, intrathecal injections of a series of DAO inhibitors (CBIO, Compound 8, AS057278, and sodium benzoate, but not NPCA) specifically and powerfully prevented and reversed formalin-induced tonic pain but not acute nociception (or carrageenan-induced acute thermal hyperalgesia) in a dose-dependent manner. The ED_{50} value of CBIO for blockade of tonic pain was as little as 0.06 μg, approximately 5- and 2-fold more potent than morphine and MK-801. Dose-response analysis revealed that analgesia potencies of these DAO inhibitors correlated well to their inhibitions of spinal cord-derived DAO enzymatic activity. Moreover, maximum

inhibition by these compounds of formalin-induced tonic pain was approximately 60%, superior or compatible to that of the NMDA receptor antagonist MK-801 (approximately 50%), suggesting that 60% of formalin-induced tonic pain is “DAO sensitive” while formalin-induced acute nociception and the remaining 40% of tonic pain are “DAO insensitive”. These results, combined with previous DAO gene mutation results, indicate spinal DAO mediates both induction and maintenance of formalin-induced tonic pain and further documents that spinal DAO is an efficacious target molecule for the treatment of chronic pain including chronic neuropathic pain.

Acknowledgements

We thank Dr George Miljanich at Airmid Inc. (Redwood City, California, USA) for edition of the manuscript.

Authorship Contributions

1 *Participated in research design:* Y.X. Wang.

2 *Conducted experiments:* Gong, Gao, Y. C. Wang, Li, Huang.

3 *Contributed new reagents or analytic tools:* Hashimoto.

4 *Performed data analysis:* Gong, Gao, Y.X. Wang.

5 *Wrote or contributed to the writing of the manuscript:* Y.X. Wang, Gong.

6 *Other:* Y.X. Wang acquired funding for the research.

References

Abbott FV, Franklin KBJ, Westbrook RF (1995). The formalin test: scoring properties of the first and second phases of the pain response in rats. *Pain*. 60: 91-102.

Adage T, Trillat AC, Quattropani A, Perrin D, Cavarec L, Shaw J *et al*. (2008). In vitro and in vivo pharmacological profile of AS057278, a selective d-amino acid oxidase inhibitor with potential anti-psychotic properties. *Eur. Neuropsychopharmacol*. 18: 200-214.

Almond SL, Fradley RL, Armstrong EJ, Heavens RB, Rutter AR, Newman RJ *et al*. (2006). Behavioral and biochemical characterization of a mutant mouse strain lacking D-amino acid oxidase activity and its implications for schizophrenia. *Mol. Cell. Neurosci*. 32: 324-334.

Basbaum AI, Bautista DM, Scherrer G, Julius D (2009). Cellular and Molecular Mechanisms of Pain. *Cell* 139: 267-284.

Chaplan SR, Malmberg AB, Yaksh TL (1997). Efficacy of spinal NMDA receptor antagonism in formalin hyperalgesia and nerve injury evoked allodynia in the Rat. *J. Pharmacol. Exp. Ther.* 280: 829-838.

Coderre TJ, Fundytus ME, McKenna JE, Dalal S, Melzack R (1993). The formalin test - a validation of the weighted-scores method of behavioral pain rating. *Pain* 54: 43-50.

Cook AJ, Woolf CJ, Wall PD, McMahon SB (1987). Dynamic receptive-field plasticity in rat spinal-cord dorsal horn following C-primary afferent input. *Nature* 325: 151-153.

Cui J-G, Meyerson BA, Linderoth B (1999). Opposite effects of spinal cord stimulation in different phases of carrageenan-induced hyperalgesia. *Eur. J Pain* 3: 365-374.

D'Aniello, G. D'Onofrio, M. Pischetola, G. D'Aniello, A. Vetere, L. Petrucelli *et al*. (1993). Biological role of d-amino acid oxidase and d-aspartate oxidase, *J. Biol. Chem.* 268: 26941–26949.

Dirig DM, Isakson PC, Yaksh TL (1998). Effect of COX-1 and COX-2 inhibition on

induction and maintenance of carrageenan-evoked thermal hyperalgesia in rats. *J. Pharmacol. Exp. Ther*. 295: 1031-1038.

Duplantier AJ, Becker SL, Bohanon MJ, Borzilleri KA, Chrunyk BA, Downs JT *et al*. (2009). Discovery, SAR, and pharmacokinetics of a novel 3-hydroxyquinolin-2(1H)-one series of potent D-amino acid oxidase (DAAO) inhibitors. *J. Med. Chem.* 52: 3576-3585.

Fang QK, Hopkins S, Jones S, Fang Q (2005). New pyrazole derivatives, useful to e.g. treat ataxia, schizophrenia and neuropathic pain, are D-amino acid oxidase inhibitors. *Patent*: US2005143443-A1.

Ferraris D, Duvall B, Ko YS, Thomas AG, Rojas C, Majer P *et al*. (2008). Synthesis and biological evaluation of D-amino acid oxidase inhibitors. *J. Med. Chem.* 51: 3357-3359.

Hargreaves K, Dubner R, Brown F, Flores C, Joris J (1988). A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. *Pain* 32: 77-88.

Hashimoto K, Fujita Y, Horio M, Kunitachi S, Iyo M, Ferraris D *et al*. (2009). Co-administration of a D-amino acid oxidase inhibitor potentiates the efficacy of D-serine in attenuating prepulse inhibition deficits after administration of dizocilpine. *Biol. Psychiatry* 65: 1103-1106.

Hedo G, Laird JMA, Lopez-Garcia JA (1999). Time-course of spinal sensitization following carrageenan-induced inflammation in the young rat: a comparative electrophysiological and behavioural study in vitro and in vivo. *Neuroscience* 92: 309-318.

Horiike K, Ishida T, Tanaka H, Arai R (2001). Distribution of D-amino-acid oxidase and D-serine in vertebrate brains. *J. Mol. Catal. B-Enzymatic* 12: 37-41.

Horio M, Fujita Y, Ishima T, Iyo M, Ferraris D, Tsukamoto T *et al*. (2009). Effects of D-amino acid oxidase inhibitor on the extracellular D-alanine levels and the efficacy of D-alanine on dizocilpine-induced prepulse inhibition deficits in mice. *Open Clin. Chem. J.* 2: 16-21.

Hulsebosch CE, Hains BC, Crown ED, Carlton SM (2009). Mechanisms of chronic central neuropathic pain after spinal cord injury. *Brain Res. Rev.* 60: 202-213.

Jett MF, McGuirk J, Waligora D, Hunter JC (1997). The effects of mexiletine, desipramine and fluoxetine in rat models involving central sensitization. *Pain* 69: 161-169.

Kapoor R, Kapoor V (1997). Distribution of D-amino acid oxidase (DAO) activity in the medulla and thoracic spinal cord of the rat: implications for a role for D-serine in autonomic function. *Brain Res*. 771: 351-355.

Kocher L, Anton F, Reeh PW, Handwerker HO (1987). The effect of carrageenan-induced inflammation on the sensitivity of unmyelinated skin nociceptors in the rat. *Pain* 29: 363-373.

Konno R, Yasumura Y (1983). Mouse mutant deficient in d-amino acid oxidase activity. *Genetics* 103: 277– 285.

Kubota K, Ishizaki T (1991). Dose-dependent pharmacokinetics of benzoic-acid following oral-administration of sodium benzoate to humans. *Eur. J. Clin. Pharmacol*. 41: 363-368.

MacArthur RB, Altincatal A, Tuchman M (2004). Pharmacokinetics of sodium phenylacetate and sodium benzoate following intravenous administration as both a bolus and continuous infusion to healthy adult volunteers. *Mol. Genet. Metab.* 81: S67-S73.

Mattevi A, Vanoni MA, Todone F, Rizzi M, Teplyakov A, Coda A *et al*. (1996). Crystal structure of D-amino acid oxidase: a case of active site mirror-image convergent evolution with flavocytochrome b2. *Proc. Natl. Acad. Sci. USA* 93: 7496–7501.

Moreno S, Nardacci R, Cimini A, Ceru MP (1999). Immunocytochemical localization of D-amino acid oxidase in rat brain. *J .Neurocytol.* 28: 169-185.

Moses J, Siddiqui A, Silverman PB (1996). Sodium benzoate differentially blocks circling induced by D- and L-dopa in the hemi-Parkinsonian rat. *Neurosci. Lett.* 218: 145-148.

Nasstrom J, Karlsson U, Post C (1992). Antinociceptive actions of different classes of

excitatory amino acid receptor antagonists in mice. *Eur. J. Pharmacol.* 212: 21-29.

