

# 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

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**Abstract:** D-Alanine, one of D-amino acids present in the mammalian brain, is a selective and potent agonist at the N-methyl-D-aspartate (NMDA) receptors. Like D-serine, D-alanine is reported to be effective in the treatment of schizophrenia. However, orally given D-alanine is metabolized substantially by D-amino acid oxidase (DAAO), diminishing its oral bioavailability. In this study, we studied the effects of oral D-alanine administration with or without the novel DAAO inhibitor, 5-chloro-benzo[d]isoxazol-3-ol (CBIO), on the extracellular D-alanine levels in the brain and on the prepulse inhibition (PPI) deficits after administration of the NMDA receptor antagonist dizocilpine. Co-administration of CBIO (30 mg/kg) with D-alanine (100 mg/kg), but not D-alanine (100 mg/kg) alone, significantly attenuated dizocilpine (0.1 mg/kg)-induced PPI deficits in mice. The *in vivo* microdialysis study of the conscious and free moving mice revealed that co-administration of CBIO (30 mg/kg) significantly increased extracellular levels of D-alanine in the frontal cortex after oral administration of D-alanine (100 mg/kg). These findings suggest that co-administration of CBIO can increase the bioavailability of D-alanine after oral administration of D-alanine, and that co-administration of CBIO can enhance the efficacy of D-alanine on dizocilpine-induced PPI deficits. Therefore, combination of D-alanine and a DAAO inhibitor such as CBIO offers new therapeutic potential for treatment of schizophrenia.

**Key Words:** D-Alanine, D-amino acid oxidase, NMDA receptors, Prepulse inhibition, Schizophrenia, Bioavailability.

## INTRODUCTION

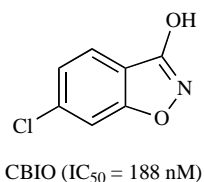
Several lines of evidence suggest that a dysfunction in the glutamatergic neurotransmission *via* the N-methyl-D-aspartate (NMDA) receptors might be involved in the pathophysiology of schizophrenia [1-9]. Based on the hypofunction hypothesis of NMDA receptors in schizophrenia, the drugs which can stimulate the function of NMDA receptors would be attractive drugs for novel treatment of schizophrenia [10-15].

D-Alanine is the first D-amino acid found in serum of guinea pig and mice [16]. Subsequent studies demonstrated that free D-alanine is one of the D-amino acids naturally occurring in the mammalian tissues including brain and blood [17-22]. D-Alanine, like D-serine, is also a selective and potent agonist at the glycine-modulatory site on the NMDA receptors [23-25]. Accumulating evidence suggests that, similar to D-serine, D-alanine also might be involved in the pathophysiology of schizophrenia. First, bilateral injection of D-alanine, but not L-alanine, into the lateral ventricle significantly blocked hyperlocomotion in rats after administration of the NMDA receptor antagonist phencyclidine (PCP) [26]. Second, in a 6-week double-blind, placebo-

controlled study, Tsai *et al.* [27] reported that D-alanine (100 mg/kg/day) significantly improved schizophrenic symptoms when used as adjunctive to conventional antipsychotic drugs. Third, mRNA expression and activity of D-amino acid oxidase (DAAO), which can metabolize D-alanine, is increased in the postmortem brain of schizophrenic patients [28, 29]. Fourth, the G72 gene on the chromosome 13q was significantly associated with schizophrenia [30]. The G72 gene is given the designation DAAO activator since G72 protein was shown to interact with physically with DAAO [30]. A recent meta-analysis shows highly significant evidence of association between nucleotide variations in the G72/G30 region and schizophrenia [31]. Taken all together, it is likely that alterations in brain D-alanine levels may be implicated in the pathophysiology of schizophrenia.

In animals, D-alanine, like D-serine, is suggested to be metabolized substantially by DAAO in peripheral organs, diminishing its oral bioavailability [32, 33]. These findings prompted us to identify small molecule DAAO inhibitors that can be co-administered with D-amino acids (e.g., D-serine and D-alanine) to minimize its metabolism by DAAO. Recently, we reported that oral administration of the novel and potent DAAO inhibitor, 5-chloro-benzo[d]isoxazol-3-ol (CBIO) (Fig. 1), in conjunction with D-serine could enhance the plasma and brain levels of D-serine in rats and mice compared to the oral administration of D-serine alone [34, 35].

