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Inhibitory effects of benzoate on chiral inversion and clearance of N^G-nitro-arginine in conscious rats

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Running title:

a) Benzoate on chiral inversion of N^G-nitro-arginine

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d) **ABBREVIATIONS**: NNA, N^G-nitro-arginine; L-NNA, N^G-nitro-L-arginine; D-NNA, N^G-nitro-D-arginine; DAAO, D-amino acid oxidase; CEC, capillary electrochromatography.

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Abstract

N^G-nitro-arginine (NNA) is known to exhibit stereoselective pharmacokinetics in which D-NNA has a faster clearance rate than L-NNA in anesthetized rats, and D-NNA undergoes unidirectional chiral inversion. It was postulated that chiral inversion of D-NNA was performed in a two-step pathway by D-amino acid oxidase (DAAO) followed by an unidentified transaminase. Such chiral inversion contributes (at least partially) to the pharmacokinetic stereoselectivity of NNA. This study used the selective inhibitor of DAAO, sodium benzoate, to test the above hypothesis. I.V. bolus injection of D-NNA (32 mg/kg) and L-NNA (16 mg/kg) in conscious rats exhibited biphasic disposition with different pharmacokinetic parameters in a stereospecific manner (approximately 5-10 fold differences). Unidirectional chiral inversion of D-NNA but not L-NNA was found from these animals. In addition to its similar inhibitory effects on the D-NNA conversion and DAAO activity in kidney homogenates, sodium benzoate completely blocked chiral inversion of D-NNA and led to smaller stereospecific difference, reflected by nearly 50% reduction of D-NNA clearance and 2-fold increase in $T_{1/2}$ and AUC of D-NNA in benzoate-pretreated rats. The results suggest that DAAO plays an essential role in chiral inversion of D-NNA and chiral inversion contributes mostly to the pharmacokinetic stereospecificity of NNA.

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Introduction

N^G-nitro-arginine (NNA) is an inhibitor of nitric oxide synthase, exhibiting stereospecificity such that the L- but not the D-enantiomer inhibits nitric oxide synthesis *in vitro* (Moncada *et al*., 1991; Wang *et al*., 1999). However, *in vivo* administration of the inactive D-enantiomer (D-NNA) produced the same biological effects as its L-enantiomer (L-NNA), such as raising blood pressure that was blocked by administration of L-arginine but not D-arginine (Wang *et al*., 1991, 1993), leading to a notion that D-NNA was converted into L-NNA *in vivo* (Wang *et al*., 1993). Wang *et al* (1999) from another laboratory demonstrated that D-NNA was indeed unidirectionally converted into L-NNA *in vivo* by using chiral HPLC. Furthermore, the kidney was confirmed to be the major organ accounting for approximately 80% of chiral inversion of D-NNA *in vivo* (Xin *et al*., 2005).

It was hypothesized that D-NNA unidirectionally converts to L-NNA by a two-step pathway involving the oxidation of D-NNA to N^G-nitro-5-gunidino-2-oxopentanoic acid by D-amino acid oxidase (DAAO) followed by amination to L-NNA by an unidentified transaminase (Wang *et al*., 1999; Xin *et al*., 2006). Several lines of evidence support the above hypothesis. (1) DAAO incubation with D-NNA *in vitro* reduced D-NNA content (Wang *et al*., 1999); (2) the chiral inversion rate of D-NNA was parallel with the DAAO activity in tissues homogenates (Xin *et al*, 2005); (3) injection of the inhibitor of DAAO benzoate completely blocked the pressor response to naive

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D-NNA in conscious rats (Xin *et al.*, 2005). However, further studies such as testing DAAO inhibitors on chiral inversion of D-NNA *in vitro* and *in vivo* are needed to confirm the above hypothesis.

It is known that chiral drugs may exhibit stereoselective pharmacokinetics. Stereoselective disposition of NNA was observed in anesthetized rats where D-NNA had a faster clearance than that of L-NNA (Wang *et al.*, 1999). Chiral inversion is one type of drug metabolism which contributes to drug clearance. However, it is not yet known whether chiral inversion of D-NNA accounts for pharmacokinetics stereoselectivity of NNA.