Pollegioni L, Piubelli L, Sacchi S, Pilone MS, Molla G (2007). Physiological functions of D-amino acid oxidases: from yeast to humans. *Cell. Mol. Life Sci.* 64: 1373-1394.

Sawynok J, Reid A (2002). Modulation of formalin-induced behaviors and edema by local and systemic administration of dextromethorphan, memantine and ketamine. *Eur. J. Pharmacol.* 450: 153-162.

Sawynok, J, Liu XJ (2003). The formalin test: characteristics and usefulness of the model. *Rev. Anal.* 7: 145-163.

Smith SM, Uslaner JM, Yao L, Mullins CM, Surles NO, Huszar SL *et al.* (2009). The behavioral and neurochemical effects of a novel D-amino acid oxidase inhibitor compound 8 [4H-thieno [3,2-b]pyrrole-5-carboxylic acid] and D-serine. *J. Pharmacol. Exp. Ther.* 328: 921-930.

Sparey T, Abeywickrema P, Almond S, Brandon N, Byrne N and Campbell A *et al.* (2008) The discovery of fused pyrrole carboxylic acids as novel, potent D-amino acid oxidase (DAO) inhibitors. *Bioorg. Med. Chem. Lett.* 18:3386-3391.

Tao F, Tao Y-X, Mao P, Zhao C, Li D, Liaw W-J *et al*. (2003). Intact carrageenan-induced thermal hyperalgesia in mice lacking inducible nitric oxide synathase. *Neuroscience* 120: 847-854.

Vaccarino AL, Marek P, Kest B, Weber E. Keana JFW, Liebeskind JC (1993). NMDA receptor antagonists, MK-801 and ACEA-1011, prevent the development of tonic pain following subcutaneous formalin. *Brain Res.* 615: 331-334.

Vanconi MA, Cosma A, Mazzeo D, Mattevi A, Todone F, Curti B *et al.* (1997). Limited proteolysis and X-ray crystallography reveal the origin of substrate specificity and of the rate-limiting product release during oxidation of D-amino acids by mammalian D-amino acid oxidase. *Biochemistry* 36: 5624–5632.

Verrall L, Burnet PWJ, Betts JF, Harrison PJ (2009). The neurobiology of D-amino acid oxidase and its involvement in schizophrenia. *Mol. Psychiatry* 15: 1–16.

Wake K, Yamazaki H, Hanzawa S, Konno R, Sakio H, Niwa A *et al*. (2001).

Exaggerated responses to chronic nociceptive stimuli and enhancement of N-methyl-D-aspartate receptor-mediated synaptic transmission in mutant mice lacking D-amino-acid oxidase. *Neurosci. Lett.* 297: 25-28.

Wang Y-X, Gao D, Pettus M, Phillips C, Bowersox SS (2000). Interactions of intrathecally administered ziconotide, a selective blocker of neuronal N-type voltage-sensitive calcium channels, with morphine on nociception in rats. *Pain* 84: 271-281.

Wang Y-X, Pang CCY (1993). Functional integrity of the central and sympathetic nervous systems is a prerequisite for pressor and tachycardic effects of diphenyleneiodonium, a novel inhibitor of nitric oxide synthase. *J. Pharmacol. Exp. Ther*. 265: 263-272.

Wei H, Zhao WJ, Wang Y-X, Pertovaara A (2007). Pain-related behavior following REM sleep deprivation in the rat: Influence of peripheral nerve injury, spinal glutamatergic receptors and nitric oxide. *Brain Res.* 1148: 105-112.

Wheeler-Aceto H, Cowan A (1993). Naloxone causes apparent antinociception and pronociception simultaneously in the rat paw formalin test. *Eur. J. Pharmacol.* 236: 193-199.

Williams M (2009). Genome-based CNS drug discovery: D-Amino acid oxidase (DAAO) as a novel target for antipsychotic medications: Progress and challenges. *Biochem. Pharmacol.* 78: 1360-1365.

Williams RE, Lock EA (2005). Sodium benzoate attenuates D-serine induced nephrotoxicity in the rat. *Toxicology* 207: 35-48.

Wong EHK, Kemp JA, Priestley T, Knight AR, Woodruff GN, Iversen LL (1986). The anticonvulsant MK-801 is a potent N-methyl-D-aspartate antagonist. *Proc. Natl. Acad. Sci. USA* 83: 7104-7108.

Woolf CJ, Shortland P, Sivilotti LG (1994). Sensitization of high mechanothreshold superficial dorsal horn and flexor motor-neurons following chemosensitive primary afferent activation. *Pain* 58: 141-155.

Wu M, Zhou X-J, Konno R, Wang Y-X (2006). D-dopa is unidirectionally converted to L-dopa by D-amino acid oxidase, followed by dopa transaminase. *Clin. Exp.*

Pharmacol. Physiol. 33: 1042-1046.

Xin Y-F, Li X, Hao B, Gong N, Sun W-Q, Konno R *et al*. (2010). Indispensable but insufficient role of renal D-amino acid oxidase in chiral inversion of NG-nitro-D-arginine. *Chem. Biodivers*. 7: 1413-1423.

Xin Y-F, Zhou X-J, Cheng X< Wang Y-X (2005). Renal D-Amino Acid Oxidase Mediates Chiral Inversion of NG-nitro-D-arginine. *J. Pharmacol. Exp. Ther*. 312: 1090-1096.

Xin Y-F, Zhou X-J, Lu J, Wang Y-X (2007). Inhibitory effects of benzoate on chiral inversion and clearance of N-G-nitro-arginine in conscious rats. *Drug Metab. Dispos*. 35: 331-334.

Yamamoto T, Yaksh TL (1992). Comparison of the antinociceptive effects of pre- and posttreatment with intrathecal morphine and MK801, an NMDA antagonist, on the formalin test in the rat. *Anesthesiology* 77: 757-63.

Yoshikawa M, Oka T, Kawaguchi M, Hashimoto A (2004). MK-801 upregulates the expression of D-amino acid oxidase mRNA in rat brain. Mol. *Brain Res*. 131: 141-144.

Zhao W-J, Gao Z-Y, Wei H, Nie H-Z, Zhao Q, Zhou X-J *et al*. (2010). Spinal D-Amino acid oxidase contributes to neuropathic pain in rats. *J. Pharmacol. Exp. Ther*. 332: 248-254.

Zhao W-J, Konno R, Zhou X-J, Yin M, Wang Y-X (2008). Inhibition of D-amino-acid oxidase activity induces pain relief in mice. *Cell. Mol. Neurobiol.* 28: 581-591.

Footnotes

a. This study was supported by National Natural Science Foundation of China (No: 81072623 and No: 30973581) and the Mega New Drug Development Program of China (No: 2009ZX09301-007).

b. Both Nian Gong and Zhen-Yu Gao are first authors with equal contributions.

Legends for figures and tables

Fig. 1 Chemical structures of D-amino acid oxidase (DAO) inhibitors: sodium benzoate (benzoic acid), CBIO (5-chlorobenzo[*d*]isoxazol-3-ol), Compound 8 (sc-203909, 4H-thieno[3,2-b]pyrrole-5-carboxylic acid) from Merck, AS057278 (5-methylpyrazole-3-carboxylic acid), NPCA (4-nitro-3-pyrazole carboxylic acid), and Compound 2 (3-hydroxyquinolin-2-(1H)-one) from Pfizer.

Fig. 2. Inhibitory effects of D-amino acid oxidase (DAO) inhibitors CBIO (5-chlorobenzo[*d*]isoxazol-3-ol), Compound 8 (sc-203909, 4H-thieno[3,2-b]pyrrole-5-carboxylic acid), AS057278 (5-methylpyrazole-3-carboxylic acid), sodium benzoate and NPCA (4-nitro-3-pyrazole carboxylic acid) on DAO enzymatic activities of rat spinal cord-derived DAO (rsDAO, A) and porcine kidney-derived (pkDAO, B). DAO enzymatic activity was assayed in homogenates from spinal cord lumbar enlargements and pure enzyme of pkDAO by using pyruvate production method. All the readings are means ± SEM of triplicates, repeated 2 - 3 times. Concentration response analysis of DAO inhibitors on DAO enzymatic activity were fitted by nonlinear least-squares method.

