

DMD#46482

Pharmacokinetics of Oral D-Serine in D-Amino Acid Oxidase Knockout Mice

Rana Rais, Ajit G. Thomas, Krystyna Wozniak, Ying Wu, Hanna Jaaro-Peled, Akira Sawa,
Christine A. Strick, Sandra J. Engle, Nicholas J. Brandon, Camilo Rojas, Barbara S. Slusher and
Takashi Tsukamoto

Departments of Neurology (R.R., B.S.S., T.T.), Brain Science Institute (A.G.T., K.W., Y.W.,
C.R.) and Psychiatry and Behavioral Sciences (H.J.P., A.S.), Johns Hopkins University School
of Medicine, Baltimore, Maryland; Neuroscience (C.A.S., N.J.B.) and Pharmacokinetics,
Dynamics and Metabolism (S.J.E.), Pfizer, Groton, Connecticut

DMD#46482

Running Title: Oral D-serine PK in mice lacking DAAO

Takashi Tsukamoto, Department of Neurology, Johns Hopkins University, 855 North Wolfe Street, Baltimore, Maryland, USA 21205, phone: 410-614-0982, Fax: 410-614-0659, E-mail: ttsukamoto@jhmi.edu

Number of text pages: 21

Number of tables: 1

Number of figures: 5

Number of references: 40

Number of words in the Abstract: 175

Number of words in Introduction: 667

Number of words in Discussion: 1557

Non-standard abbreviations: D-amino acid oxidase (DAAO), 6-chlorobenzo[d]isoxazol-3-ol (CBIO)

DMD#46482

ABSTRACT

D-Amino acid oxidase (DAAO) catalyzes the oxidative deamination of D-amino acids including D-serine, a full agonist at the glycine modulatory site of the NMDA receptor. To evaluate the significance of DAAO-mediated metabolism in pharmacokinetics of oral D-serine, plasma D-serine levels were measured in both wild-type mice and transgenic mice lacking DAAO. While D-serine levels were rapidly diminished in wild-type mice ($t_{1/2} = 1.2$ hr), sustained drug levels over the course of 4 h ($t_{1/2} > 10$ hr) were observed in mice lacking DAAO. Co-administration of D-serine with 6-chlorobenzo[d]isoxazol-3-ol (CBIO), a small molecule DAAO inhibitor, in wild-type mice resulted in the enhancement of plasma D-serine levels, though CBIO appears to have only temporary effects to sustain plasma D-serine levels due to glucuronidation of the key hydroxyl group. These findings highlight the predominant role of DAAO in clearance of D-serine from the systemic circulation. Thus, a potent DAAO inhibitor with a longer half-life should be capable of maintaining high plasma D-serine levels over a sustained period of time and may have therapeutic implication for the treatment of schizophrenia.

DMD#46482

INTRODUCTION

Cumulative evidence suggests that allosteric activation of NMDA receptors via the glycine modulatory site may have therapeutic implication for the treatment of schizophrenia (Labrie and Roder, 2010). Both glycine and D-serine, two known endogenous glycine modulatory site agonists, have been shown to ameliorate persistent negative and cognitive symptoms of the disorder (Javitt, 2010), where existing antipsychotics have failed to show significant efficacy. D-Serine is particularly promising because (i) D-serine is more permeable than glycine to the blood-brain barrier (Oldendorf, 1971) and exhibits a long half-life in cortex upon peripheral administration (Hashimoto and Chiba, 2004), (ii) D-serine is more potent than glycine at activating the glycine modulatory site of the NMDA receptors (Matsui et al., 1995), and (iii) there is no known signal transduction site modulated by D-serine other than the glycine modulatory site, minimizing the risk of off-target toxicity.

