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ORIGINAL ARTICLE

Contributions of spinal D-amino acid oxidase to bone cancer pain

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Abstract D-Amino acid oxidase (DAAO), a FAD-dependent peroxisomal flavoenzyme that catalyzes oxidation of D-amino acids to hydrogen peroxide, is distributed in the spinal cord almost exclusively expressed within astrocytes. The present study aims to explore potential contributions of spinal DAAO to the development of bone cancer pain and morphine tolerance to analgesia. Tibia inoculation of carcinoma cells produced mechanical allodynia (but not heat hyperalgesia), in synchronous with induction of DAAO expression and DAAO enzymatic activity, as well as activation of spinal astrocytes marked by GFAP. Subcutaneous and intrathecal injection of the specific DAAO inhibitor CBIO (5-chloro-benzo[*d*]isoxazol-3-ol) blocked mechanical allodynia in a dose- and time-dependent manner in tumor-bearing rats, with maximum inhibition of 40–50 %. Multi-daily intrathecal injections of the DAAO gene silencer siRNA/DAAO also yielded anti-allodynic effects by approximately 40 % and the analgesia remained for at least 6 days. Subcutaneous injection of CBIO suppressed the production of spinal hydrogen peroxide and GFAP expression. 7-Day multiple bi-daily injections of CBIO produced anti-allodynia without inducing self-tolerance to analgesia or cross-tolerance to morphine, and concurrent injections of CBIO with morphine produced apparent additive anti-allodynia and completely prevented morphine tolerance in behaviors and spinal expression of μ-opioid receptors. Our results provide the first evidence that spinal DAAO contributes to the development of morphine tolerance to analgesia and bone cancer pain accounting for 40–50 % pain status, probably via production of hydrogen peroxide leading to activation of astrocytes. The unique characterizations of DAAO inhibitors make them a potential for the treatment of cancer pain when they are administered alone or in combination with morphine.

Keywords D-Amino acid oxidase (DAAO) · Bone cancer pain · 5-Chloro-benzo[*d*]isoxazol-3-ol (CBIO) · Morphine tolerance · Gene silencer siRNA/DAAO

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Introduction

Bone metastases often occur from distant sites, such as the breast, prostate and lungs in a significant proportion of patients with advanced cancers (Goblirsch et al. 2006; Luger et al. 2001). Severe chronic pain, one of the most frequent symptoms of bone cancer, manifested as spontaneous pain, hyperalgesia and allodynia, as well as bone fractures, hypercalcemia and neurologic deficits, dramatically disrupts and interferes with patient's quality of life (Urch 2004). Although opiates, especially the μ-opioid receptor agonist morphine, are used as a conventionally prescribed analgesic drugs for the treatment of advanced bone cancer pain, patients often undergo tolerance to analgesia that is one of the reasons for limiting the long-term clinical application of opioids, and require higher doses of morphine, which consequently produce diverse and disabling side effects such as sedation, somnolence, and constipation. Thus, bone cancer pain remains one of

the most challenging symptoms to manage in patients with cancers and represents a large unmet need for improved therapies.

D-Amino acid oxidase (DAAO, EC1.4.3.3) is a FAD-dependent peroxisomal flavoenzyme, which catalyzes the oxidative deamination of neutral and polar D-amino acids to their corresponding α-keto acids and hydrogen peroxide (H_2O_2) (Angermüller et al. 2009; Kappor and Kapoor 1997; Wang et al. 2012). In mammals including human beings, it is mainly distributed in the kidney, liver and the central nervous system including the lower brainstem, spinal cord and cerebellum and is almost exclusively expressed within astrocytes (Horiike et al. 1994; Kappor and Kapoor 1997; Wang et al. 2012). We have recently discovered that spinal DAAO is involved in chronic pain conditions (Chen et al. 2012; Gong et al. 2011; Zhao et al. 2008, 2010). Spinal nerve ligation up-regulated spinal DAAO gene expression and enzymatic activity in the development of neuropathic pain (Zhao et al. 2010). Gene mutation (Zhao et al. 2008) and silencing (Chen et al. 2012) of DAAO significantly reduced formalin-induced tonic phase pain. Moreover, systemic and intrathecal administrations of a series of DAAO inhibitors, including CBIO (5-chloro-benzo[*d*]isozazol-3-ol), AS057278 (5-methylpyrazole-3-carboxylic acid), "Compound 8" (4*H*-thieno[3,2-b]pyrrole-5-carboxylic acid) and sodium benzoate, greatly blocked formalin-induced tonic phase pain (Gong et al. 2011; Lu et al. 2012; Zhao et al. 2008) and chronic neuropathic pain (Zhao et al. 2010) in rats and mice but not acute nociception such as thermal-evoked licking and tail-flicking response and formalin-induced acute phase nociception (Gong et al. 2011; Zhao et al. 2008, 2010).

These chronic pain models share a common mechanism of central sensitization which is usually defined as the change in excitability of spinal and brain neuron after persistent nociceptive stimulation (Coderre and Melzack 1992; Cook et al. 1987; Latremoliere and Woolf 2009; Woolf et al. 1994). Chronic bone cancer pain (Yanagisawa et al. 2010) and morphine tolerance to analgesia (Mao et al. 1995; Mayer et al. 1999) are thought to be at least partly due to central sensitization. Thus, it is hypothesized that spinal DAAO contributes to the induction and maintenance of bone cancer pain and morphine tolerance to analgesia. The present study aimed to explore the possible anti-allodynic and morphine tolerance blockade effects in the rat model of bone cancer pain by inhibition of spinal DAAO enzymatic activity employing CBIO and knockdown of spinal DAAO expression employing siRNA/DAAO. CBIO is known to be one of the most potent DAAO inhibitors with IC_{50} values of 90–188 nM (Ferraris et al. 2008; Gong et al. 2011; Smith et al. 2010). siRNA is a class of double-stranded RNA molecules interfering with and silencing expression of its specific gene of interest (Chakraborty 2007). Multi-daily intrathecal injections of the siRNA/DAAO have been previously shown to be effective to silence spinal DAAO expression and inhibit DAAO enzymatic activity (Chen et al. 2012). This study included the following procedures: (1) examining changes of DAAO expression and enzymatic activity in the development of bone cancer pain after cancer cell inoculation; (2) testing anti-allodynic actions of systemic and intrathecal administrations of CBIO, as well as multi-daily intrathecal injections of the siRNA/DAAO in the rat bone cancer pain model; (3) exploring whether CBIO exerts its anti-allodynic effect on bone cancer pain by reduction of spinal hydrogen peroxide via inhibition of DAAO resulting in blockade of the activation of spinal astrocytes; and (4) finally determining whether CBIO produces self-tolerance to analgesia or cross-tolerance to morphine and particularly prevents morphine tolerance to analgesia in the rat bone cancer pain model. The preliminary results were presented in the 14th National Conference of Neuropharmacology of China in an abstract form (Huang et al. 2010).

Materials and methods

Drugs, synthesis of double stranded RNA, animals and cells

CBIO (5-chloro-benzo[*d*]isozazol-3-ol) and morphine sulfate were obtained from Maybridge PLC (Cornwall, U.K.) and Northeast Pharmaceutical Group Co. (Shanghai, China), respectively. Both drugs were freshly dissolved in sterile saline solution (Sinopharm Group Chemical Reagent Co., Shanghai, China) with pH adjusted to 7.4 by 0.5 M NaOH as needed.

The double-stranded small interfering RNAs targeting the sequence of the rat DAAO mRNA (GeneBank accession NM–_053626) were designed and submitted to a Blast-Search (http://blast.ncbi.nlm.nih.gov) to analyze and confirm that only one gene was being targeted. siRNA/DAAO sense and antisense sequences were as follows: 5′-GGAGU GAAGUUCAUCCAUCUU-3′ and 5′-GAUGGAUGAACU UCACUCCUU-3′; The nonspecific oligonucleotide was also additionally designed as a control. The 19-nucleotide duplex and 2-unpaired nucleotides overhang of 3′end were as follows: nonspecific oligonucleotide sense: 5′-UUCUCC GAACGUGUCACGUUU-3′; nonspecific oligonucleotide antisense: 5′-ACGUGACACGUUCGGAGAAUU-3′. All siRNAs were chemically synthesized by Shanghai GenePharma Co., Ltd (Shanghai, China). Linear polyetherimide (PEI) (PolyScience Corporation, Niles Illinois, USA) was dissolved in 5 % dextrose in water (2 mg/ml, pH 7.0). 1 mg of RNA was dissolved in 1.25 mg of PEI in a PEI: RNA ratio of six equivalents of PEI nitrogen per RNA phosphate (Goula

et al. 1998; Tan et al. 2005) to form RNA-polymer complexes for 10 min at room temperature.