Fig. 3. Differentially preventive effects of intrathecal injection of the D-amino acid oxidase (DAO) inhibitor CBIO (5-chlorobenzo[*d*]isoxazol-3-ol) on formalin-induced acute nociception and tonic pain in rats (**A**). Intrathecally cannulated rats received intrathecal injection of normal saline (10 μl) or CBIO (0.01, 0.03, 0.1, 0.3, 1, or 3 μg) 30 minutes before subcutaneous injection of 50 μl of 5% formalin. Nociceptive behavior was quantified by counting the number of the formalin-injected paw flinches supplemented by licking duration (considered as 1 count per second matching the duration of the typical flinching behavior) in 1-min epochs. Data are presented as means ± SEM; n = 5 - 6 in each group. The analgesic effect was statistically significant ($P < 0.05$ by a two-way repeated measures ANOVA). (**B**) Dose response analysis of CBIO on formalin-induced tonic pain measured by $AUC_{12\text{-}96\ min}$, best fitted

by nonlinear least-squares method.

Fig. 4. Effects of intrathecal injections of the D-amino acid oxidase inhibitors CBIO (5-chlorobenzo[*d*]isoxazol-3-ol) and NPCA (4-nitro-3-pyrazole carboxylic acid) at different time points (marked by↓) on 50 μl of 1% or 5% formalin-induced acute nociception and tonic pain in rats. (**A**) Rats received intrathecal injection of 10 μl normal saline or 3 μg CBIO 90 minutes before subcutaneous injection of 5% formalin. (**B**) Rats received intrathecal injection of 10 μl normal saline or 3 μg CBIO 30 minutes before 1% formalin injection. (**C**) Rats received intrathecal injection of 10 μl normal saline or 1 μg CBIO 24 minutes post 5% formalin injection. (**D**) Rats received intrathecal injection of 30 μl of 50% DMSO in normal saline or 750 μg NPCA 30 minutes before 5% formalin injection. Nociceptive behavior was quantified by counting the number of the formalin-injected paw flinches supplemented by licking duration (considered as 1 count per second matching the duration of the typical flinching behavior) in 1-min epochs. Data are presented as means ± SEM; n = 4 - 5 in each group. * denotes statistically significant difference ($P < 0.05$) compared with the respective normal saline control group (Two-way repeated measures ANOVA followed by post-hoc two-tailed Student's t-tests).

Fig. 5. Differential effects of intrathecal injection of the D-amino acid oxidase (DAO) inhibitor Compound 8 (sc-203909, 4H-thieno[3,2-b]pyrrole-5-carboxylic acid) on formalin-induced acute nociception and tonic pain in rats (**A**). Intrathecally cannulated rats received intrathecal injection of normal saline (10 μl) or Compound 8 (0.1, 1, 3, or 10 μg) 30 minutes before subcutaneous injection of 50 μl of 5% formalin. Nociceptive behavior was quantified by counting the number of the formalin-injected paw flinches supplemented by licking duration (considered as 1 count per second matching the duration of the typical flinching behavior) in 1-min epochs. Data are presented as means ± SEM; n = 3 - 4 in each group. The analgesic effect was statistically significant ($P < 0.05$ by a two-way repeated measures ANOVA). (**B)** Dose response analysis of Compound 8 on formalin-induced tonic pain measured by

$AUC_{12\text{-}96\ min}$, best fitted by nonlinear least-squares method.

Fig. 6. Differential effects of intrathecal injection of the D-amino acid oxidase (DAO) inhibitor AS057278 (5-methylpyrazole-3-carboxylic acid) on formalin-induced acute nociception and tonic pain in rats (**A**). Intrathecally cannulated rats received intrathecal injection of normal saline (10 μl) or AS057278 (0.3, 1, 3, or 10 μg) 30 minutes before subcutaneous injection of 50 μl of 5% formalin. Nociceptive behavior was quantified by counting the number of the formalin-injected paw flinches supplemented by licking duration (considered as 1 count per second matching the duration of the typical flinching behavior) in 1-min epochs. Data are presented as means ± SEM; n = 6 in each group. The analgesic effect was statistically significant ($P < 0.05$ by a two-way repeated measures ANOVA). (**B)** Dose response analysis of AS057278 on formalin-induced tonic pain measured by $AUC_{12\text{-}96\ min}$, best fitted by nonlinear least-squares method.

Fig. 7. Differential effects of intrathecal injection of the D-amino acid oxidase (DAO) inhibitor sodium benzoate on formalin-induced acute nociception and tonic pain in rats (**A**). Intrathecally cannulated rats received intrathecal injection of normal saline (10 μl) or sodium benzoate (3, 10, 30, 100, or 300 μg) 30 minutes before subcutaneous injection of 50 μl of 5% formalin. Nociceptive behavior was quantified by counting the number of the formalin-injected paw flinches supplemented by licking duration (considered as 1 count per second matching the duration of the typical flinching behavior) in 1-min epochs. Data are presented as means ± SEM; n = 6 in each group. The analgesic effect was statistically significant ($P < 0.05$ by a two-way repeated measures ANOVA). (**B)** Dose response analysis of sodium benzoate on formalin-induced tonic pain measured by $AUC_{12\text{-}96\ min}$, best fitted by nonlinear least-squares method.

Fig. 8. Differential effects of intrathecal injection of the NMDA receptor antagonist MK-801 on formalin-induced acute nociception and tonic pain in rats (**A**).

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Intrathecally cannulated rats received intrathecal injection of normal saline (10 μl) or MK-801 (0.03, 0.1, 0.3, 1, 3, or 10 μg) 30 minutes before subcutaneous injection of 50 μl of 5% formalin. Nociceptive behavior was quantified by counting the number of the formalin-injected paw flinches supplemented by licking duration (considered as 1 count per second matching the duration of the typical flinching behavior) in 1-min epochs. Data are presented as means ± SEM; n = 4 in each group. The analgesic effect was statistically significant ($P < 0.05$ by a two-way repeated measures ANOVA). (**B)** Dose response analysis of MK-801 on formalin-induced tonic pain measured by $AUC_{12\text{-}96\ min}$, best fitted by nonlinear least-squares method.

Fig. 9. Correlation between prevention of formalin-induced tonic by intrathecal injections of D-amino acid oxidase (DAO) inhibitors (CBIO, Compound 8, AS057278, sodium benzoate, and NPCA) to their inhibitory effects on rat spinal cord-derived DAO (rsDAO, **A**) or porcine kidney-derived (pkDAO, **B**) DAO enzymatic activity. The IC_{50} values were derived from Fig. 2, while ED_{50} values were derived from Figs 3, 5, 6 and 7. As NPCA (4-nitro-3-pyrazole carboxylic acid) was not effective in prevention of pain up to the maximally allowable dose (750 μg) (Fig. 4C) and thus no ED_{50} value was obtained, the maximal allowable dose was plotted as >* instead. Correlation coefficient was calculated and tested statistically by unpaired and two-tailed Student t-test.

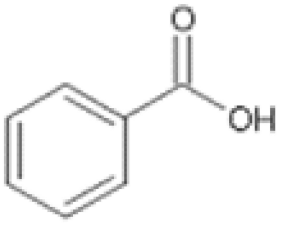
Fig. 10. Ineffectiveness of intrathecal injection of the D-amino acid oxidase (DAO) inhibitor CBIO (5-chlorobenzo[*d*]isoxazol-3-ol) on radial heat-induced acute nociception and carrageenan-induced thermal hyperalgesia in rats. Intrathecally cannulated rats received intrathecal injection of normal saline (10 μl) or CBIO (1 μg) 2 hours post intraplantar injection of 100 μl of 2% carrageenan (marked by↓). Withdrawal latency was measured in both paws 2.5 hours before carrageenan injection, before and 0.5, 1, 2, and 3 hours after control and test article intrathecal administrations. Data are presented as means ± SEM; n = 4 in each group. Withdrawal latency in the carrageenan paw was significantly lower than that of the normal paw (P

< 0.05 by two-way repeated measures ANOVA).

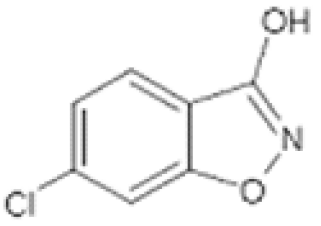
Table 1 Summary from different studies of maximum inhibitory effects of D-amino acid oxidase (DAO) gene mutation and DAO inhibition, on formalin-induced tonic pain, compared with the NMDA receptor antagonist MK-801.

DAO Inhibition Phenotype	E_{max} (%)	References
DAO mutation (entire loss in DAO activity) in ddY mice	60	Zhao *et al*., 2008
Systemic DAO inhibition		
Swiss mice (Benzoate, maximum dose)	63	Zhao *et al.*, 2008
Balb/c mice (CBIO, maximum dose)	68	Gong *et al.*, 2010
Wistar rats (CBIO, E_{max})	63	Gong *et al.*, 2010
Swiss mice (CBIO, E_{max})	50	Gong *et al.*, 2010
Spinal DAO inhibition		
CBIO (E_{max})	67	Present study
Compound 8 (E_{max})	67	Present study
AS057278 (E_{max})	56	Present study
Benzoate (E_{max})	58	Present study
Apparent average	61	
MK-801	51	Present study

Fig. 1.