This study aimed to test whether the DAAO inhibitor sodium benzoate blocks the chiral inversion of D-NNA in rat kidney homogenates and in conscious rats. This study also intended to confirm pharmacokinetic stereospecificity of NNA in conscious rats, as well as deduce whether chiral inversion of D-NNA contributes to this stereospecificity. Our results showed that benzoate completely blocked chiral inversion of D-NNA *in vitro* and *in vivo*, suggesting that D-NNA conversion is *via* the metabolism of DAAO. Moreover, NNA exhibited its pharmacokinetics in a stereospecific manner in which the D-enantiomer cleared much faster (five-fold higher) due to its chiral inversion because administration of sodium benzoate mostly reduced the clearance of D-NNA.

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Materials and Methods

Drugs and reagents D-NNA was obtained from Bachem Bioscience (King of Prussia, PA), and L-NNA and aspartame were purchased from Acros Organics (Geel, Belgium). All other reagents were purchased from Shanghai Chemical Reagents Co. (Shanghai, China). D-NNA and L-NNA were dissolved in 0.9% saline solution, and the dissolution required 20 minutes of ultrasonication.

Animal preparation Male Sprague-Dawley rats (350 ~ 400 g), from Fudan University Medical Animal Center (Shanghai, China), were anesthetized with sodium pentobarbital (65 mg/kg, i.p.). A polyethylene cannula (PE50, Becton Dickinson Co., USA) was inserted into the left femoral vein for the collection of blood samples. The vascular cannula were tunneled subcutaneously and exteriorized at the back of the neck. The rats were given at lease 24 h recovery from anesthesia and surgery before use.

Preparation of tissue homogenates Rats were sacrificed and the kidneys were removed and washed with Tris-HCl buffer (4 °C, 0.1 M, pH 8.2). Tissue samples (1 g each) were homogenized in Tris-HCl buffer (3 ml, pH 8.2) at 4 °C with a homogenizer (Fluko Equipment Co., Shanghai, China). The homogenates were centrifuged at 1,500 rpm for 10 minutes, and the supernatants were used for the measurements of the DAAO activity and D-NNA conversion.

Determination of the DAAO activity The activity of DAAO was

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determined according to the 'keto acid method' (D'Aniello *et al*., 1996; Sarower *et al.*, 2003). The supernatants (200 µl) of the homogenates were incubated with 0.1 M D-alanine (200 µl, dissolved in the above Tris-HCl buffer) plus additional sodium benzoate solution (100 µl, concentration range 0.00025 to 500 mg/ml) for 30 min in a shaking bath (700 rpm) at 37 °C. The control samples were treated similarly, except the benzoate solution (100 µl) was replaced by Tris-HCl buffer (100 µl). Afterwards, trichloroacetic acid (200 µl, 25%) was added to the incubated mixture, mixed again, and centrifuged at 12,000 rpm for 10 minutes. The supernatant (400 µl) was mixed with 2,4-dinitrophenylhydrazine (400 µl of 1 mM in 1 M HCl) and incubated at 37 °C for 10 minutes. Subsequently, NaOH (800 µl of 1.5 M) was added and mixed. The mixture was kept at room temperature for 10 min and the absorbance was read at 445 nm against a blank sample consisting of the same mixture without D-alanine. The activity of DAAO in the homogenates was quantified against the standard curve of pyruvic acid (from 0.35 nM to 2.80 nM, $R^2 > 0.99$).

Tissue homogenates incubation with D-NNA The effects of kidney homogenates (n = 4) on the conversion of D-NNA into L-NNA were determined. The incubation systems of D-NNA and kidney homogenates for conversion were treated similarly with that for DAAO activity detection, except that in the D-alanine solution (200 µl of 0.1 M), the substrate of DAAO, was replaced by D-NNA solution (200 µl of 50 mM, dissolved in the same Tris-HCl buffer). In some experiments, the homogenate were first denatured through heating (10

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min at 100 °C). The incubation supernatants were used for *ex vivo* determination of D-NNA and L-NNA by capillary electrochromatography (CEC).