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**Fig. (1). Chemical structure of CBIO.**

The IC<sub>50</sub> value of CBIO for DAAO was 188 nM [34].

The prepulse inhibition (PPI) deficits have been widely used as an animal model of schizophrenia [36, 37]. In the present study, we examined the effects of oral D-alanine administration with or without CBIO on the PPI deficits in mice after administration of the NMDA receptor antagonist dizocilpine. Furthermore, using the *in vivo* microdialysis method, we measured the extracellular levels of D-alanine in the frontal cortex after oral administration of D-alanine with or without CBIO.

## METHODS

### Animals

Male Slc:ddY mice (6 weeks old) weighing 25–30 g were purchased from SLC Japan (Hamamatsu, Shizuoka, Japan). The mice were housed in clear polycarbonate cages (22.5×33.8×14.0 cm) and in groups of 5 or 6 mice under a controlled 12/12-h light–dark cycle (light from 7:00 AM to 7:00 PM), with room temperature at 23 ± 1°C and humidity at 55 ± 5%. The mice were given free access to water and food pellets. The experimental procedure was approved by the Animal Care and Use Committee of Chiba University.

### Drugs and Drug Administration

(+)-MK-801 hydrogen maleate (dizocilpine) (0.1 mg/kg as a hydrogen maleate salt, Sigma-Aldrich Corporation., St. Louis, MO, USA), dissolved in physiological saline, was injected subcutaneously (s.c.) in a volume of 10 ml/kg. The dose (0.1 mg/kg) of dizocilpine was selected because this dose caused PPI deficits in mice as reported previously [35, 38]. D-Alanine (100, 300 or 1000 mg/kg, Wako Pure Chemical Industries, Ltd., Tokyo, Japan), dissolved in 0.5% carbomethoxycellulose (CMC: Wako Pure Chemical Co., Tokyo, Japan), was administered orally in a volume of 10 ml/kg. CBIO (30 mg/kg) [34, 35] was suspended in 0.5% CMC, was administered orally in a volume of 10 ml/kg. The other chemicals used were purchased from commercial sources.

### Measurement of Acoustic Startle Reactivity and Prepulse Inhibition of Startle

The mice were tested for their acoustic startle reactivity (ASR) in a startle chamber (SR-LAB, San Diego Instruments, CA, USA) using standard methods described by Swerdlow and Geyer [37]. After an initial 10-min acclimation period in the chamber, the test sessions began. They consisted of six trial types: (1) pulse alone, 40 ms broadband burst; pulse preceded 100 ms by a 20 ms prepulse that was (2) 4 dB, (3) 8 dB, (4) 12 dB, or (5) 16 dB over background (65 dB); and (6) background only (no stimulus). The amount of prepulse inhibition (PPI) is expressed as the percentage

decrease in the amplitude of the startle reactivity caused by presentation of the prepulse (% PPI).

D-Alanine (100, 300 or 1000 mg/kg) with or without CBIO (30 mg/kg) [34, 35], or vehicle (0.5% CMC) (10 ml/kg) were administered 60 min (including 10-min acclimation period) before the machine records, and dizocilpine (0.1 mg/kg) or saline (10 ml/kg) was administered s.c. 10 min (including 10-min acclimation period) before. The PPI test lasted 20 min in total.

### *In Vivo* Microdialysis Study in Free-Moving Mice

Mice were anesthetized with sodium pentobarbital prior to the stereotaxic implantation of a probe into the left frontal cortex (+2.1 mm anteroposterior, +1.0 mm mediolateral from the bregma, and -1.2 mm dorsoventral with respect to dura). Probes were secured onto the skull using stainless-steel screws and dental acrylic. Twenty-four hours after surgery, *in vivo* microdialysis was performed on conscious and free-moving mice. Probes were perfused continuously with artificial CSF (147 mM NaCl, 4 mM KCl, and 2.3 mM CaCl<sub>2</sub>) at a rate of 2 µl/min. D-Alanine (100 mg/kg) with or without CBIO (30 mg/kg) was orally administered into mice. The dialysate was collected in 30-min fractions, and then stored at -80°C before use.

Measurement of total, D- and L-alanine levels was carried out using a column-switching high performance liquid chromatography (HPLC) system (Shimadzu Corporation, Kyoto, Japan). To the 20 µl of dialysis sample, 20 µl of 0.1 M borate buffer (pH 8.0) and 60 µl of 50 mM 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F; Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan) in CH<sub>3</sub>CN (HPLC grade) were added. The reaction mixture was then heated at 60°C for 1 min, and immediately supplemented with 100 µl of H<sub>2</sub>O/CH<sub>3</sub>CN (90/10) containing 0.1 % trifluoroacetic acid to stop the reaction.