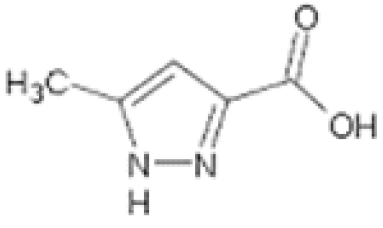


Benzoic Acid



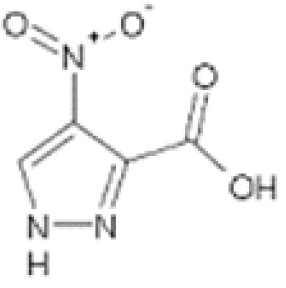
CBIO

(5-chlorobenzo[*d*]isoxazol-3-ol)



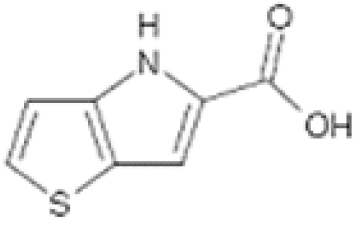
AS057278

(5-methyl-3-pyrazole-carboxylic acid)



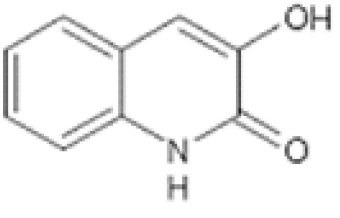
NPCA

(4-nitro-3-pyrazole carboxylic acid)



Compound 8 (sc-203909)

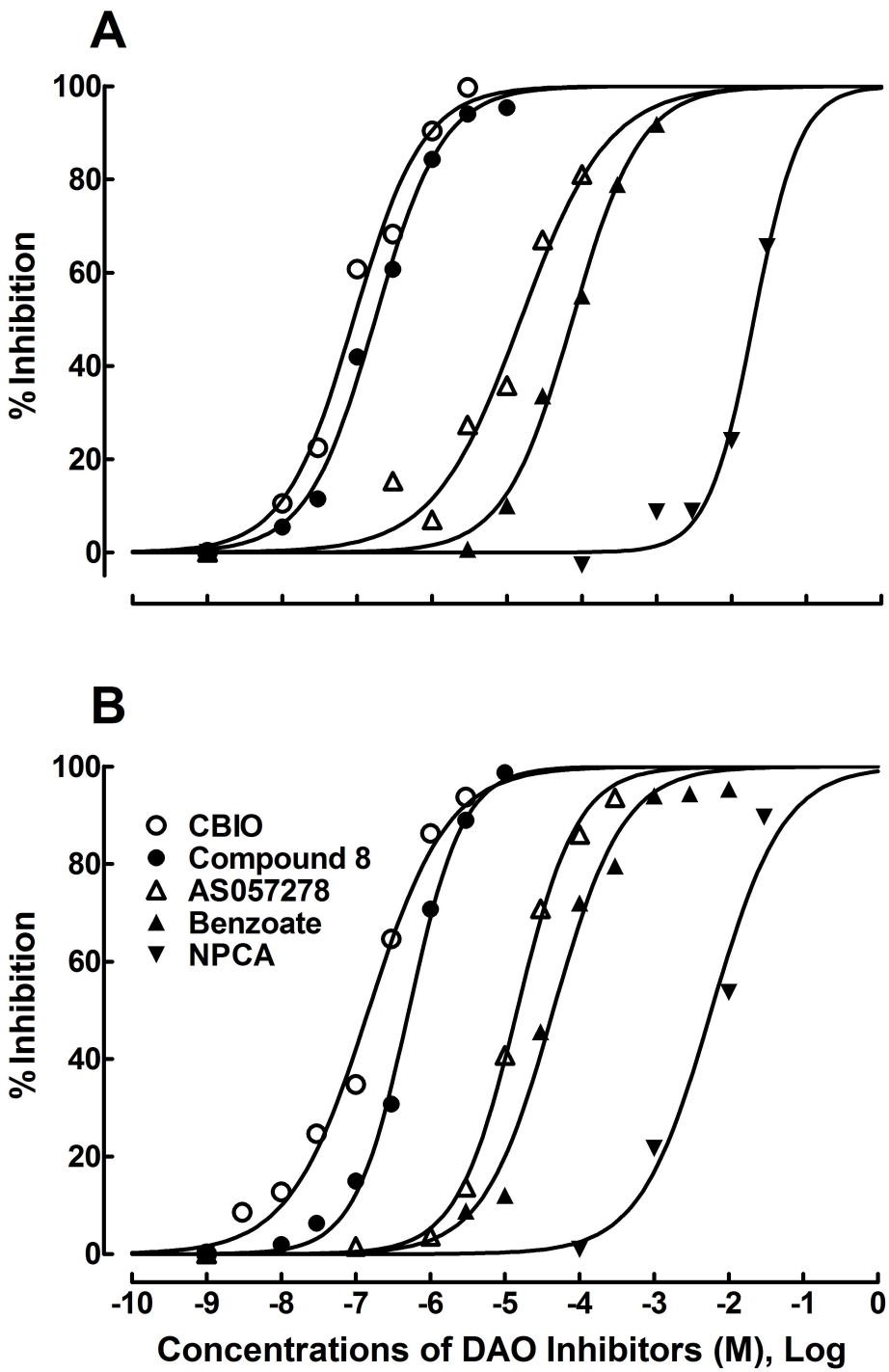
([4H]-thieno[3,2-b]pyrrole-5-carboxylic acid)



Compound 2

(3-hydroxyquinolin-2-[1H]-one)

Fig. 2.



	IC_{50} (μM)	
	rsDAO	pkDAO
CBIO	0.09	0.15
Compound 8	0.17	0.52
AS057278	15.41	14.57
Benzoate	75.43	44.63
NPCA	20030	5876

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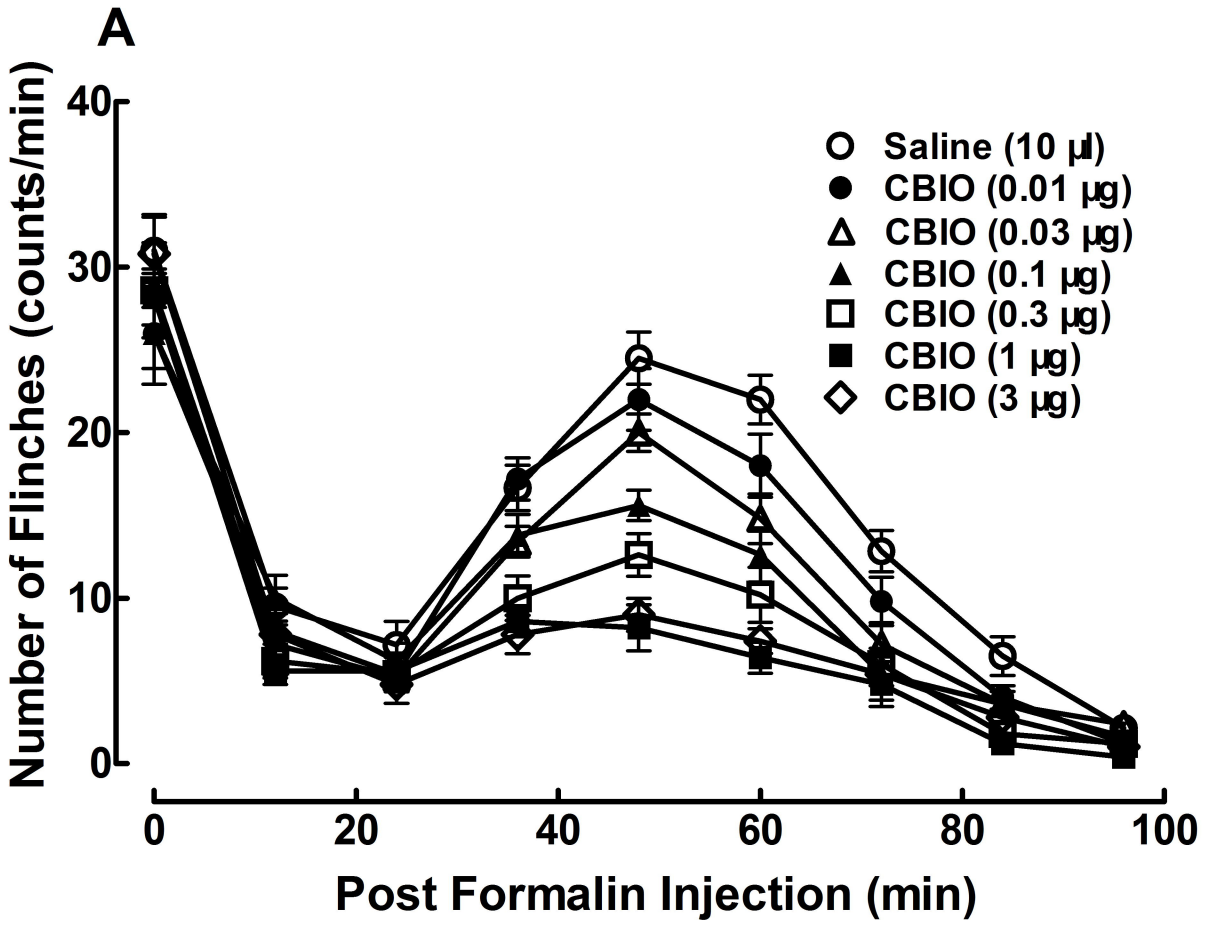
Fig. 3.