Measurement of D-NNA and L-NNA in biological samples D-NNA and L-NNA were measured by CEC in the chiral ligand exchange mode (Xin *et al*., 2005). Plasma samples (100 µl) or incubation samples (100 µl) of D-NNA or L-NNA were deproteinated through mixing for 20 minutes with methanol/acetone (1000 µl; v/v = 1:1). After centrifugation at 10,000 rpm for 20 minutes, the organic layer was dried at -20 °C, and the residue was dissolved in acetate buffer (pH 5.0, plasma in 100 µl and incubation samples in 5 ml). After mixing for 1 minute, a 10 nl aliquot of the sample was injected into the CEC system (Unimicro Tech. Co., CA, USA). Cupric acetate (1 mM) and aspartame (2 mM) were dissolved in the methanol/acetate buffer (pH 5.0, v/v = 1:20) in order to allow the formation of diastereomeric pairs with D-NNA or L-NNA. The diastereomeric pairs were separated through a reverse phase C18 column (75 µm × 20 cm; Unimicro Tech. Ltd, Shanghai, China) at detection wavelength of 280 nm. The retention times for L-NNA and D-NNA were 17 and 19 minutes, respectively. The samples were quantified against standard curves of L-NNA and D-NNA that ranged from 0.025 to 0.75 mM ($r^2 > 0.98$). The between-day (n = 5) precision values measured by the time and the area under curve were 3.5% and 3.9% for L-NNA and L-NNA, respectively. The within-day precision of the quality control samples was 2% for both L-NNA and D-NNA.

Pharmacokinetics studies Conscious rats were divided into four groups

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(n = 5 in each group). Two groups of rats were injected with D-NNA (32 mg/kg) or L-NNA (16 mg/kg), respectively. The other two groups of rats were pretreated with injection of sodium benzoate (400 mg/kg), a selective inhibitor of DAAO and effectively blocks DAAO activity at this dose (Moses *et al.*, 1996; Xin *et al*., 2005, 2006). 20 minutes later, the benzoate-pretreated group was injected with D-NNA (32 mg/kg) or L-NNA (16 mg/kg), respectively. All the drugs were i.v. bolus injected *via* the indwelling cannula. The cannula was flushed with saline solution (600 µl, 0.9%) to ensure no contamination during blood sampling. Blood samples (200 µl) were obtained at 5, 20, 40, 60, 90, 120, 180, 240, 300, 420 minutes after administration. Blood volume was replaced by injection of an equal volume of saline solution (0.9% NaCl) after each sampling. Plasma samples obtained were stored at -20 °C for later analysis by CEC.

Pharmacokinetics Analyses Pharmacokinetics parameters were obtained from the plasma concentration–time curves using non-compartmental model. Data was weighted $1/c^2$. The elimination half-life ($T_{1/2}$) was calculated by $0.693/K_{el}$. The chiral inversion rate of D-NNA to L-NNA was calculated using the following formula (Pang and Kwan, 1993):

$$F_{D\text{-}NNA \rightarrow L\text{-}NNA} = \frac{AUC_{L\text{-}NNA \text{ from } D\text{-}NNA} / \text{ Dose}_{D\text{-}NNA}}{AUC_{L\text{-}NNA} / \text{ Dose}_{L\text{-}NNA}}$$

The area under the curve (AUC) was calculated by the trapezoidal rule.

Data Analyses IC_{50} values for benzoate to block DAAO and D-NNA conversion were determined by using a curve fitting program, DoseResp of

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Origin 7.5 (Northampton, MA, USA). All results were expressed as means ± S.E.M. and analyzed by the analysis of variance followed by Duncan's Multiple Range test. Differences with values of $P < 0.05$ were considered statistically significant.

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Results and Discussion

Previous findings suggested that chiral inversion of D-NNA was metabolized by kidney DAAO (Wang *et al*., 1999; Xin *et al*., 2005, 2006). However, further experiments such as those using DAAO inhibitors are needed to confirm the above hypothesis. Sodium benzoate was selected as a DAAO inhibitor for this study to examine whether DAAO inhibitors block chiral inversion of D-NNA *in vitro* and *in vivo*.