For culture and growth of Walker 256 mammary gland cancer cells, the experiments were carried out in female Wistar rats (weighing 50–60 g for cancer cell harvest) and Sprague–Dawley rats (weighing 180–250 g for the bone cancer pain model), purchased from Shanghai Experimental Animal Center (Shanghai, China). The rats were kept under controlled conditions (22 ± 2 °C) in a vivarium with a 12-h alternating light–dark cycle (lights on 7:00 AM) and ad libitum access to food and water. Animals were put in the laboratory environment for 5–7 days in order to accommodate to it before experiments. The research protocol, approved by the Animal Care and Welfare Committee of Shanghai Jiao Tong University School of Pharmacy, followed the animal care guidelines of the National Institutes of Health. 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.

Walker 256 mammary gland carcinoma cells derived from the Sprague–Dawley rat were kindly provided by Dr. Quan-Hai Liu (Shanghai Institute of Pharmaceutical Industry, Shanghai, China). The cancer cells were prepared using the previously described method (Mao-Ying et al. 2006) with slight modifications. Briefly, 1×10^7 Walker 256 tumor cells in 0.5 ml of sterile PBS were injected into the abdominal cavity of female Wistar rats. Six or seven days later, carcinoma cells were collected by centrifugation at 1,500 rpm for 2.5 min. The pellet was washed with sterile PBS and re-centrifuged at 1,500 rpm for 2.5 min. This procedure was repeated 2–3 times until hetero cells were removed, with the final concentration of 4×10^7 cells/ml in PBS counted by a haemocytometer.

Bone cancer pain model and intrathecal catheterization

The bone cancer pain procedure followed the protocol described in detail by Medhurst et al. (2002). Briefly, female Sprague–Dawley rats were anesthetized by sodium pentobarbital (50 mg/kg, i.p.). A 1-cm long rostra-caudal incision was made in the medial tibial skin. Using a 23-gauge needle, a hole was drilled 1 cm below the knee joint distal to the epiphysial growth plate at the medial flat of the bone. 4×10^5 Walker 256 cancer cells in 10 μl of sterile PBS in a 50-μl microsyringe (Shanghai Anting Micro-injector Factory, Shanghai, China) were then injected into the tibia. The injection pore was closed with aseptic bone wax. In some sham experiments, the same operation was carried out on the contralateral tibia with PBS only without cancer cells.

Intrathecal catheterization is described in detail elsewhere (Gong et al. 2011; Størkson et al. 1996). Briefly, under sodium pentobarbital anesthesia, a polyethylene catheter (PE-10: 0.28 mm i.d. and 0.61 mm o.d., Clay Adams, Parsippany, NJ, USA) was inserted into the rat lumbar subarachnoid space for intrathecal drug administrations. Upon recovery from anesthesia, 10 μl of 2 % lidocaine hydrochloride followed by 15 μl of saline was administrated to verify whether the catheter was indeed intraspinally located. Following intrathecal administration of lidocaine, an immediate onset of a transient bilateral paralysis of hind limbs was considered to be indicative of correct intraspinal location of the tube. Only those rats that had no motor impairment before lidocaine injection but had bilateral paralysis of hindlimbs after lidocaine were selected for further study and the successful rate in our laboratory was 100 %. The control and test drugs were injected intrathecally with a 50 μl microsyringe in a volume of 10 μl, followed by 15 μl of normal saline to flush the catheter.

Tests of thermal hyperalgesia and mechanical allodynia

In order to assess the paw withdrawal latency to radiant heat, rats were put in a plexiglass box on the elevated glass surface. Following an adaption period of 30 min at least, radiant heat was applied to the plantar medial surface of each hind paw until the animal suddenly withdrew or licked its paw from glass (Model 390G Plantar Test Analgesia Meter, IITC Life Science Inc., CA, USA). The maximal intensity of radiant heat was set to 50 %. A cut-off time was 20 s to minimize tissue damage upon the stimulation. The paw withdrawal latency was defined as the time from the onset of radiant heat application to the withdrawal of hind paws. Both hind paws were tested independently for three times with a 10-min interval between trials (Gong et al. 2011; Mao-Ying et al. 2006).

To evaluate mechanical allodynia, animals were acclimated for at least half an hour to the test environment, namely a plexiglass box on a metal grid (0.5 × 0.5 cm). The hind paw withdrawal threshold was measured by a 2450 CE Electronic Von Frey Anesthesiometer (IITC Life Science Inc., CA, USA). An electronic hand-held transducer with a No. 15 monofilament was applied perpendicularly to the medial, surface of the hind paws with the force increasing until the rat suddenly withdrew or licked the hind paw. The minimal value to evoke withdrawal response was considered to be the nocifensive threshold. Bilateral paws of each rat were examined for three times with a 10-min interval (Miao et al. 2010).

Real-time quantitative PCR and Western blot analysis of spinal DAAO expression, and measurements of spinal DAAO enzymatic activity and hydrogen peroxide level

Sham and tumor-bearing rats were killed by decapitation. For real-time quantitative PCR analysis, the ipsilateral spinal

lumbar enlargements were collected and mechanically homogenized using electronic micro-homogenizer at 10,000 rpm for 30 s in TRIzol (Invitrogen, Grand Island, NY, USA) on ice. According to manufacturer's instruction, total RNA of the lumbar intumescentia of the spinal cord was purified from individual sample by use of the TRIzol reagent. The purity and integrity of the prepared total RNA were identified with spectroscopicometer and gel electrophoresis before carrying out next steps. One microgram of total RNA sample was reversely transcribed using ReverTra Ace qPCR RT-Kit (Toyobo Co., Ltd., Osaka, Japan). Real-time quantitative PCR was carried out with Mastercycler® ep realplex (Eppendorf AG, Hamburg, Germany) using Real master Mix (SYBR Green I) (Tiangen Biotech Co., Ltd., Beijing, China). PCR conditions were optimized in a preliminary experiment to achieve a linear relationship between the initial RNA concentration and the PCR product. Triplicates of each real time PCR reaction were performed according to the following protocol: 3-min pre-denaturation for 1 cycle at 95 °C, 30-s denaturation at 95 °C, 30-s annealing at 58 °C followed by a 30-s extension at 72 °C. The forward and reverse primers for DAAO, GFAP, μ-opioid receptor and GAPDH were synthesized by Shanghai DNA Bio Technologies Co., Ltd. (Shanghai, China) and listed in Table 1. Amplification of the housekeeping gene GAPDH was measured for each sample as an internal control for sample loading and normalization. The specificity of the primers was checked by examining the melting curve. To quantity the relative amount of gene expression for the target and housekeeping genes, the $2^{-\Delta\Delta Ct}$ calculations were done where $\Delta\Delta Ct = (Ct,_{Target} - Ct,_{Gapdh})_{cancer\ \ samples} - (Ct,_{Target} - Ct,_{Gapdh})_{control\ samples}$ and Ct was the cycle threshold (Livak and Schmittgen 2001). The percentage of the genes of interest relative to housekeeping gene was calculated as following: % mRNA expression $= 100\ \% \times 2^{-\Delta\Delta Ct}$.

For the Western blot, ipsilateral spinal lumbar enlargements were homogenized and lysed in a lysis buffer NP-40 (10 µl/mg). The homogenate was centrifuged for 10 min at 12,000 rpm at 4 °C. The supernatant was sucked to a new clean tube, and the protein concentration was determined by the Bradford's method (Bradford 1976). Proteins were separated by SDS-PAGE (12 %) and then transferred to a PVDF membrane by electrophoretic methods. The membrane was blocked in 5 % skim milk powder in PBST at room temperature for 1 h, and then incubated with primary antibody raised against DAAO protein (Novus Biologicals, LLC, Littleton, USA) and GFAP protein (Millipore Corporation, Billerica, USA) with a dilution of 1:250 or 1:1,000 overnight at 4 °C. Antibody binding was visualized using a horseradish peroxidase-conjugated secondary antibody and a DAB detection system. The bands were scanned using an image scanning densitometer (Tanon Science & Technology Co., Shanghai, China). To control sampling errors, the percentage of DAAO/β-actin or GFAP/β-actin band intensity was obtained to quantify relative protein expression levels and selected as the expression of target proteins.