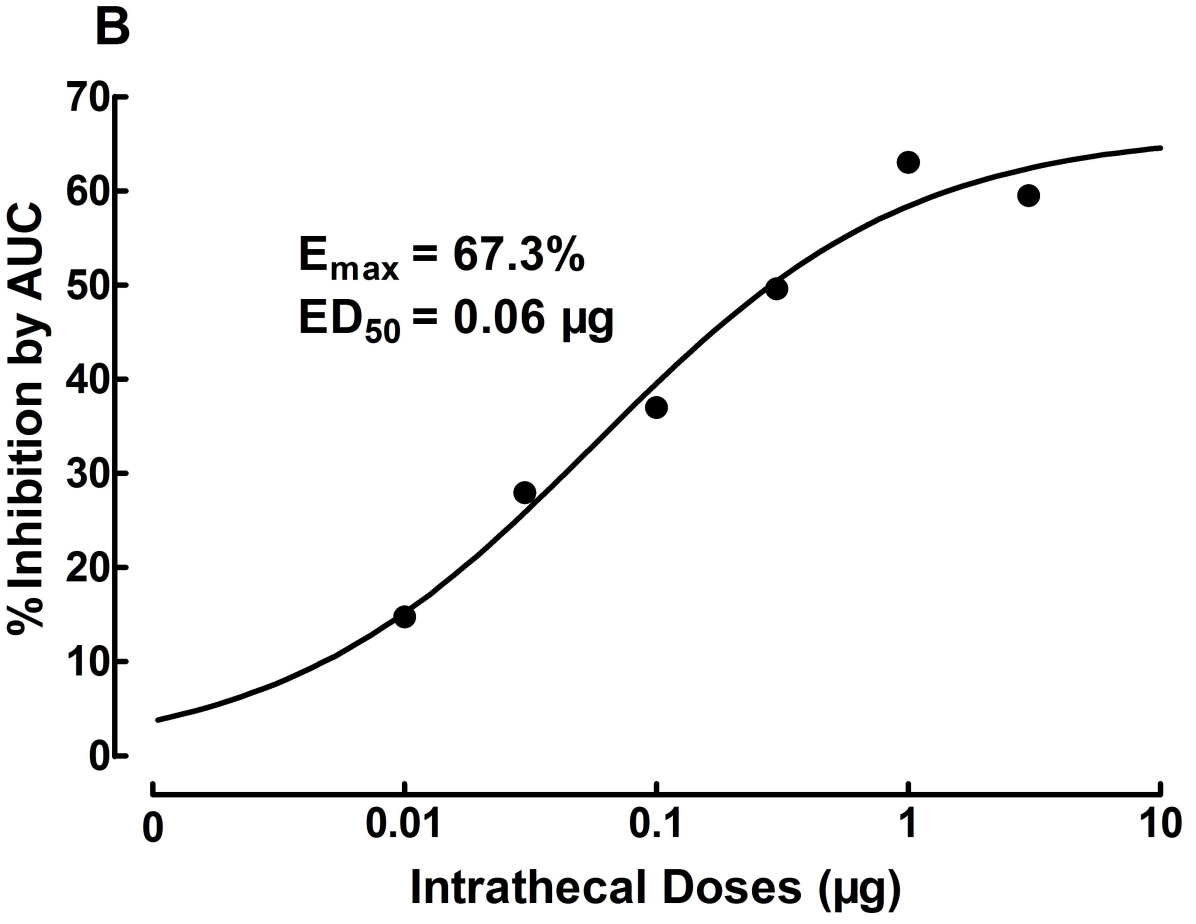


Fig. 4.

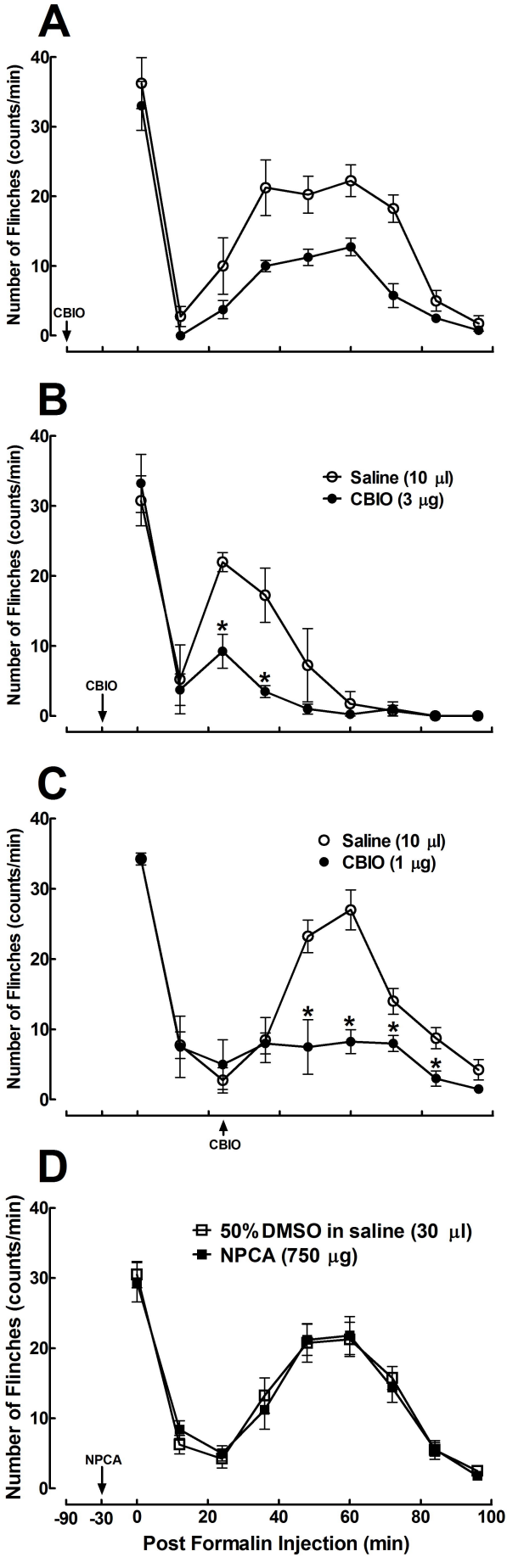


Fig. 5.