Our previous study indicated that D-NNA but not L-NNA was the substrate of DAAO and the K_{cat}/K_m of DAAO to D-NNA was about 10% of that to D-alanine, the optimum substrate for kidney DAAO (Xin *et al*., 2006). In kidney homogenates, DAAO activity was measured by using 'keto acid method' where 0.1 M D-alanine was used (D'Aniello *et al*., 1996; Sarower *et al.*, 2003). Sodium benzoate inhibited DAAO activity in a sigmoid concentration-response manner (Fig. 1), with the IC_{50} value of 6.7 mM (95% confidence interval, 5.72 to 7.68 mM). Our results, together with a previous report that the K_d for benzoate to inhibit DAAO was approximately 0.2 mM (Pilone, 2000), suggest that benzoate is a weak inhibitor of DAAO. Moreover, the chiral inversion of D-NNA in this study was observed at 50 mM D-NNA, in contrast to the measurement of DAAO activity where 0.1 M D-alanine was used. Benzoate also blocked the chiral inversion of D-NNA in kidney homogenates, with the concentration-response curve correlating very well to that of the inhibition of DAAO activity (Fig. 1). The IC_{50} value for benzoate to block D-NNA disposition

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was 2.1 mM (95% confidence interval, 1.31 to 2.88 mM), which was close to the inhibition of the DAAO activity. These results suggest benzoate blocks D-NNA conversion *via* the inhibition of the DAAO activity in kidney homogenates.

Pharmacokinetics of D-NNA was studied in two groups of conscious rats (n = 5 in each group) pretreated with the vehicle or benzoate. I.V. bolus injection of D-NNA (32 mg/kg) exhibited a biphasic disposition curve while D-NNA was almost undetectable in plasma after 3 hours of administration (Fig. 2A), with calculated CL and $T_{1/2}$ of 0.98 ± 0.07 L/h/kg and 0.55 ± 0.33 h, respectively (Table 1). Meanwhile L-NNA was immediately detected in the plasma following D-NNA injection and the plasma concentration of L-NNA exceeded that of D-NNA at 1.5 hours and reached the peak at 2 hours after D-NNA dosage (Fig. 2A). In order to observe whether inhibition of the DAAO activity blocked the chiral inversion of D-NNA *in vivo*, conscious rats were pretreated with benzoate at 400 mg/kg, a dosage selected based on the biological effects of D-dopa (Moses *et al*., 1996), as well as results from a previous study that showed benzoate led to the inhibition of DAAO activity (Williams and Lock, 2005). As seen in Fig. 2B, benzoate completely blocked D-NNA conversion *in vivo*, in agreement with our previous study that showed benzoate abolished the pressor response to naive D-NNA (Xin *et al*., 2005). In contrast to control rats, where nearly no D-NNA was detected in plasma samples at 3 hours after administration, plasma D-NNA was kept at ≈10 μg/ml at 3 hours and was still detectable at 7 hours after D-NNA dosing in benzoate-pretreated rats. As a

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result of the blockade of chiral inversion of D-NNA, the mean clearance of D-NNA was significantly reduced ($P < 0.05$) by nearly 50% from 0.98 ± 0.07 L/h/kg to 0.49 ± 0.12 L/h/kg upon coadministration (Table 1). Table 1 also shows that benzoate significantly increased $T_{1/2}$ of D-NNA and approximately doubled AUC of D-NNA. The effectiveness of benzoate to block chiral inversion of D-NNA and partially reduce clearance of D-NNA is in agreement with the previous report that pretreatment of sodium benzoate (1000 mg/kg, i.p.) led to a 40% reduction of D-ethionine disposition in 4 hours in rats (Brada and Bulba, 1980). Similar results have also been demonstrated in the study of D-methionine metabolism where intraperitoneal treatment with sodium benzoate (0.9 g/kg) reflected a gradual clearance of D-methionine from the blood and a lower of hepatic metabolism to D-methionine (London and Gabel, 1988).