A 20 µl aliquot of the resultant solution was injected into the HPLC system. A reversed-phase ODS column [TSKgel ODS-80Ts (Tosoh Corporation, Tokyo, Japan) as Column 1] was used for the separation and quantification of total (D- and L-) alanine, and the gradient elution of the mobile phase was maintained at a constant flow rate of 0.8 mL/min. Mobile phase 1a consisted of H<sub>2</sub>O/CH<sub>3</sub>CN (90/10) containing 0.1% TFA, and phases 1b and 1c, of H<sub>2</sub>O/CH<sub>3</sub>CN (10/90) containing 0.1% TFA and CH<sub>3</sub>CN, respectively. The time program for gradient elution was as follows: 0 - 40 min 1a : 1b : 1c = 80 : 20 : 0, 40 - 55 min 1a : 1b : 1c = 0 : 100 : 0, and 55 - 57 min, 1a : 1b : 1c = 0 : 0 : 100. The chiral column (Column 2) used for the separation and quantification of D- and L-alanine with NBD-F comprised two Sumichiral OA-2500 columns (S) (Sumika Chemical Analysis Service Ltd., Osaka, Japan), which were connected in tandem. The mobile phase was 15 mM citric acid in MeOH. The flow rate was isocratically pumped at 0.8 mL/min. The column temperature of all columns was maintained at 35°C. Fluorescence detection was performed at 530 nm with an excitation wavelength at 470 nm.

### Statistical Analysis

The data are presented as the mean ± standard error of the mean (S.E.M.). PPI was calculated as the percent inhibi-

tion of the startle amplitude evoked by the pulse alone: % PPI =  $100 \times (\text{magnitude on pulse alone trial} - \text{magnitude on prepulse + pulse trial} / \text{magnitude on pulse alone trial})$ . The PPI data were analyzed by multivariate analysis of variance (MANOVA). When appropriate, group means at individual dB levels were compared by one-way ANOVA, followed by Bonferroni/Dunn test. The results of the *in vivo* microdialysis were analyzed by two-way analysis of variance (ANOVA) for repeated measures, with treatment as the between-subjects factor, and time as the within-subjects factor. When appropriate, group means at individual time points were compared by unpaired Student t-test. Significance for the results was set at  $p < 0.05$ .

## RESULTS

### Effects of D-Alanine with or without CBIO on PPI Deficits after a Single Administration of Dizocilpine

Fig. (2) shows the effects of D-alanine (100, 300 or 1000 mg/kg) with or without CBIO (30 mg/kg) on dizocilpine (0.1 mg/kg)-induced PPI deficits in mice. The MANOVA analysis of all PPI data revealed that there was a significant effect [Wilks lambda = 0.384,  $P < 0.001$ ]. Subsequent ANOVA analysis revealed the significant differences at all dB groups (69, 73, 77, and 81 dB). A *posteriori* analysis indicated a significant ( $P < 0.001$ ) difference between vehicle + vehicle group and vehicle + dizocilpine (0.1 mg/kg) group (Fig. 2). Higher doses (300 or 1000 mg/kg) of D-alanine alone significantly attenuated PPI deficits induced by dizocilpine (0.1 mg/kg) (Fig. 2). However, the low dose (100 mg/kg) of D-

alanine alone did not alter PPI deficits induced by dizocilpine. Interestingly, co-administration of CBIO (30 mg/kg) with D-alanine (100 mg/kg) significantly attenuated dizocilpine-induced PPI deficits at 73 dB ( $P = 0.006$ ), and 77 dB ( $P = 0.009$ ), and 81 dB ( $P < 0.001$ ) (Fig. 2). In contrast, CBIO (30 mg/kg) alone did not alter PPI in control mice (Fig. 2). Treatment with CBIO (30 mg/kg) alone was no effect on dizocilpine-induced PPI deficits in mice, as reported previously [35].