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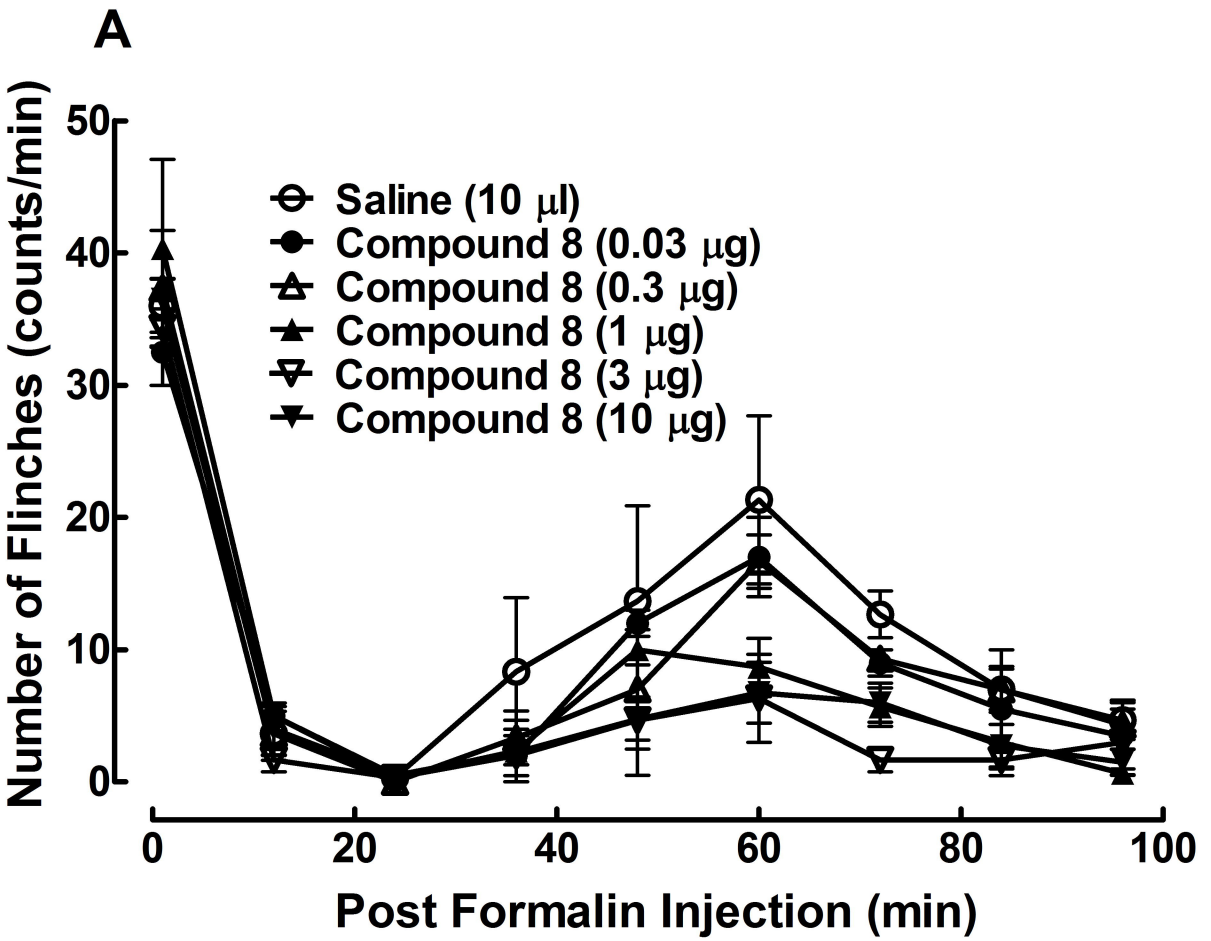

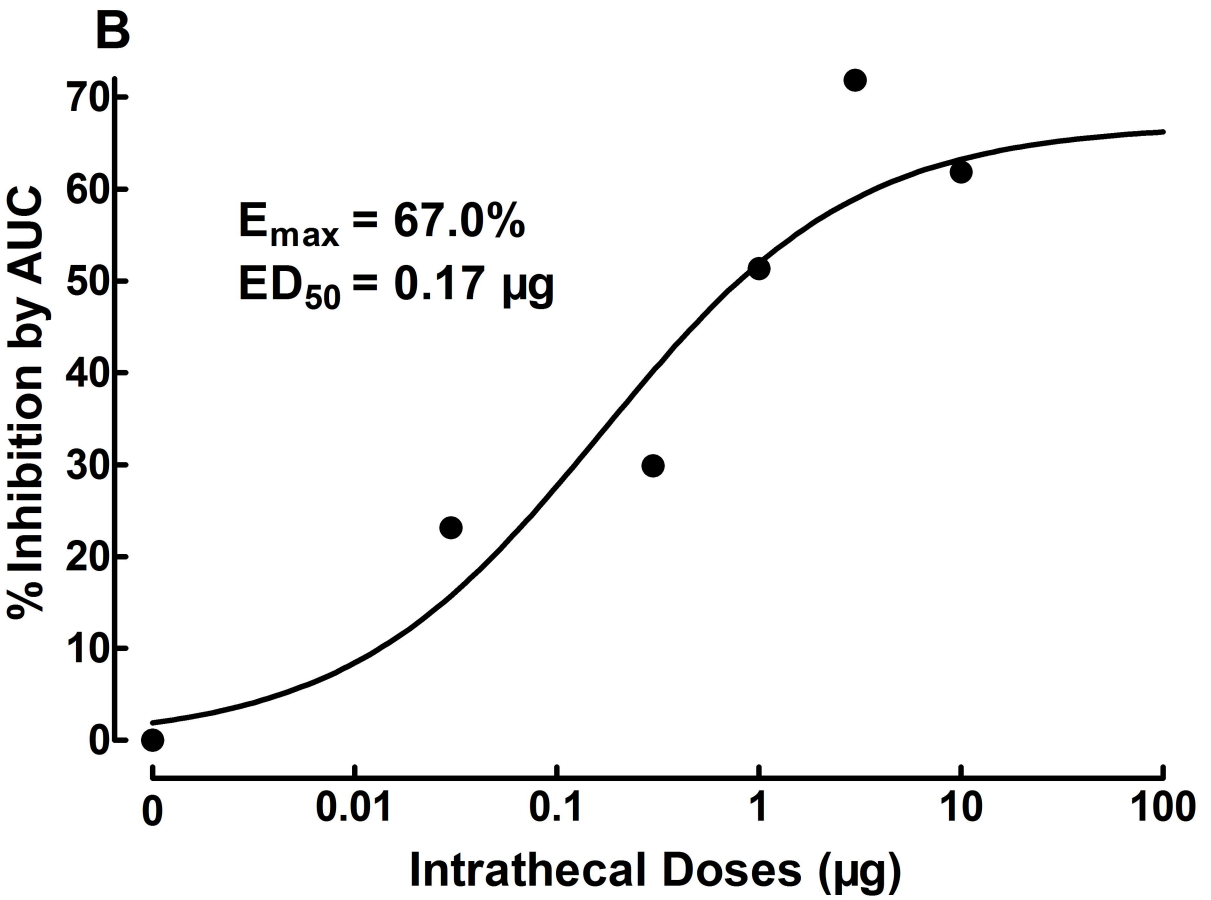

Fig. 6.