In order to find out whether the effect of benzoate on pharmacokinetics of D-NNA was specific on chiral inversion blockade, the effect of benzoate on L-NNA pharmacokinetics was tested in two groups of conscious rats (n = 5 in each group). I.V. bolus injection of L-NNA (16 mg/kg) also exhibited a biphasic disposition curve in control rats (Fig. 3A,), with calculated CL and $T_{1/2}$ of 0.19 L/hr/kg and 5.21 hours, respectively (Table 1). These parameters clearly showed a stereoselectivity in pharmacokinetics of NNA in which CL and $T_{1/2}$ of L-NNA had approximately 5-fold and 10-fold differences from those of D-NNA (Table 1). The results are consistent with previous findings that showed the

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stereoselective disposition of NNA in anesthetized rats (Wang *et al*., 1999). Unlike in D-NNA injection, in which L-NNA was immediately detected, the D-enantiomer of L-NNA was not detected in plasma samples during the period observed after L-NNA administration, confirming that chiral inversion of D-NNA was unidirectional (Wang *et al*., 1999). On the other hand, benzoate pretreatment, as shown in Fig 3B, did not significantly affect pharmacokinetic parameters of L-NNA (Table 1), excluding the possibility that the effect of benzoate on D-NNA chiral inversion and clearance was non-specific. Moreover, in the presence of benzoate for complete blockade of chiral inversion, the difference in pharmacokinetics parameters between D-NNA and L-NNA became significantly smaller (only approximately 2 folds) (Table 1). The results suggest that chiral inversion mostly contributes to clearance stereoselectivity of NNA.

According to the 'Pang and Kwan formula' (1993), the calculated inversion rate of D-NNA to L-NNA was 36.7 ± 0.6%. Chiral inversion ratio of D-NNA to L-NNA calculated from this study was in agreement with the potency ratio (40%) of L-NNA/D-NNA to increase blood pressure in conscious rats (Wang *et al*., 1991), as well as chiral inversion of D-NNA in anesthetized rats (Wang *et al.,* 1999).

It is well known that most chiral drugs exhibit stereospecificity in their pharmacokinetics. Enantioselectivity of pharmacokinetics may be influenced by enzymatic metabolism, carrier transport, protein binding, distribution, and

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elimination of different stereoisomers (Burke *et al*., 2002). In this study, we suggest that chiral inversion is an additional factor which contributes to pharmacokinetics stereospecificity, particularly in clearance. It was recently reported that many chiral pharmaceuticals undergo chiral inversion *in vivo*, such as certain D-amino acids (Hasegawa *et al*., 2000; Lehmann *et al*., 1983), 2-arylpropionic acid analogs (Drummond *et al.*, 1990), the new quinoxaline toposomerase poison 2-[4-(7-chloro-2-quinoxaliny)oxy] phenoxy propionic acid (Zheng *et al*., 2002), and thalidomide (Eriksson *et al.*, 1998). Therefore, chiral inversion may be an important contributing factor for pharmacokinetics stereospecificity of these chiral drugs.

In summary, D-NNA and L-NNA in conscious rats exhibited biphasic disposition in conscious rats with different pharmacokinetics parameters. Unidirectional chiral inversion of D-NNA but not L-NNA was found in animals. In addition to its inhibition of D-NNA conversion in kidney homogenates, DAAO inhibitor sodium benzoate completely blocked chiral inversion of D-NNA and partially reduced clearance of D-NNA. The results suggest DAAO plays an essential role in chiral inversion of D-NNA and chiral inversion contributes mostly to the pharmacokinetic stereospecificity of NNA.

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Footnotes

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Legends for figures

Fig 1 Inhibitory effects of sodium benzoate on the D-amino acid oxidase (DAAO) activity (■) and the chiral inversion of N^G-nitro-D-arginine (D-NNA) (●) in rat kidney homogenates (n = 4 samples in each point). All readings are mean ± S.E.M.

Fig 2 Mean plasma concentration–time curves of N^G-nitro-arginine (NNA) enantiomers (□ L-NNA; ● D-NNA) after i.v. bolus injection of D-NNA (32 mg/kg) in conscious rats (n = 5 in each group) pretreated with the vehicle (0.9% NaCl saline, 4 ml/kg, i.v., A) or sodium benzoate (400 mg/kg, i.v., B). All readings are mean ± S.E.M.

Fig 3 Mean plasma concentration–time curves of N^G-nitro-L-arginine (L-NNA) after i.v. bolus injection of L-NNA (16 mg/kg) in conscious rats (n = 5 in each group) pretreated with the vehicle (0.9% NaCl saline, 4 ml/kg, i.v., A) or sodium benzoate (400 mg/kg, i.v., B). No N^G-nitro-D-arginine (D-NNA) was detected from plasma samples of these rats. All readings are mean ± S.E.M.