Clinical development of D-serine, however, could be hampered by the high doses of D-serine (120 mg/kg) required for the optimal treatment of schizophrenia (Kantrowitz et al., 2010). Furthermore, high doses of D-serine have been reported to cause selective necrosis to the pars recta region of the renal proximal tubules in the rat (Ganote et al., 1974). Moreover, one patient receiving high dose of D-serine (120 mg/kg) showed a nephrotoxic-like pattern in an open label clinical trial (Kantrowitz et al., 2010). Studies using a D-amino acid oxidase (DAAO) inhibitor (Williams and Lock, 2005) and rats lacking DAAO activity (Maekawa et al., 2005) suggest that the mechanism of D-serine-induced nephrotoxicity is associated with oxidative stress caused by hydrogen peroxide, a by-product of DAAO-mediated metabolism of D-serine in the kidneys (Figure 1). DAAO (EC 1.4.3.3) is a flavoenzyme that catalyzes the oxidative deamination of D-

DMD#46482

amino acids including D-serine and produces the corresponding α -keto acids, ammonia, and hydrogen peroxide (Dixon and Kleppe, 1965a; Dixon and Kleppe, 1965b).

In mammals, DAAO is present in kidneys, liver, and brain. Interestingly, two recent independent studies demonstrated that DAAO expression and activity are elevated in schizophrenia (Burnet et al., 2008; Madeira et al., 2008). Since the highest DAAO activity is found in the kidneys (Curti et al., 1992), a substantial amount of orally administered D-serine is metabolized there. This contributes to D-serine's rapid clearance and consequently the high dose required for efficacy. These findings suggest that inhibition of DAAO would exert dual beneficial effects on D-serine therapy, by (i) enhancement of D-serine exposure and (ii) suppression of hydrogen peroxide generation in the kidneys. Thus, DAAO inhibitors might address the issues associated with clinical use of D-serine and salvage the most clinically efficacious glycine modulatory site agonist. 6-Chlorobenzo[d]isoxazol-3-ol (CBIO) is a potent competitive inhibitor of DAAO with a K_i value of 100 nM for porcine DAAO (Ferraris et al., 2008). While its toxicity profile has not been fully established, CBIO has been tested in both mice and rats as treatment for pain with no apparent toxicity (Gong et al., 2011; Lu et al., 2011). Oral co-administration of CBIO with D-serine enhanced oral bioavailability of D-serine and its levels in prefrontal cortex (Ferraris et al., 2008). Subsequent studies demonstrated that oral co-administration of CBIO with D-serine normalized prepulse inhibition (PPI) deficits induced by MK801 in a preclinical model of schizophrenia at all prepulse intensities to a degree similar to that of 10-fold higher dose of D-serine alone (Hashimoto et al., 2009).

Little is understood, however, as to what extent DAAO-mediated metabolism is involved in the overall clearance of D-serine in vivo. Such information should provide insights into the extent of the benefits provided by DAAO inhibitors as pharmac-enhancers for orally dosed D-serine. In

DMD#46482

the present study, we compared plasma D-serine levels in mice with or without DAAO activity following oral administration of D-serine. The primary objective of this study is to identify, if any, other D-serine clearance pathways and assess the degree of pharmaco-enhancement achieved by DAAO inhibition on oral D-serine pharmacokinetics. In addition, the ability of CBIO to enhance D-serine pharmacokinetics was evaluated in the light of its in vitro metabolic stability in plasma and liver microsomes.

MATERIALS AND METHODS

Chemicals

CBIO was obtained from Maybridge (Cornwall, UK). Boric acid, *o*-phthaldialdehyde (OPA), sodium acetate and all amino acids except D-serine and D-alanine were obtained from Sigma (St. Louis, MO). D-Serine and D-alanine were obtained from Bachem Bioscience (King of Prussia, PA).). Liver microsomes from mouse and human, the NADPH regenerating system and UGT reaction mix were purchased from BD Biosciences (San Jose, CA). All solvents (HPLC grade) and *N*-*tert*-butyloxycarbonyl-L-cysteine (Boc-L-Cys) were purchased from EMD Biosciences (San Diego, CA) and Novabiochem (a subsidiary of EMD Biosciences, L  ufelfingen, Switzerland), respectively. Glacial acetic acid, sodium hydroxide and ultrapure water were procured from J. T. Baker (Phillipsburg, NJ).