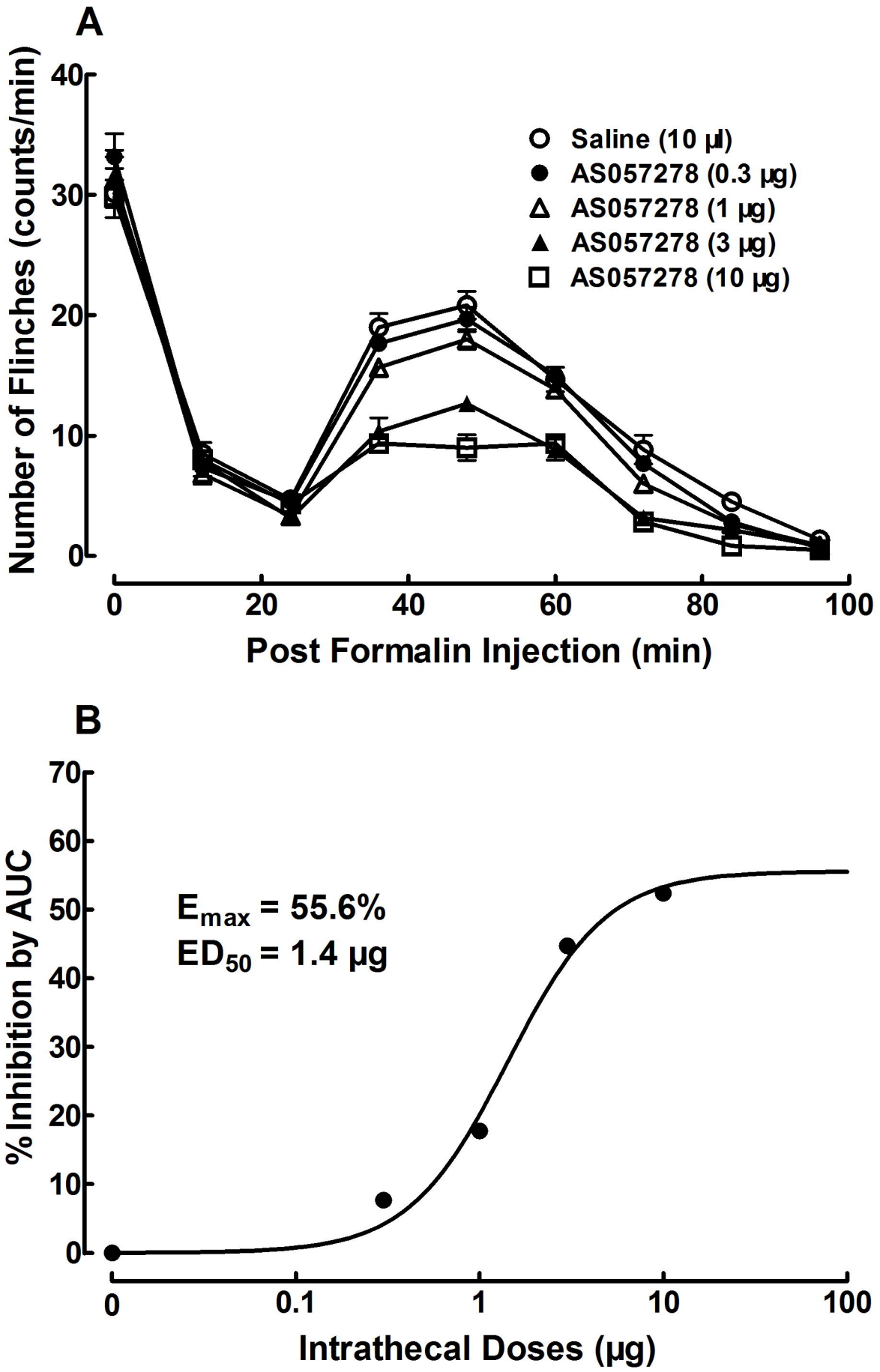

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Fig. 7.

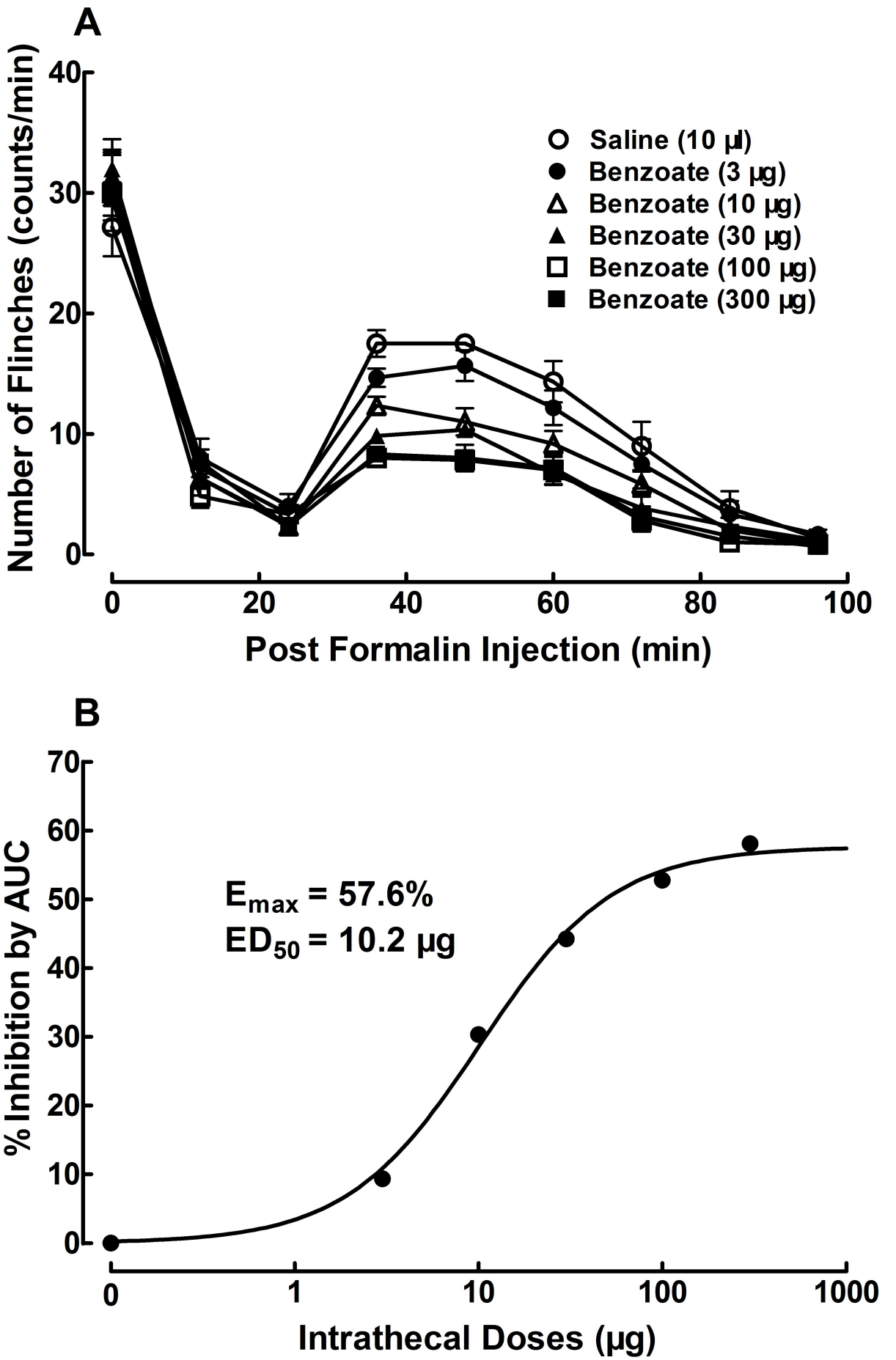

Fig. 8.

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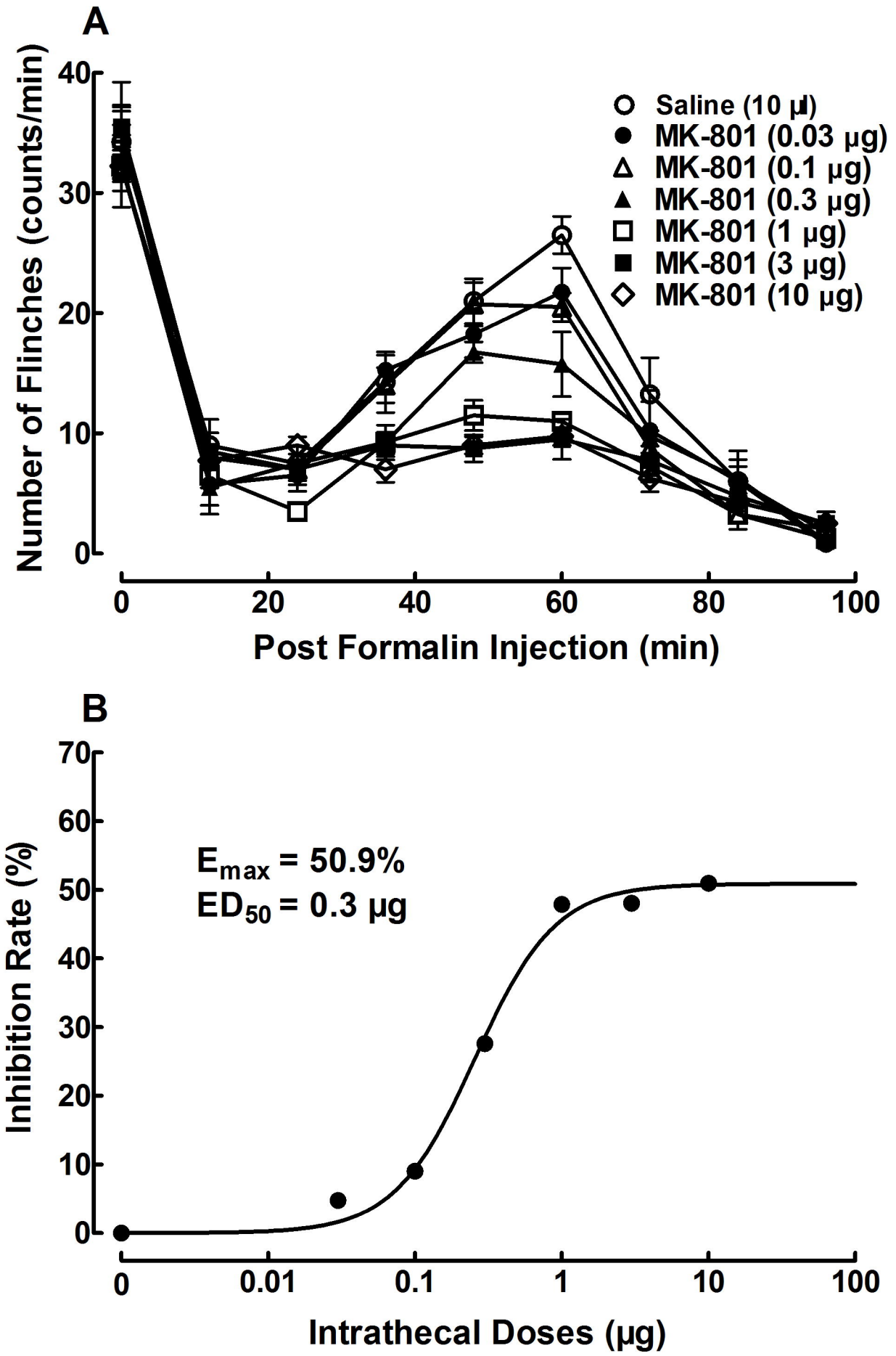


Fig. 9.