The spinal DAAO activity was examined according to the "keto acid method" (D'Aniello et al. 1993; Xin et al. 2005, 2010). One gram of spinal lumbar enlargement tissue was homogenized (10,000 rpm for 15 s) using a homogenizer with 3 ml of 100 mM Tris–HCl buffer, pH 8.2, at 2–4 °C and centrifuged at a speed of 4,000 rpm for 10 min at 4 °C. 50 µl of 100 mM D-alanine (dissolved in 0.1 M Tris–HCl buffer, pH 8.2) was incubated with 50 µl of the supernatant for 60 min at 37 °C on the Thermomixer (Eppendorf AG, Hamburg, Germany). 50 µl of 25 % trichloroacetic acid was mixed with the assay mixture and centrifuged at 14,000 rpm for 5 min. 50 µl of the supernatant was added with 50 µl of 2,4-dinitrophenylhydrazine (in 1 M HCl) and incubated (700 rpm) at 37 °C for 10 min. The absorbance was read at 450 nm in a Thermo Labsystems-Multiskan MK3 (Thermo Fisher Scientific Inc., Waltham, USA) against a blank sample consisting of the same homogenate but heated with 100 mM D-alanine. The enzymatic activity of DAAO in the homogenates was quantified against the standard curve of pyruvic acid (from 0-800 µM, $R^2 > 0.99$). The specific enzymatic activity was expressed as pyruvate production per milligram protein per minute. The protein concentration of the homogenates was measured by the Bradford's method.

The spinal hydrogen peroxide level was determined using the FOX-1 assay (Jiang et al. 1990; Lu et al. 2012). Briefly, spinal enlargements were removed from rats, weighed and stored on ice. The homogenization was executed for them in 3 ml of homogenization solution (100 mM Tris–HCl, pH 8.2) per gram spinal cord tissue. Homogenates were centrifuged at a speed of 12,000 rpm for 5 min at 4 °C. Supernatants were subsequently transferred to new corresponding tubes. Samples and hydrogen peroxide standards were assayed at 560 nm in the spectrophotometer.

Data analysis and statistics

For analysis of the dose–response curve of CBIO against bone cancer 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 individual dose–response curves. To determine the parameters of dose–response curves, values of response (Y) were fitted using the Program of GraphPad Prism 5.01 version (GraphPad Software Inc., San Diego, CA, USA), 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).

Table 1 The sequences of forward and reverse primers used for amplifying the genes of DAAO (D-amino acid oxidase), GFAP (glial fibrillary acidic protein), μ-opioid receptor and GAPDH (glyceraldehyde-3-phosphate dehydrogenase), as well as the product length of gene fragments of interest which were obtained by amplification using these paired primers

Gene name	Primers	Product length (base pair)
DAAO	Forward primer: 5′-CCC TTT CTG GAA AAG CAC AG-3′	209
	Reverse primer: 5′-CTC CTC TCA CCA CCT CTT CG-3′	
GFAP	Forward primer: 5′-ACA TCG AGA TCG CCA CCT AC-3′	219
	Reverse primer: 5′-ACA TCA CAT CCT TGT GCT CC-3′	
μ-Opioid receptor	Forward primer: 5′-CAC GTT GAT GGC AAC CAG TC-3′	213
	Reverse primer: 5′-GTT GGT GGC AGT CTT CAT TTT G-3′	
GAPDH	Forward primer: 5′-CGG CAA GTT CAA CGG CAC AG-3′	146
	Reverse primer: 5′-AGA CGC CAG TAG ACT CCA CGA C-3′	

To define % maximum possible effect (MPE), data were analyzed according to the modified method of Chaplan et al. (1994). Paw withdrawal thresholds were transformed into a % MPE which was 100*[New threshold (g)-baseline threshold (g)]/[Contralateral threshold (g)-baseline threshold (g)]. % MPE values near 100 manifest normal mechanical thresholds (i.e., at or near contralateral thresholds) while values near 0 indicate allodynia.

The results were expressed as the mean values and the corresponding standard errors (mean ± SEM). Statistical analysis was done by a one-way or two-way ANOVA followed by post hoc Dunnett's test, two-tailed Student t test. In all cases, the statistical significance criterion P value was set at 0.05.

Results

Induction of spinal DAAO expression and enzymatic activity in the development of bone cancer pain

One group of rats ($n = 6$) were inoculated with 10 µl PBS or 4×10^5 Walker 256 cancer cells into the contralateral and ipsilateral tibiae, respectively. Rats exhibited general good health conditions with no signs of distress during the experimental observation period. Enlargement around the tibia became visible in most cancer-bearing rats by day 15. This swelling of the tibia was considered to be signs of tumor growth and indication of hyperalgesia and/or allodynia as described previously (Zhang et al. 2005). After inoculation of Walker 256 cancer cells (from Shanghai Institute of Pharmaceutical Industry, Shanghai, China), rats displayed progressive and significant mechanical allodynia in the ipsilateral paw without changing paw withdrawal thresholds in the contralateral paw. As shown in Fig. 1a, there was no marked differentiation in the basal paw withdrawal thresholds between the ipsilateral and contralateral paws before inoculation. After inoculation of Walker 256 cancer cells, the paw withdrawal thresholds in the ipsilateral paws significantly and progressively decreased on day 10 and reached to the plateau on day 20 and afterwards, compared to those of the contralateral hind paws which remained at the pre-injection level ($P < 0.05$ by a two-way ANOVA followed by post hoc Dunnett's test). In contrast, rats inoculated with Walker 256 carcinoma cells showed no detectable difference in radiant heat-induced paw withdrawal responses between both hind paws throughout the study period (Fig. 1b). Mechanical allodynia was then selected for the afterward behavior studies.

In order to examine spinal DAAO expression during the development of bone cancer pain, 6 groups of rats ($n = 5$ in each group) were inoculated with PBS or Walker 256 cancer cells and one group of five rats were used as pre-surgery control. Ipsilateral spinal lumbar enlargements were collected before surgery and on days 7, 18 and 25 after inoculation. Expressions of DAAO mRNA and protein, as well as its enzymatic activity were measured using real-time quantitative PCR, Western blot and the "keto acid" method, respectively. As shown in Fig. 1c, the spinal DAAO mRNA expression relative to the GADPH gene was maintained to the same level during the experimental period in sham rats. Compared to sham rats, inoculation of Walker 256 cancer cells, however, progressively up-regulated spinal DAAO gene expression, with a plateau effect of approximately 160 % on day 15 and afterwards ($P < 0.05$ by a two-way ANOVA followed by post hoc Dunnett's test). Furthermore, spinal DAAO protein expression relative to β-actin protein (Fig. 1d) and DAAO enzymatic activity (Fig. 1e) were also progressively and significantly up-regulated, with plateau effects of approximately 60 % on day 15 and afterwards ($P < 0.05$). Thus, induction of spinal DAAO expression and enzymatic activity was correlated to the development of bone cancer pain (mechanical allodynia) in a time-dependent manner.