Animals.

All mice were maintained in accordance with the Institutional Animal Care and Use Committee (IACUC) at Johns Hopkins University School of Medicine. To generate the DAO^{null} targeting vector, recombineering (Liu et al., 2003) was used to replace 1957 bp encompassing exons 7 and 8 of *Dao* ([ENSMUSG00000042096](#)) with a neomycin phosphotransferase cassette in a C57BL/6

DMD#46482

BAC (RP23-450E21, Invitrogen). The deletion removes the glycine that is mutated in the naturally-occurring DAAO-deficient (ddY) mice and the tyrosine that is thought to be catalytically important. Splicing of exon 6 to exon 9 results in a reading frame shift leading to a truncated protein with no homology past exon 6. The mutant allele was introduced into 129SvEv mouse embryonic stem cells (TG-ES01-01 ESM07, Eurogentec) using standard homologous recombination techniques (Joyner, 2000). Quantitative PCR of *Dao* gene copy number was used to identify targeted clones (DAOF: 5'-CCCATGATCCTAGCCTTGGTATC-3'; DAOR: 5'-CCCCTTGTATGACCTTAGGTCAGT-3'; DAO probe: 5'-AACTCTCCGTACATCATCCCAGGGTAAACTCC-3'; PPIAF: 5'-GCCAGGGTGGTGACTTTACAC-3'; PPIAR: 5'-GACAAGATGCCAGGACCTGTATG-3'; PPIA probe: 5'-TGGCGGCAGGTCCATCTACGG-3'). Fluorescent in situ hybridization using the wild-type *Dao* containing BAC as a probe were used to confirm targeting and the single integration of the targeting vector. Male chimeric mice were generated by injection of the targeted ES cells into C57Bl/6J blastocysts (Nagy et al., 2003). Chimeric mice were bred with 129SvEv mice to produce F1 heterozygotes. Germline transmission was confirmed by PCR analysis (G3: 5'-CAGGGCAAAGGGACTGAATA-3'; G4: 5'-CACTCCACCACCATCGATTA-3'; dNEO2: 5'-ACATAGCGTTGGCTACCCGTGATA-3'). F1 heterozygous males and females were mated to produce F2 wild-type, heterozygous and homozygous null mutant animals. The colony was maintained on a background of 129SvEv under specific pathogen free conditions with unrestricted access to food and water. Mice for experiments were obtained by heterozygous × heterozygous matings. Genotyping was done by Transnetyx, Inc. (Cordova, TN) using automated real time PCR. Preliminary behavioral characterization of the DAAO KO mice revealed only one significant difference compared to the wild-type, decreased center path in the

DMD#46482

open field test, indicating increased anxiety. However, this was not corroborated by the outcome of the elevated plus maze test (P. A. Seymour, personal communications).

Animal study.

Mice (n = 3-6 for each time point for each group except for wild-type mice treated with D-serine and CBIO wherein n = 2 for T = 120 and 240 min) were orally dosed (10 mL/kg) with either D-serine (30 mg/kg) alone or D-serine (30 mg/kg) in combination with CBIO (30 mg/kg in 10% DMSO: 0.9% saline (w/v)). The mice were then euthanized at 30, 60, 120 and 240 minutes post dosing. Approximately 1 mL of whole blood was collected from each animal by cardiac puncture into heparinized microcentrifuge tubes, capped, gently inverted a few times and stored on wet ice until centrifugation (10 min at 800 g, 4°C). Thereafter, the top layer of each tube (~ 400 µl plasma) was aspirated via transfer pipette, dispensed into a clean non-heparinized microcentrifuge tube and stored at -80°C until subsequent analyses. Additionally, mouse brains were quickly excised, the cerebellum and the frontal cortex isolated, weighed and stored at -80°C until the time of analyses.