### Effects of CBIO on Extracellular Levels of D-Alanine in the Frontal Cortex after a Single Oral Administration of D-Alanine

In order to explore the effects of CBIO on the extracellular levels of D-alanine in the brain, we used an *in vivo* microdialysis technique to examine in extracellular D-alanine levels in the frontal cortex of conscious mice. Two-way repeated ANOVA analysis revealed significant differences among the two groups [ $F(1,8) = 14.39$ ,  $p = 0.005$ ]. Co-administration of D-alanine (100 mg/kg) and CBIO (30 mg/kg) significantly increased the extracellular D-alanine levels in the mouse frontal cortex as compared with D-alanine (100 mg/kg) alone treated group (Fig. 3).

## DISCUSSION

The present findings suggest that administration of DAAO inhibitor CBIO could potentiate the bioavailability of D-alanine in mice after oral administration. In this study, we found that co-administration of CBIO (30 mg/kg) potentiated

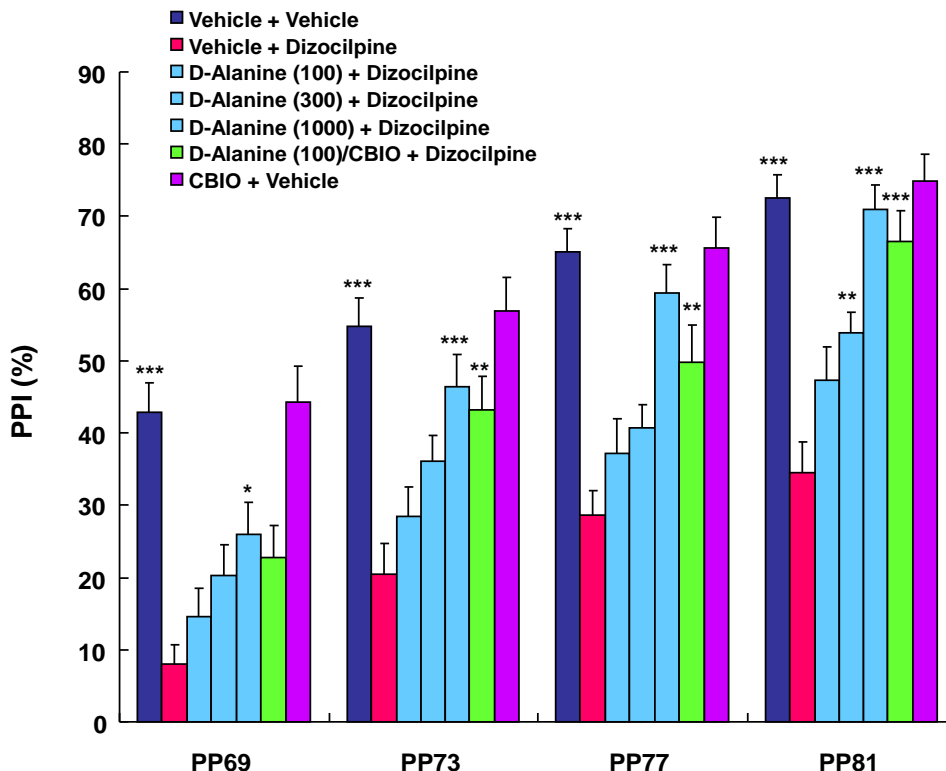
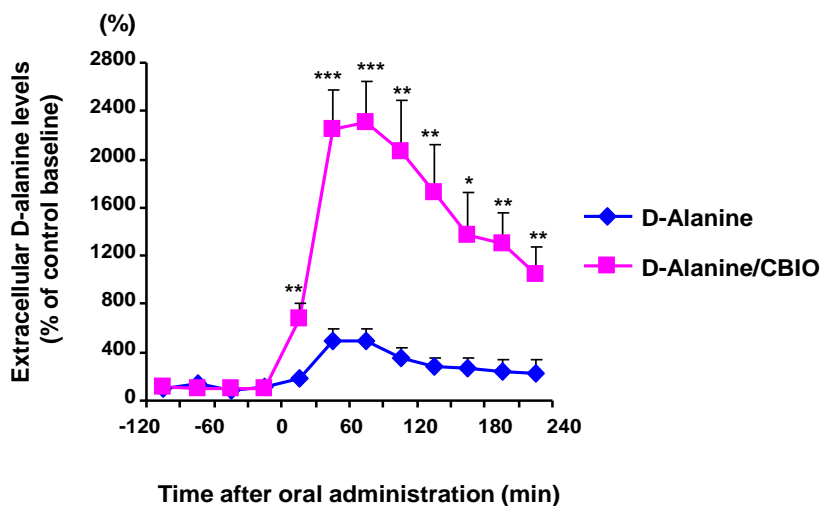


Fig. (2). The effect of D-alanine with or without CBIO on dizocilpine-induced PPI deficits in mice.