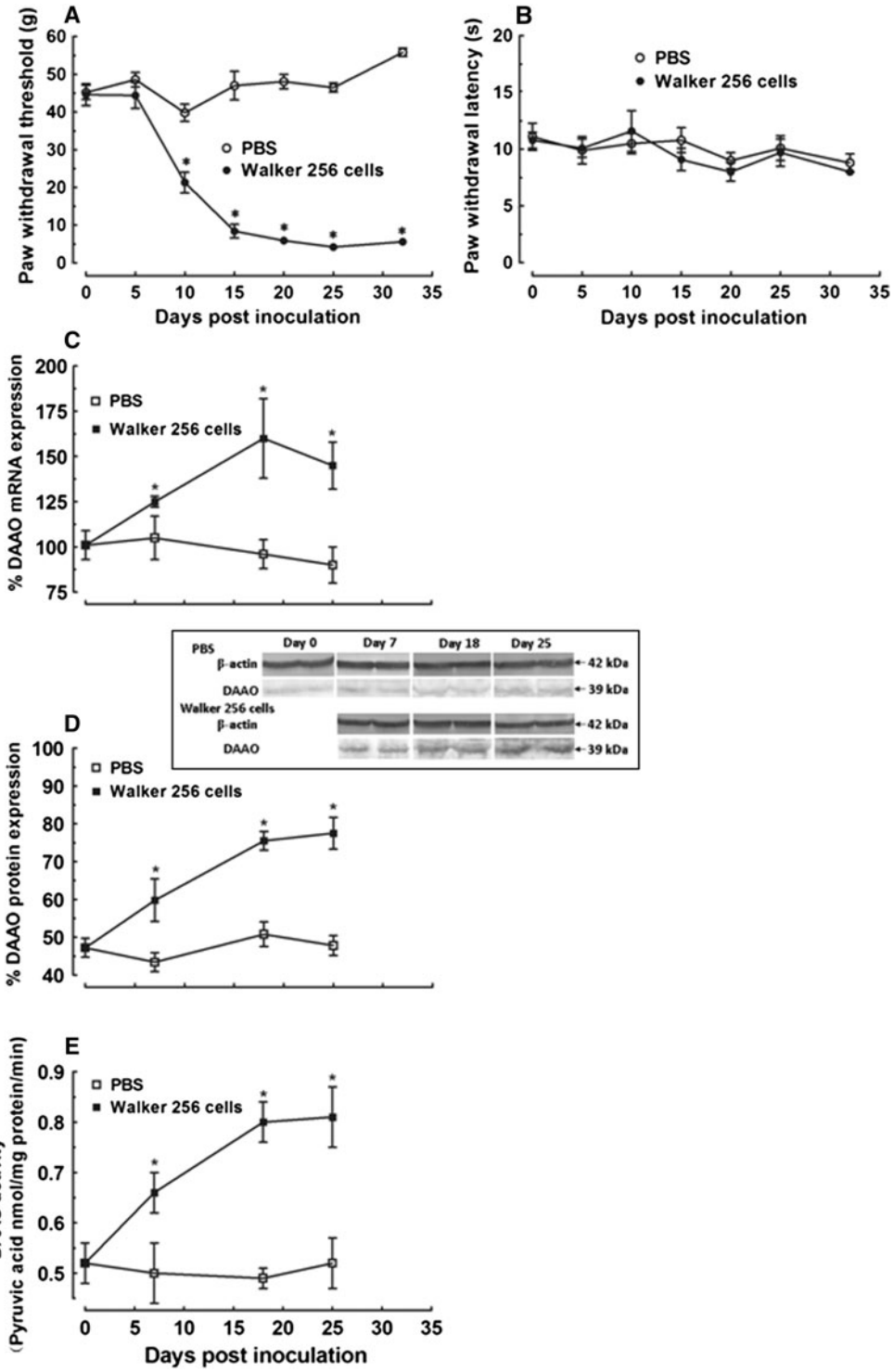

Fig. 1 Time courses of mechanical allodynia (**a**), thermal hyperalgesia (**b**), spinal DAAO (D-amino acid oxidase) mRNA expression (**c**), protein expression (**d**) and enzymatic activity (**e**) in rats inoculated by Walker 256 cancer cells. Mechanical allodynia was measured by electronic Von Frey filaments and thermal hyperalgesia was measured by radial heat. DAAO (mRNA and protein) expression and enzymatic activity was detected using real-time PCR, Western blot and the "keto acid" method where GAPDH (glyceraldehyde-3-phosphate dehydrogenase) gene and β-actin protein were used as internal references. In D, DAAO and β-actin bands were scanned and the DAAO/β-actin band intensity ratio was presented while the representative Western blot images of two samples were inserted in the box. Data are presented as mean ± SEM ($n = 5$–6 in each group). *Statistically significant difference compared to sham controls ($P < 0.05$ by a two-way ANOVA followed by post hoc Dunnett's test)

Inhibitory effect of the DAAO inhibitor CBIO on bone cancer pain

In order to examine the anti-allodynic effects of inhibition of DAAO enzymatic activity on mechanical hyperalgesia/allodynia in rats with bone cancer pain, we first tested the effect of CBIO on spinal DAAO enzymatic activity. One group of five rats received tibia inoculation of PBS whereas other three groups of rats ($n = 5$ in each group) received cancer cells. On day 18 after inoculation, the rats received subcutaneous injection of saline (1 ml/kg) or CBIO (10 or 30 mg/kg). Spinal enlargements were collected 1 h after injection and their DAAO enzymatic activities were measured. As shown in Fig. 2a, systemic injection of CBIO (10

and 30 mg/kg) significantly suppressed upregulated spinal DAAO enzymatic activity by 50.7 and 65.7 %, respectively, ($P < 0.05$ by a two-way ANOVA followed by post hoc Dunnett's test).

Six groups of rats ($n = 5$–6 in each groups) received single subcutaneous injection of saline (1 ml/kg) or CBIO (1, 3, 10, 30 or 100 mg/kg). There were no significant changes in mechanical hyperalgesia/allodynia in bilateral hind paws during the 4-h observation period after saline injection (Fig. 2b, c). In addition, as shown in Fig. 2b, single injection of CBIO up to 100 mg/kg did not significantly alter withdrawal thresholds in contralateral paws compared to the saline control group. Subcutaneous injection of CBIO reversed bone cancer mechanical allodynia in the ipsilateral paws, in a time-dependent manner, compared to the saline control ($P < 0.05$ by a two-way ANOVA followed by Dunnett's test). The duration of CBIO's anti-allodynia lasted for longer than 2 h (Fig. 2c). No apparent sedation and motor adverse effects were observed up to 100 mg/kg CBIO. The anti-allodynic effect of CBIO was dose-dependent and dose–response analysis by best fit indicated that the E_{max} value was 45.5 % MPE and the ED_{50} value was 8.0 mg/kg (95 % confident limits: 0.7–86.5 mg/kg) at time point of 1 h after injection (Fig. 2d).

To confirm intrathecal injection of CBIO produced analgesia in bone cancer pain, four groups of tumor-bearing rats ($n = 6$ in each group) received single intrathecal bolus injection of 10 µl saline or CBIO (1, 3 or 10 µg). Intrathecal CBIO produced a time-dependent inhibition of cancer-induced pain behavior in the ipsilateral paws but did not affect the behaviors of the contralateral paws, with duration of longer than 2 h ($P < 0.05$ by a two-way ANOVA followed by Dunnett's test) (Fig. 3a, b). No apparent sedation and motor adverse effects were observed up to 10 µg of CBIO, consistent with our previous findings