Bioanalysis of D-serine

Methanol was used to extract amino acids (AA) from both plasma and brain samples. Plasma samples were mixed with methanol (20 × volumes, v/v), vortexed briefly and allowed to stand at room temperature for 2 min. AA extraction procedures from brain tissues were adapted from previously published methods (Morikawa et al., 2001). Brain tissues were homogenized and sonicated in 20 × volumes (w/v) of methanol. Proteins in both matrices were removed upon centrifugation at 50,000 g for 15 min at 4°C. Aliquots (plasma - 100 µl, brain - 20 µl) of the supernatant were evaporated to dryness using a vacuum lyophilizer operated at 30°C.

DMD#46482

Subsequently, the residues were reconstituted in ultrapure water (50 μ l) and prepped for AA derivatization.

Amino acid derivatization was carried out based on the previously reported methods (Hashimoto et al., 1992). Sodium-borate buffer was made using 0.4 M boric acid and pH-adjusted to 9.0 with sodium hydroxide. On the day of the analysis, 10 mg each of OPA and Boc-L-Cys were dissolved in 1 ml of methanol and 3.5 ml of borate buffer added to the Boc-L-Cys-OPA mixture (derivatization reagent). A 45- μ l volume of derivatization reagent was then added to a vial containing 5 μ l of either the AA standard or the sample. After 2 minutes of derivatization at room temperature, an aliquot (10 μ l) of the derivatized material was introduced into the HPLC system described below.

The HPLC system consisted of a degasser (DGU-14A, Shimadzu, Columbia, MD), pumps (LC-10ADVP, Shimadzu, Columbia, MD), an autoinjector (SIL-10ADVP, Shimadzu, Columbia, MD), a column oven (CTO-10ACVP, Shimadzu, Columbia, MD), and a fluorescence detector (RF-10AXL, Shimadzu, Columbia, MD). Mobile phase A was made up of 0.1 M sodium acetate buffer (pH 6.0), acetonitrile and tetrahydrofuran (90:7:3, v/v) and mobile phase B was made up of 0.1 M sodium acetate buffer (pH 6.0), acetonitrile and tetrahydrofuran (50:47:3, v/v). Amino acids were resolved using a C₁₈ Nova-Pak analytical column (3.9 \times 300 mm, 4 μ m; Waters, Milford, MA) maintained at 30°C, with a linear gradient from mobile phase A to B in 120 min, and operated at a constant flow-rate of 0.8 ml/min. Fluorescence detection was carried out at 443 nm with excitation at 344 nm. Data were processed using a system controller from Shimadzu (SCL-10AVP, Columbia, MD).

PK analysis

DMD#46482

Plasma concentrations of D-Serine were analyzed using non-compartmental methods as implemented in the computer software program WinNonlin version 5.2 (Pharsight, Inc., Mountain View, CA, USA). The maximum plasma concentration (C_{\max}) and time to C_{\max} (T_{\max}) were the observed values. Half-life ($t_{1/2}$) was calculated as 0.693 divided by λ_z (the elimination rate constant) using a uniform weighting. The area under the plasma concentration time curve (AUC) value was calculated to the last quantifiable sample (AUC_{last}) by using the log-linear trapezoidal rule. The AUC values, wherever applicable, were extrapolated to infinity (AUC_{∞}), by dividing the last quantifiable concentration by the terminal disposition rate constant (λ_z), which was determined from the slope of the terminal phase of the concentration-time profiles. The percent extrapolated was determined using the equation $AUC_{0-\infty} = AUC_{0-t} + C_{\text{last}}/\lambda_z$, where C_{last} was the final quantifiable concentration. Acceptance criteria for the model were percent AUC extrapolated $\leq 25\%$ or the r^2 on the $\lambda_z \geq 0.9$. The method of Bailer was used to estimate the variance of AUC_{last} based on the variance of the mean concentration at each time point (Bailer, 1988). To determine whether there was a significant difference between exposure as expressed by AUCs of D-serine in three cohorts i.e. wild type, wild type with DAAO inhibitor, and the DAAO-KO (DAAO knock out), a pairwise comparison was performed using a Z test (Yuan, 1993). The a priori level of significance was $p < 0.05$.