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Tables

Table 1 Pharmacokinetic parameters of N^G-nitro-D-arginine (D-NNA) and N^G-nitro-L-arginine (L-NNA) after i.v. bolus injection of D-NNA (32 mg/kg) or L-NNA (16 mg/kg) in conscious rats (n = 5 in each group) pretreated with the vehicle (0.9% NaCl saline, 4 ml/kg, i.v.) or sodium benzoate (400 mg/kg, i.v.). All readings are mean ± S.E.M.

Parameters	D-NNA	D-NNA (benzoate)	L-NNA	L-NNA (benzoate)
$AUC_{(0\text{-}7h)(mg/L/h)}$	31.32 ± 2.36	54.34 ± 8.05 [a]	52.58 ± 2.60	58.82 ± 17.00
$CL_{(L/h/kg)}$	0.98 ± 0.07	0.49 ± 0.12 [a]	0.19 ± 0.04 [a]	0.16 ± 0.03 [b]
$T_{1/2\ (h)}$	0.58 ± 0.04	1.37 ± 0.32 [a]	5.21 ± 1.69 [a]	6.31 ± 1.97 [b]
$AUC_{(0\text{-}7h)\ (mg/L/h)}$ (*L*-NNA after *D*-NNA)	28.98 ± 3.72			
T_{max}	2.20 ± 0.20			
C_{max}	6.73 ± 0.96			
$T_{1/2\ (h)}$	8.60 ± 2.58			

[a] and [b] denote significant ($P < 0.05$) differences from respective D-NNA group and D-NNA + benzoate group, respectively.

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Fig. 1

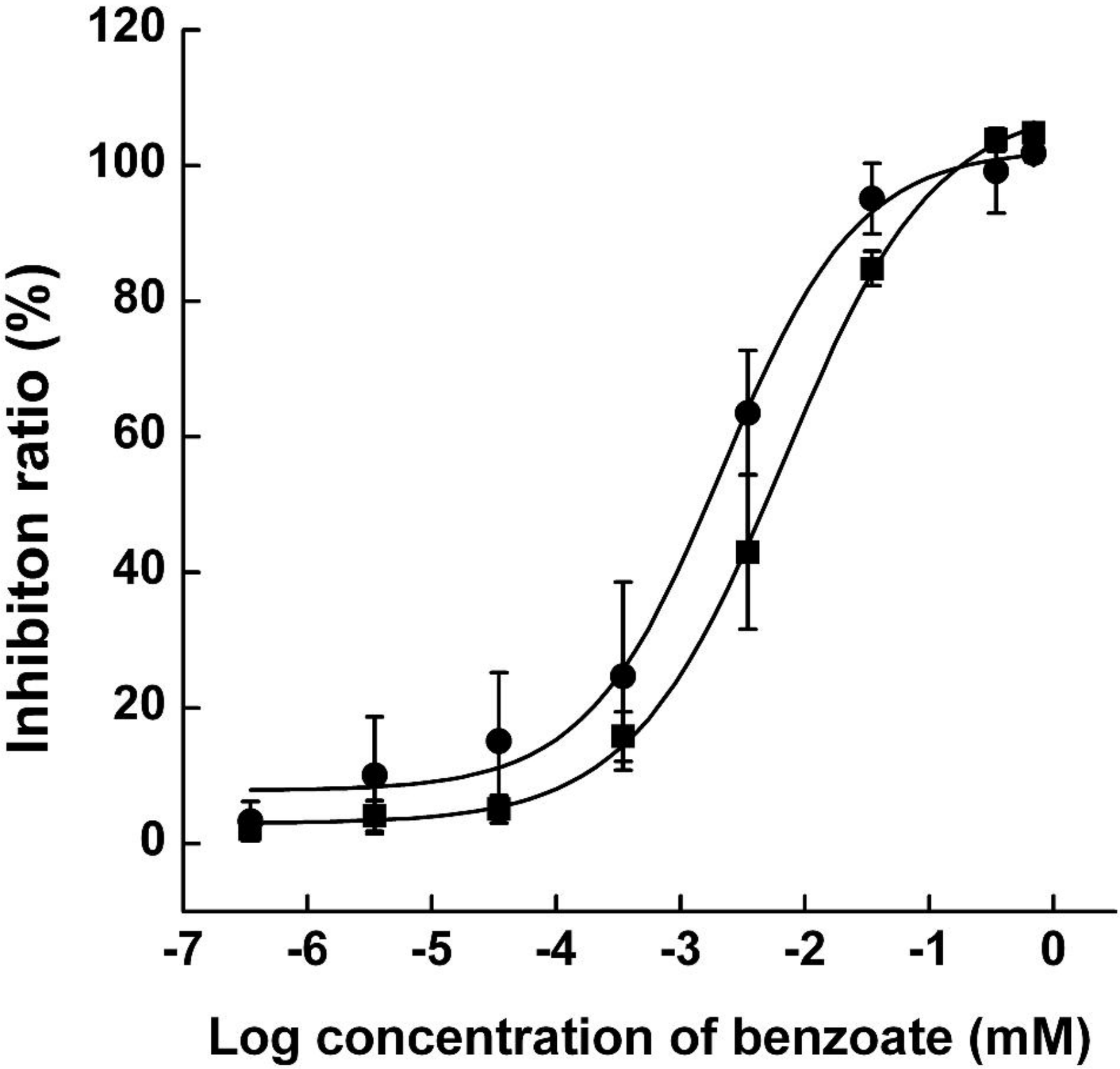
120
100
80
60
40
20
0
Inhibiton ratio (%)
-7
-6
-5
-4
-3
-2
-1
0
Log concentration of benzoate (mM)