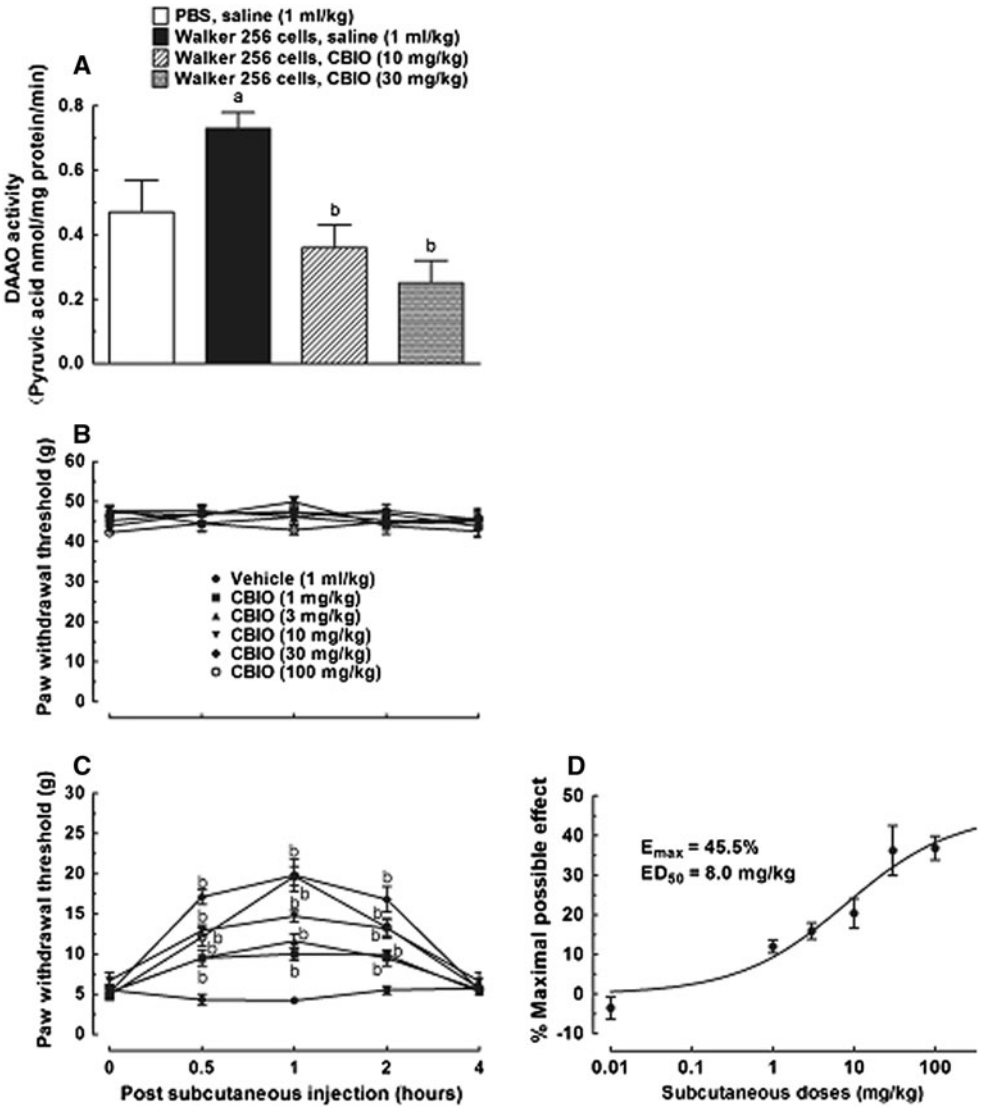

Fig. 2 Effects of single subcutaneous injection of the DAAO (D-amino acid oxidase) inhibitor 5-chloro-benzo [*d*]isozazol-3-ol (CBIO) on spinal DAAO enzymatic activity (**a**), paw withdrawal thresholds in the contralateral paw (**b**) and ipsilateral paw (**c**) in rats bearing cancer. **d** Dose–response analysis of subcutaneous administered CBIO on cancer cell-induced mechanical allodynia. The DAAO enzymatic activity was measured using the "keto acid" method and mechanical hyperalgesia/allodynia was measured by electronic Von Frey filaments. Data are presented as mean ± SEM ($n = 5$–6 in each groups). [a] and [b] denote statistically significant difference compared to sham + saline group and the bone cancer pain + saline group, respectively ($P < 0.05$ by a one-way ANOVA followed by post hoc Dunnett's test

where intrathecal CBIO did not affect the motor coordination or locomotor activity in normal animals (Gong et al. 2011). The anti-allodynic effect of intrathecal CBIO was dose-dependent and 10 μg of CBIO produced a MPE of 40.2 % at 1 h after injection which was very close to the E_{max} value (45.5 % MPE) obtained by its subcutaneous injection. Thus the projected ED_{50} value for intrathecal CBIO would be roughly 1 μg when the E_{max} value was assumed to be 40 % MPE.

Inhibitory effect of the DAAO gene silencer on bone cancer pain

In order to further test the role of spinal DAAO expression on the maintenance of bone cancer pain, gene silencing technology using siRNA was employed. Multi-daily intrathecal injections of the designed siRNA/DAAO in PEI complexation for 7 days have been previously shown to block DAAO gene and protein expression and inhibit DAAO enzymatic activity by 50–80 %, compared to the saline or nonspecific oligonucleotide control (Chen et al. 2012). In this study, two groups of rats ($n = 7$ in each group) were implanted with Walker 256 cancer cells in the tibia and received multi-daily intrathecal injections of the nonspecific oligonucleotide (5 μg/day) and siRNA/DAAO (5 μg/day) for 11 days starting from the day 9 after inoculation of cancer cells. The paw withdrawal thresholds were measured in both contralateral and ipsilateral paws for an experimental period of longer than 30 days. As shown in Fig. 4a, multi-daily intrathecal administrations of nonspecific oligonucleotide did not significantly affect the paw withdrawal thresholds in bilateral paws compared to the pre-injection control. Intrathecal injections of siRNA/DAAO produced anti-allodynic effect in a time-dependent fashion, beginning from day 4 after injection and plateau day 6 and afterwards by approximately 40 % MPE, compared to the nonspecific oligonucleotide control ($P < 0.05$ by a two-way ANOVA followed by post hoc Dunnett's test). The anti-allodynic effect still lasted for approximately 6 days after stopping injections of siRNA/DAAO. However, intrathecal treatment with siRNA/DAAO did not significantly affect contralateral paw withdrawal thresholds. In addition, no evident sedation and motor adverse effects were observed after continuous intrathecal administrations of siRNA/DAAO.

In order to test whether the anti-allodynic effect of CBIO is specifically via inhibition of the spinal DAAO, we further examined the effect of CBIO on bone cancer pain in the above two groups of rats on the 10th day postdose of oligonucleotides. Both groups of rats received intrathecal single bolus injection of CBIO (10 μg) and their mechanical withdrawal thresholds were measured for 4 h. As shown in Fig. 4b, CBIO blocked mechanical allodynia in a time-dependent manner in ipsilateral paws by approximately 40 % MPE at peak compared to the pre-injection control in nonspecific oligonucleotide-treated rats ($P < 0.05$ by a two-way ANOVA followed by post hoc Dunnett's test). However, CBIO was not effective in increasing mechanical withdrawal thresholds in rats multi-daily pretreated with siRNA/DAAO. The results suggest that CBIO produced analgesic effects on bone cancer allodynia specifically via inhibition of spinal DAAO enzymatic activity.

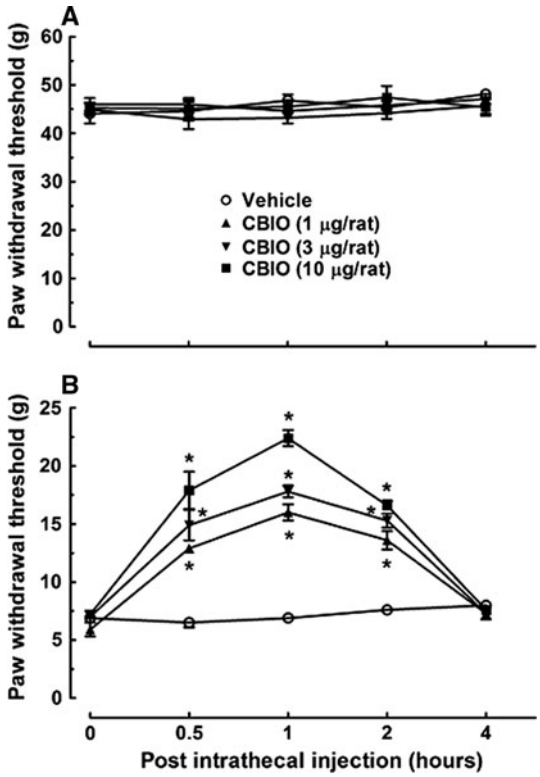

Fig. 3 Effects of single intrathecal bolus injection of the DAAO (D-amino acid oxidase) inhibitor 5-chloro-benzo[*d*]isozazol-3-ol (CBIO) on paw withdrawal thresholds in the contralateral paw (**a**) and ipsilateral paw (**b**) in rats bearing cancer. Mechanical allodynia was measured by electronic Von Frey filaments. Data are presented as mean ± SEM ($n = 6$ in each group). *Statistically significant difference compared to the saline control group ($P < 0.05$ by a two-way ANOVA followed by Dunnett's test)

Preventive effect of CBIO on morphine tolerance to analgesia in bone cancer pain

To test whether concurrent injections of CBIO with morphine prevented morphine tolerance to analgesia, four groups of rats with bone cancer pain ($n = 6$ in each group) received two treatments for 7 days. They were: (1) saline (1 ml/kg) + saline (1 ml/kg), (2) CBIO (10 mg/kg) + saline (1 ml/kg), (3) saline (1 ml/kg) + morphine (5 mg/kg), (4) CBIO (10 mg/kg) + morphine (5 mg/kg), each twice a day (8:00 AM and 8:00 PM). Measurements of paw