Sixty minutes after oral administration of vehicle (10 ml/kg), D-alanine (100, 300 or 1000 mg/kg), D-alanine (100 mg/kg) plus CBIO (30 mg/kg) or CBIO (30 mg/kg) alone, dizocilpine (0.1 mg/kg) or vehicle (10 ml/kg) was administered s.c. to the mice. Each value is the mean  $\pm$  S.E.M. ( $n = 15$  or  $16$  per group). \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  as compared with vehicle + dizocilpine treated group.



**Fig. (3). The effects of CBIO on extracellular D-alanine levels in the frontal cortex after oral administration of D-alanine.**

Extracellular levels of D-alanine in the frontal cortex after oral administration of D-alanine (100 mg/kg) with or without CBIO (30 mg/kg) were measured by *in vivo* microdialysis in conscious and free-moving mice. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  compared with the D-alanine (100 mg/kg)-treated group. Each value is the mean  $\pm$  S.E.M. ( $n = 6$  per group).

the efficacy of D-alanine (100 mg/kg) on dizocilpine-induced PPI deficits although D-alanine (100 mg/kg) alone was no effective in this model.

The *in vivo* microdialysis study in conscious and free-moving mice revealed that the extracellular levels of D-alanine in the frontal cortex after a single oral administration of D-alanine with CBIO were significantly higher than those of D-alanine alone group, indicating the increased oral bioavailability by the DAAO inhibitor CBIO. Thus, it is likely that enhancement of D-alanine by CBIO on dizocilpine-induced PPI deficits may be due to increased D-alanine levels in the brain. Furthermore, we reported that treatment with CBIO (30 mg/kg) alone was no effect on the extracellular D-serine levels in the rat brain because the permeability of CBIO into brain is not good [34]. Therefore, a combination therapy of D-alanine and a DAAO inhibitor (e.g., CBIO) could reduce the dose of D-alanine in human since the dose (100 mg/kg for human) of D-alanine for treatment of schizophrenic patients is high [27].

Dr. Adage's group reported a slight increase in D-serine levels in rat brain following intravenous administration of a pyrazole-3-carboxylate based DAAO inhibitor (AS057278) alone [39], indicating that this effect may be due to its ability to penetrate the blood-brain barrier. Furthermore, Smith *et al.* [40] have developed the novel DAAO inhibitor 4H-thieno[3,2-b]pyrrole-5-carboxylic acid ( $IC_{50} = 145$  nM for human,  $IC_{50} = 114$  nM for rat). This compound failed to significantly influence amphetamine-induced psychomotor activity, nucleus accumbens dopamine release, or (+)-MK-801 (dizocilpine)-induced deficit in novel object recognition in rats, suggesting that acute inhibition of DAAO by this compound appears not to be sufficient to increase D-serine to concentrations required to produce antipsychotic and cognitive enhancing effects similar to those observed after administration of high doses of D-serine [40]. In contrast, there is no correlation between the distribution of DAAO and NMDA receptors in the brain [41, 42]. Therefore, even a

brain-penetrable DAAO inhibitor may not be able to significantly enhance NMDA receptor-mediated neurotransmission by itself [34, 35].

## CONCLUSION

In this study, we found that administration of DAAO inhibitor CBIO could enhance the oral bioavailability of D-alanine in mice, and that co-administration of D-alanine with CBIO significantly increased the extracellular D-alanine levels in the mouse frontal cortex as compared with D-alanine alone group. In conclusion, co-administration of D-alanine and a DAAO inhibitor would be a new approach for the treatment of schizophrenia.

## ACKNOWLEDGEMENTS

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## ABBREVIATIONS

ANOVA	=	Analysis of variance
CBIO	=	5-Chloro-benzo[ <i>d</i> ]isoxazol-3-ol
CMC	=	Carbomethoxycellulose
CSF	=	Cerebrospinal fluid
DAAO	=	D-amino acid oxidase
HPLC	=	High performance liquid chromatography
MANOVA	=	Multivariate analysis of variance
NBD-F	=	4-Fluoro-7-nitro-2,1,3-benzoxadiazole
NMDA	=	N-Methyl-D-aspartate
PCP	=	Phencyclidine
PPI	=	Prepulse inhibition
SEM	=	Standard error of the mean

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