Fig. 2

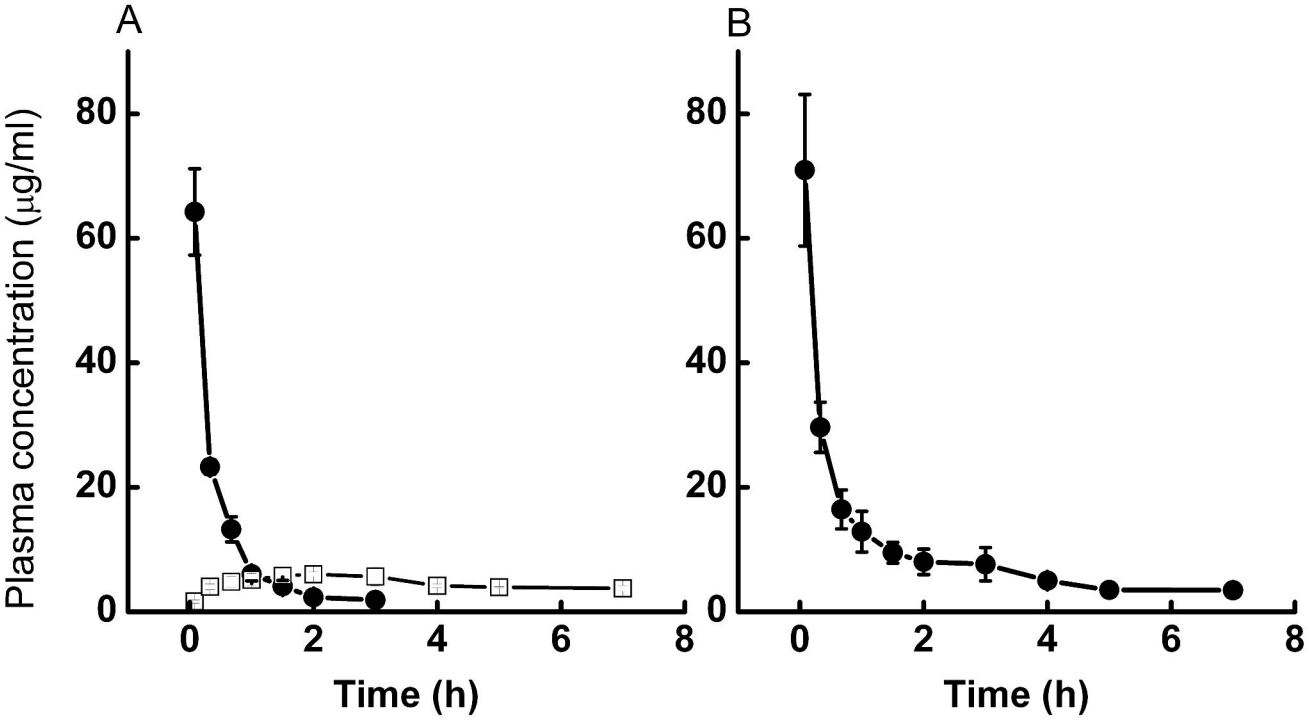