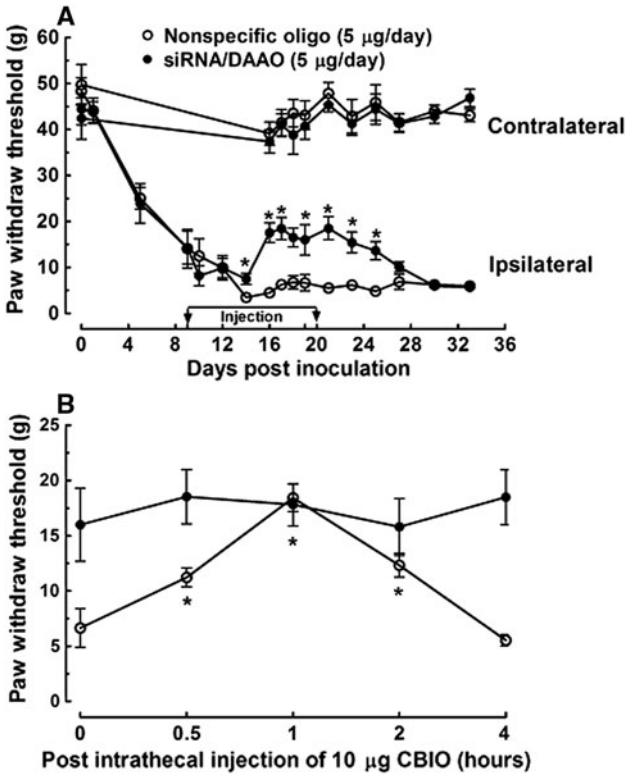

Fig. 4 **a** Inhibitory effects of multi-daily intrathecal injections of DAAO (D-amino acid oxidase) gene silencer siRNA/DAAO in naive bone cancer rats. Rats received multi-daily intrathecal injections of 5 μg nonspecific oligonucleotide or 5 μg DAAO siRNA in PEI complexation continuously for 13 days starting from day 9 post tibia implantation of Walker 256 cells. **b** Single bolus intrathecal injection of the DAAO inhibitor 5-chloro-benzo[*d*]isozazol-3-ol (CBIO) in siRNA/DAAO-pretreated bone cancer rats. CBIO (10 μg) was given on day 10 after oligonucleotide multi-daily injections in rats from **a**. Mechanical allodynia was measured by electronic Von Frey filaments. Data are presented as mean ± SEM ($n = 7$ in each group). *Statistically significant difference compared to the nonspecific oligonucleotide control in A or pre-injection control in B ($P < 0.05$ by a two-way ANOVA followed by post hoc Dunnett's test)

withdrawal thresholds were made before drug exposure and 1 h after first administration every day. Contralateral paw withdrawal thresholds were not statistically different among the four groups of rats (Fig. 5a). In contrast, as shown in Fig. 5b, multiple bi-daily subcutaneous injections of morphine resulted in potent analgesia and rapid tolerance whereas CBIO efficaciously produced sustained anti-allodynic effects with no loss of potency throughout the observation period ($P < 0.05$ by a two-way ANOVA followed by post hoc Dunnett's test). Multiple bi-daily concurrent injections of CBIO with morphine not only generated apparent additive analgesic effects but also completely prevented morphine tolerance to analgesia ($P < 0.05$). As a control, paw withdrawal thresholds in the multiple bi-daily saline-treated rats remained stable throughout the 7-day observation period.

CBIO's lack of self-tolerance and cross-tolerance to morphine analgesia was further confirmed on day 8 approximately 14 h after stopping the 7-day treatments. The above four groups of rats received single subcutaneous injection of morphine (5 mg/kg). Paw withdrawal thresholds were measured before and 1 h after morphine challenge. Baseline paw withdrawal thresholds prior to morphine in the four groups were similar and lower than 8 g (data not shown). However, single bolus injection of morphine produced similar analgesia (approximately 20 g) in all groups except for morphine tolerant rats ($P < 0.05$ by a one-way ANOVA followed by post hoc Dunnett's test) (Fig. 5c), suggesting that multiple bi-daily administrations of CBIO did not induce self-tolerance to analgesia or cross-tolerance to morphine but prevented morphine tolerance. In addition, the preventive effect of CBIO on morphine tolerance was also confirmed in spinal μ-opioid receptor mRNA expression. Ipsilateral spinal lumbar enlargements were excised from all groups of rats immediately after the behavior test. The expressions of μ-opioid receptor mRNA relative to the GADPH gene were the same both in saline and CBIO-pretreated rats. Multiple bi-daily injection of morphine resulted in down-regulation of μ-opioid receptor gene by approximately 20 %; concurrent injections of CBIO with morphine, on the other hand, completely prevented morphine-induced decrease in μ-opioid receptor expression ($P < 0.05$) (Fig. 5d).

Blockade effect of CBIO on hydrogen peroxide level and activation of astrocytes in the spinal cord

DAAO catalyzes the oxidative deamination of neutral and polar D-amino acids to produce hydrogen peroxide (Angermüller et al. 2009; Kappor and Kapoor 1997; Wang et al. 2012). To test whether CBIO affected spinal hydrogen peroxide levels, two groups of rats ($n = 6$ in each group) received inoculation of Walker 256 cancer cells, and on day 18 after inoculation received subcutaneous administration of saline (1 ml/kg) and CBIO (10 mg/kg), respectively. The spinal enlargements were moved 0.5 h after drug administration and their hydrogen peroxide levels were determined. As shown in Fig. 6a, given systemically CBIO significantly reduced spinal hydrogen peroxide levels by approximately 20 % ($P < 0.05$ by a two-tailed Student t test).

GFAP is generally accepted as a biomarker for the activation of astrocyte cells (Zhang et al. 2005) and bone implantation of cancer cells activated spinal astrocytes (Urch 2004). To test the inhibitory effect of CBIO on spinal GFAP expression, one group of five rats received tibia inoculation of PBS whereas other three groups of rats ($n = 5$ in each group) received Walker 256 cancer cells. On day 18 after inoculation, the rats received subcutaneous