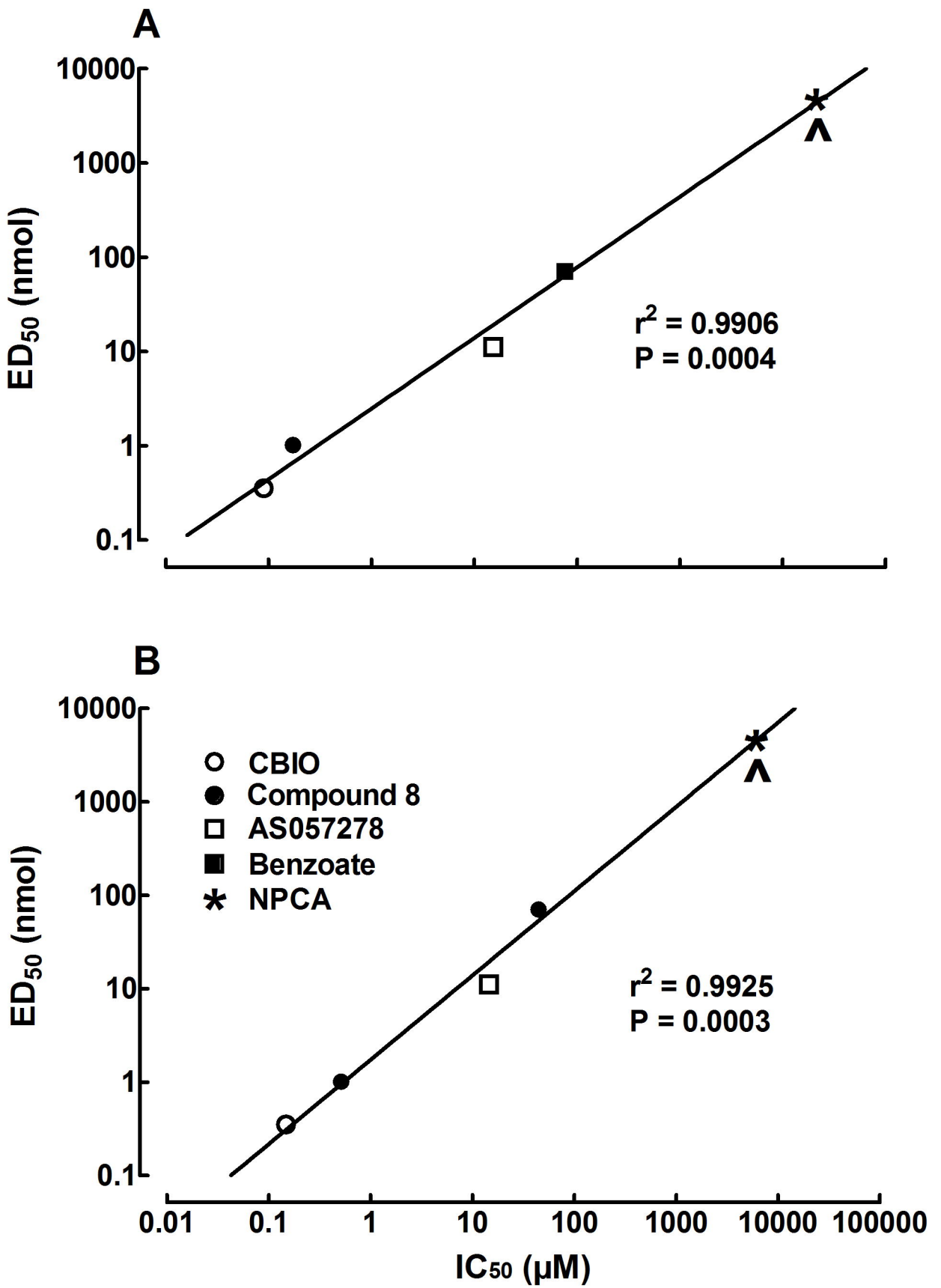
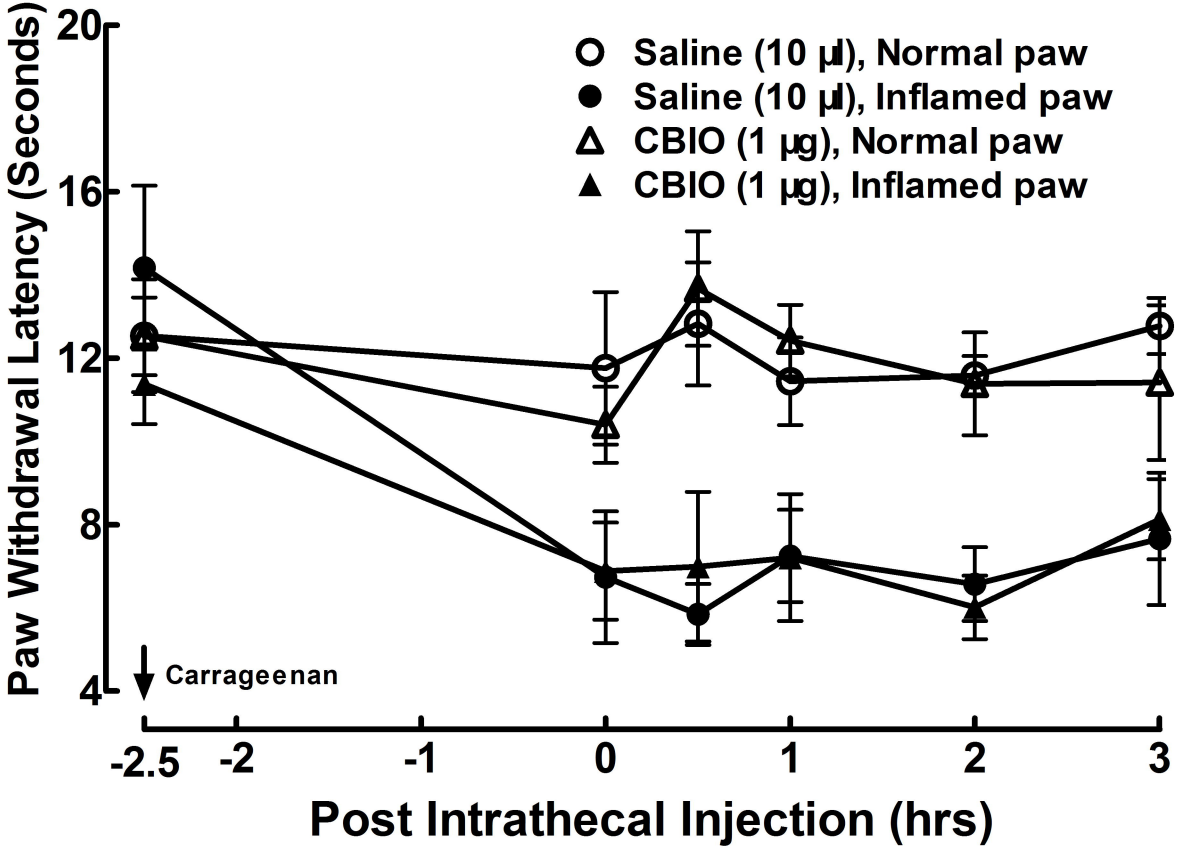

A
10000
1000
100
10
1
0.1
ED50 (nmol)
r2 = 0.9906
P = 0.0004
B
10000
1000
100
10
1
0.1
ED50 (nmol)
CBIO
Compound 8
AS057278
Benzoate
NPCA
r2 = 0.9925
P = 0.0003
0.01
0.1
1
10
100
1000
10000
100000
IC50 (μM)

Fig. 10.


Saline (10 µl), Normal paw
Saline (10 µl), Inflamed paw
CBIO (1 µg), Normal paw
CBIO (1 µg), Inflamed paw
Paw Withdrawal Latency (Seconds)
20
16
12
8
4
Carrageenan
-2.5
-2
-1
0
1
2
3
Post Intrathecal Injection (hrs)