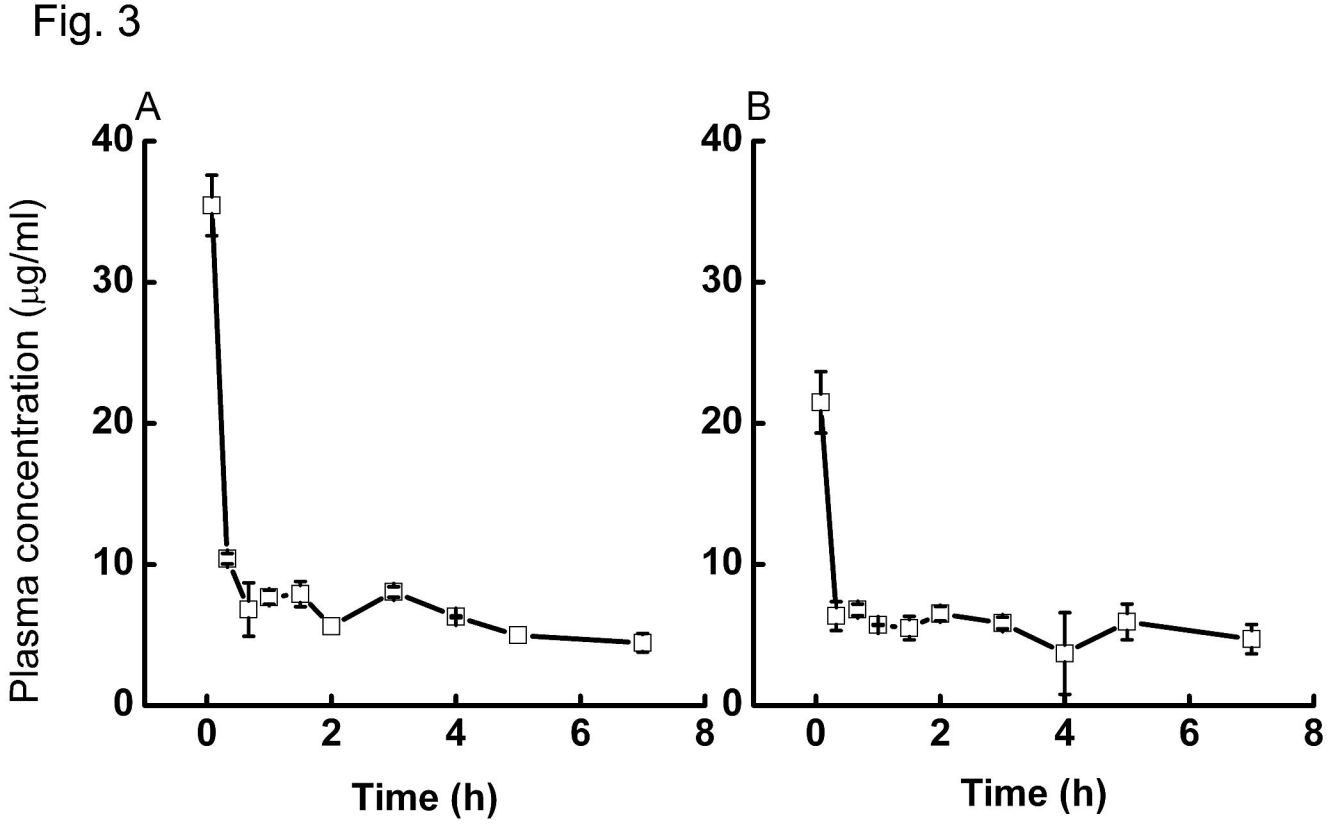
Fig. 3