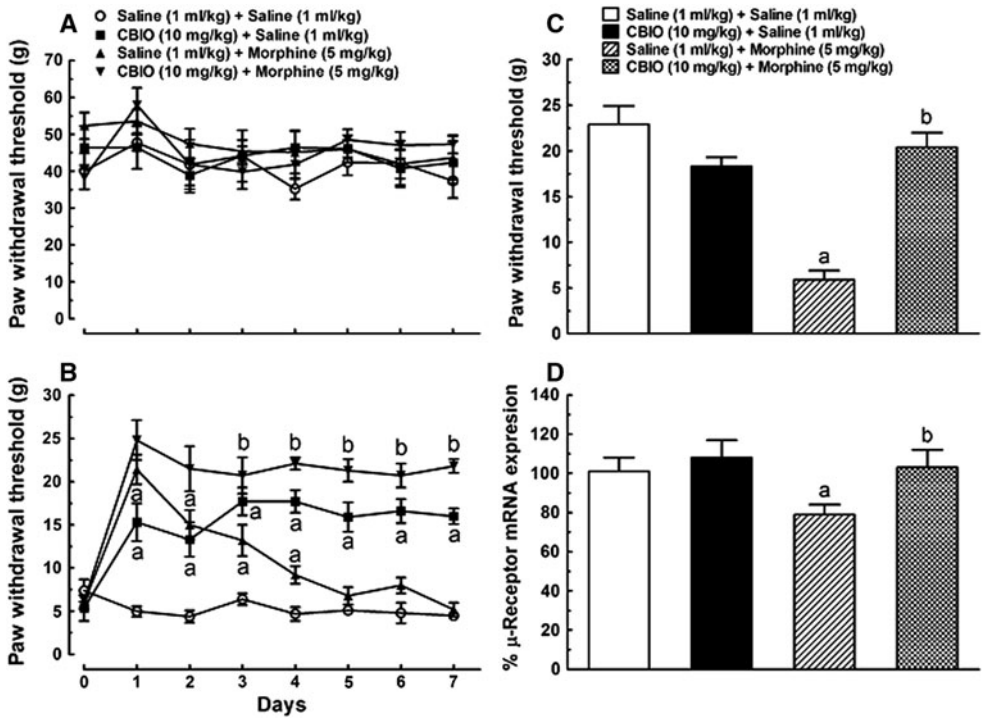

Fig. 5 Anti-allodynic effects of the DAAO (D-amino acid oxidase) inhibitor 5-chloro-benzo[*d*]isozazol-3-ol (CBIO), morphine and the combination of CBIO and morphine on paw withdrawal thresholds in the contralateral paw (**a**) and ipsilateral paw (**b**) in bone cancer pain rats. Rats bearing cancer cells received multiple bi-daily subcutaneous injections of two treatments for 7 days: saline + saline, CBIO + saline, saline + morphine, CBIO + morphine. The anti-allodynic effect of single subcutaneous injection of morphine (5 mg/kg) on ipsilateral paw withdrawal thresholds 1 h after injection (**c**) and expression of spinal μ-opioid receptor mRNA (**d**) in tumor-bearing rats from A and B on day 8 after cancer cell inoculation. Mechanical allodynia was measured by electronic Von Frey filaments. μ-Opioid receptor mRNA expression was measured using real-time PCR and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) gene was used as the internal reference. Data are expressed as mean ± SEM ($n = 6$ in each group). [a]denotes statistically significant difference compared to the saline + saline group while [b]denotes statistically significant difference compared to the saline + morphine group ($P < 0.05$ by a two-way ANOVA followed by post hoc Dunnett's test)

injection of saline (1 ml/kg) or CBIO (10 or 30 mg/kg) and the ipsilateral spinal enlargements were removed 1 h later. Measured by the real-time quantitative PCR, spinal GFAP mRNA expression relative to the GADPH gene in bone cancer pain rats was remarkably increased by 60.8 % compared with sham rats ($P < 0.05$ by a one-way ANOVA followed by post hoc Dunnett's test). Given subcutaneously CBIO (10 and 30 mg/kg) significantly down-regulated increased GFAP expression by 11.0 and 16.5 %, respectively ($P < 0.05$) (Fig. 6b).

The inhibitory effect of CBIO on spinal GFAP protein expression was also tested in these samples. As shown in Fig. 6c, expression of spinal GFAP protein relative to the β-actin protein was significantly elevated by 36.4 % in rats bearing tumors in contrast to sham rats ($P < 0.05$ by a one-way ANOVA followed by post hoc Dunnett's test). Subcutaneous injection of CBIO (10 and 30 mg/kg) effectively prevented the increased expression of spinal GFAP protein by 20.3 and 32.2 %, respectively ($P < 0.05$).

Discussion

Bone cancer pain is a severe clinical challenge to treat and a variety of bone cancer pain models which mimic clinical cancer pain have been successfully established in rats (Medhurst et al. 2002) and mice (Schwei et al. 1999) since 1990's. The models were set up in different bones, such as calcaneum (Wacnik et al. 2001), femur (Yanagisawa et al. 2010), and tibia (Hald et al. 2009; Mao-Ying et al. 2006), by implantation of various cancer cell lines, such as NCTC 2472 (Hald et al. 2009), MRMT-1 (Medhurst et al. 2002), AT-3.1 (Zhang et al. 2005), and Walker 256 cancer cells (Mao-Ying et al. 2006). After inoculation into the bones, all of these cancer cells significantly induced progressive mechanical hyperalgesia/allodynia. However, the effects of different cancer cells on heat hyperalgesia varied largely. It was reported that there were no marked alterations in paw withdrawal response to radiant heat in both hind paws during the experiment (Mao-Ying et al. 2006; Miao et al.

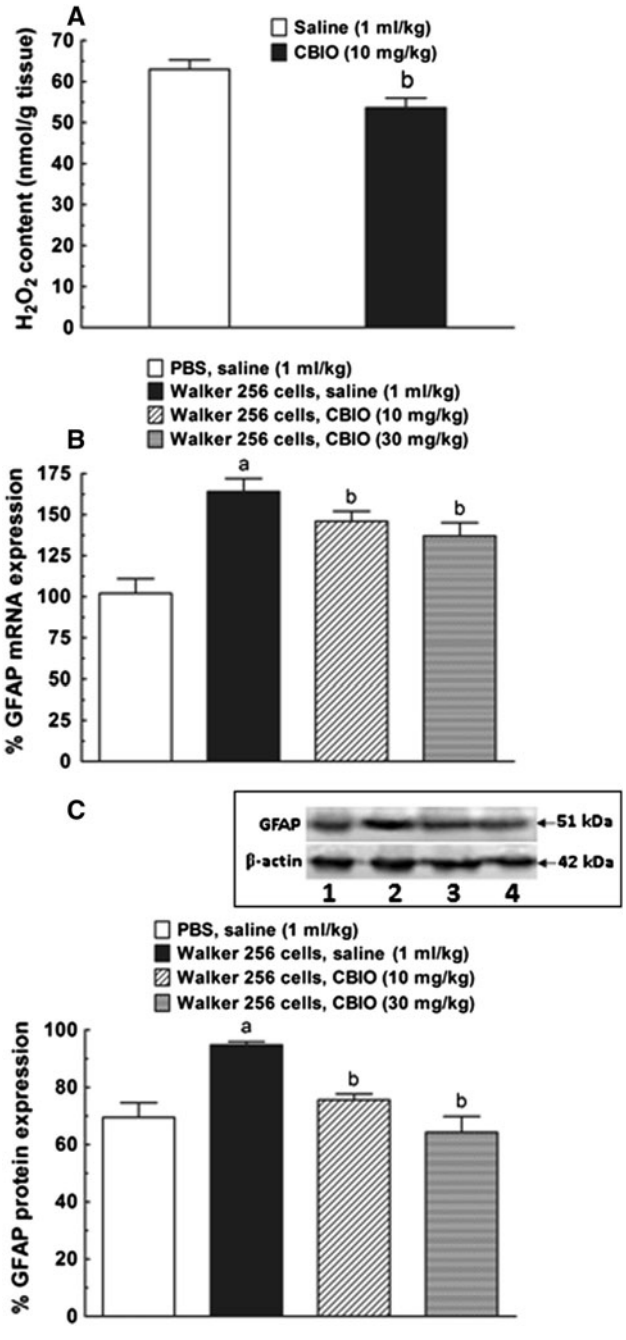

Fig. 6 Inhibitory effects of subcutaneous injection of the DAAO (D-amino acid oxidase) inhibitor 5-chloro-benzo*[d]*isozazol-3-ol (CBIO) on spinal hydrogen peroxide level (**a**), GFAP (glial fibrillary acidic protein) mRNA (**b**) and protein expression (**c**) in tumor-bearing rats. Hydrogen peroxide level, GFAP mRNA and protein expressions were measured on day 18 after inoculation of Walker 256 cancer cells and 1 h after drug injection using the FOX-1 assay, real-time PCR, Western blot where GAPDH (glyceraldehyde-3-phosphate dehydrogenase) gene and β-actin protein were used as internal references. In C, GFAP and β-actin bands were scanned and the GFAP/β-actin band intensity ratio was presented while the representative Western blot images were inserted in the box where *lane 1* PBS, saline (1 ml/kg), *lane 2* Walker 256 cells, saline (1 ml/kg), *lane 3* Walker 256 cells, CBIO (10 mg/kg), *lane 4* Walker 256 cells, CBIO (30 mg/kg). Data are presented as mean ± SEM ($n = 5$–6 in each group). [a] and [b]denote statistically significant difference compared to sham with saline rats and the bone cancer pain with saline group, respectively ($P < 0.05$ by a one-way ANOVA followed by post hoc Dunnett's test)

2010). In contrast, others showed that paw withdrawal latency was decreased gradually along with bone tumor development in mice (Gu et al. 2010). Our results are consistent with Mao-Ying et al. 2006's and Miao et al. (2010)'s findings and showed that implantation of Walker 256 cells into the tibiae did not evoke heat hyperalgesia but marked mechanical allodynia.

Chronic pain including cancer pain and neuropathic pain can not only progressively induces behavioral pain status, but also triggers neurochemical changes in the peripheral nervous system (primary afferent neurons and dorsal root ganglion) and the central nervous system (the spinal cord and brain) (Schwei et al. 1999; Honoré et al. 2000). The mechanisms that generate bone cancer pain are not fully understood because they are involved in the release of many pro-nociceptive mediates from tumor and/or inflammatory cells, nerve compression, and ongoing osteoclast activity (Luger et al. 2001). The present study systemically studied the potential role of spinal DAAO in the development of bone cancer pain. First, spinal DAAO (gene and protein) expression and DAAO enzymatic activity were consistently and remarkably up-regulated when mechanical allodynia occurred with the same time course by tibia inoculation of Walker 256 cancer cells. The results are consistent with previous studies that spinal DAAO gene expression and enzymatic activity were also significantly increased in synchronous with the development of neuropathic pain (Zhao et al. 2010). Furthermore, multi-daily intrathecal injections of siRNA/DAAO, which was previously found to effectively knock down spinal DAAO expression and enzymatic activity (Chen et al. 2012), produced anti-allodynia in the rat model of bone cancer pain by 40 % in a time-dependent manner. The results are in good agreement with other studies where intrathecal injection of the siRNA/DAAO blocked formalin-induced tonic phase pain (Chen et al. 2012). Thirdly, both systemic and intrathecal injections of specific DAAO inhibitor CBIO at effective doses to inhibit spinal DAAO enzymatic activity inhibited bone cancer allodynia by 40–50 %. Finally, intrathecal bolus injection of CBIO was not effective in siRNA/DAAO-pretreated bone cancer pain rats, indicating that CBIO exerts its anti-allodynic effects via inhibiting spinal DAAO rather than on other target molecules. In addition, systemic injection of CBIO did not alter MK801-reversible intrathecal NMDA-induced spontaneous pain (Gong et al. 2012). These results taken together provide the first evidence that spinal DAAO functionally contributes to the development of bone cancer

pain accounting for 40–50 % pain status, and may be a target molecule for treating chronic cancer pain.

In contrast, systemic and intrathecal administration of CBIO as well as intrathecal injections of siRNA/DAAO were ineffective in altering paw withdrawal thresholds in contralateral paws from bone cancer pain rats. These results, supported by previous findings that inhibition or deletion of DAAO did not lead to analgesia in acute pain such as formalin-induced acute phase nociception or thermally evoked licking/flicking responses (Gong et al. 2011; Lu et al. 2012; Zhao et al. 2008, 2010), indicated that spinal DAAO is specifically involved in chronic bone cancer pain. Furthermore, dose–response analysis showed that maximal inhibition of CBIO on bone cancer allodynia was 40–50 % while its ED_{50}s were 8 mg/kg and approximately 1 μg/rat for subcutaneous and intrathecal administration, respectively. For a comparison, our previous data showed that subcutaneous and intrathecal injection of CBIO blocked formalin-induced tonic pain, with the E_{max} values of 62–67 % and the ED_{50} values of 0.9 mg/kg and 0.06 μg/rat, respectively (Gong et al. 2011; Lu et al. 2012). The approximately 10-folds less effectiveness of CBIO on bone cancer pain than formalin-induced tonic pain may be due to that bone cancer pain exhibits more primary and/or central sensitization (Honoré et al. 2000; Luger et al. 2002; Yanagisawa et al. 2010) and may represent a more severe pain condition.

One of the main disadvantages of the clinical application of opiates is their property of inducing the development of tolerance to analgesia. This study confirmed the tolerance phenomenon by the behavior test and μ-opioid receptor expression. It has been shown that morphine generated tolerance to analgesia due to μ-opioid receptor endocytosis, desensitization of G-protein coupling (Martini and Whistler 2007) and decreased μ-opioid receptor expression in the spinal cord (Bhargava and Gulati 1990; Zhang et al. 1998). In contrast, 7-day multiple bi-daily subcutaneous injections of CBIO induced neither self-tolerance to analgesia nor cross-tolerance to morphine. The lack of tolerance property of DAAO inhibition was also confirmed by that the anti-allodynic effect of siRNA/DAAO remained the same for at least 6 days during the multi-daily injections. Concurrent injections of CBIO with morphine produced apparent additive anti-allodynic effect and completely prevented morphine tolerance to analgesia in bone cancer pain, demonstrated by blockade of mechanical allodynia behaviors and reduced spinal μ-opioid receptor expression. The apparent additive analgesic and morphine tolerance blockade effects of CBIO are consistent with our recent results in the formalin test (Gong et al. 2012). By sharing a common mechanism of central sensitization (Mao et al. 1995; Mayer et al. 1999; Yanagisawa et al. 2010), the unique combination of analgesic and morphine tolerance blockade effects make DAAO inhibitors a possibility for being developed to novel medicines for the treatment of cancer pain in clinic when they are administered alone or in combination with morphine. Almost exclusively expressed in astrocytes within peroxisomes, DAAO is a flavoprotein which catalyzes oxidation of neutral and polar D-amino acids to the byproduct hydrogen peroxide (Angermüller et al. 2009; Pollegioni et al. 2007; Wang et al. 2012) which is a less active but stable reactive oxygen species (ROS). The mechanism by which inhibition of DAAO enzymatic activity produced analgesia and blockade of morphine tolerance in the rat model of bone cancer pain is not completely understood but may be due to its prevention of spinal hydrogen peroxide production leading to inactivation of activated astrocytes. The hypothesis is supported by the following findings. (1) Subcutaneous injection of CBIO effectively inhibited DAAO enzymatic activity in the spinal cord in rats bearing tumoral cells whose spinal DAAO expression and enzymatic activity were significantly up-regulated. (2) CBIO significantly reduced production of spinal hydrogen peroxide by approximately 20 % which was presumably stimulated by inoculation of cancer cells. This is consistent with our previous study that paw formalin increased spinal hydrogen peroxide level by approximately 30 % in synchronous with time-course of tonic phase pain, and that the increased but not the basic level of hydrogen peroxide was completely prevented by systemic injection of CBIO (Lu et al. 2012). In addition, we have also demonstrated that multi-daily injections of morphine produced tolerance to analgesia and an increase in spinal hydrogen peroxide both of which were completely blocked by application of CBIO (Gong et al., unpublished data, 2012). (3) We have previously shown that intrathecal injection of hydrogen peroxide potentiated formalin-induced tonic phase pain (Lu et al. 2012). (4) It was well documented that bone implantation of cancer cells activated spinal astrocytes admittedly marked by expression of GFAP (Peters et al. 2005; Urch 2004), which is confirmed in this study. (5) Systemic injection of CBIO significantly reduced increased spinal GFAP expression in bone cancer rats, consistent with the results that multi-daily intrathecal injections of siRNA/DAAO inhibited spinal GFAP expression in formalin-challenged rats (Chen et al. 2012). It is thus postulated that in probably every process where astrocytes are activated contributing to central sensitization the inhibition of DAAO enzymatic activity would be analgesic or block morphine tolerance to analgesia. However, hydrogen peroxide also comes from other sources, such as peroxidase, xanthine oxidase, cytochrome P450, L-amino acid oxidase, monoamino oxidase and glycollate oxidase (Halliwell 1992), which are not CBIO-sensitive. The existence of non-DAAO-mediated hydrogen peroxide in addition to other

mechanisms mediating central sensitization would explain the partial (40–50 %) effect by blocking DAAO on bone cancer pain.

In summary, tibia inoculation of cancer cells yields mechanical allodynia but not heat hyperalgesia, which is correlated to the induction of spinal DAAO expression and enzymatic activity. The development and maintenance of bone cancer allodynia are blocked by 40–50 % by knockdown of spinal DAAO expression or inhibition of DAAO enzymatic activity. Additionally, unlike opioid receptor agonists such as morphine, multiple injections of CBIO do not induce tolerance to analgesia but yield an apparent additive anti-allodynia without cross-tolerance to morphine when administered in combination with morphine and more importantly prevent morphine tolerance to analgesia. The results suggest that spinal DAAO significantly contributes to the development of bone cancer pain partially (40–50 %) accounting for bone cancer pain status and morphine tolerance, probably via inhibition of spinal hydrogen peroxide subsequently blocking the hypertrophy of astrocytes. The results also provide a potential for DAAO inhibitors to treat chronic cancer pain when they are administered alone or in combination of morphine due to their unique characterizations.

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Conflict of interest All authors report no conflict of interest.

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