Metabolic stability of CBIO in plasma and liver microsomes

The metabolic stability of CBIO was evaluated using mouse and human plasma and liver microsomes. For plasma stability, 5 μM compound was spiked in plasma and reaction (150 μL) was stopped at 0, 15, 30 and 60 min by addition of acetonitrile (300 μL) spiked with internal standard (0.1 mM phenyl acetic acid).

DMD#46482

Phase I and phase II metabolic stability assay for CBIO was conducted in mouse and human liver microsomes. For phase I metabolism, the reaction was carried out with 100 mM potassium phosphate buffer, pH 7.4, in the presence of NADPH regenerating system (1.3 mM NADPH, 3.3 mM glucose 6-phosphate, 3.3 mM MgCl₂, 0.4 U/mL glucose-6-phosphate dehydrogenase, 50 μM sodium citrate). Reactions in triplicate were initiated by addition of the liver microsomes (mouse or human) to the incubation mixture (compound final concentration was 10 μM; 0.5 mg/mL microsomes). For phase II glucuronidation reaction, CBIO was added to TRIS-HCl buffer (50 mM, pH 7.5) with microsomes (0.5 mg/mL), along with MgCl₂ (8 mM), and alamethicin (25 μg/mL) and pre-incubated at 37°C. The reaction was initiated (in triplicate) with UDPGA at a final concentration of 2 mM. Controls in the absence NADPH and UDPGA were carried for both phase I and phase II metabolism respectively, to determine the specific cofactor free degradation. At predetermined times (0, 15, 30 and 60 min) aliquots of the mixture were removed and the reaction quenched by addition of two times the volume of ice cold acetonitrile spiked with the internal standard. Compound disappearance was monitored over time using a liquid chromatography and tandem mass spectrometry (LC/MS/MS) method.

Separation of the analyte from potentially interfering material was achieved using a Waters X-Terra™ (50 × 2.1mm id) column packed with 3.5μm C₁₈ stationary phase protected by a guard column packed with 3.5 μm RP₁₈ material. The mobile phase used composed of acetonitrile/water (70:30, v/v) containing 0.1% formic acid delivered isocratically at a flow rate of 0.2 mL/minute for a total run time of 5 minutes. The retention time for CBIO and the IS was 1.2 ± 0.3 minute. The column effluent was monitored using a Micromass Quattro triple-quadrupole mass spectrometric detector, equipped with waters HPLC, in the negative ionization

DMD#46482

mode. The spectrometer was programmed to allow the [M-1] mass transitions of CBIO at m/z 168>132 and m/z 135>90.8 for the internal standard.

RESULTS

Plasma Pharmacokinetics of D-serine in Wild-type and DAAO knockout Mice

D-Serine concentration-time profiles in plasma following oral administration of D-serine are shown in Figure 2 and the pharmacokinetic parameters are summarized in Table 1. The basal plasma levels of D-serine were 3.5 and 10.8 μ M in wild-type and DAAO KO mice, respectively. Similar D-serine levels were reported for serum samples from ddY mice with and without DAAO activity (Morikawa et al., 2001). After oral administration of D-serine (30 mg/kg), D-serine was rapidly absorbed with T_{max} occurring within 0.7 ± 0.3 hr in wild type mice. When D-serine was given in conjunction with CBIO, a potent DAAO inhibitor, T_{max} shifted to 1.0 hr with an increased C_{max} and AUC. In DAAO KO mice, T_{max} was further delayed to 2.7 ± 0.9 hr and additional increases were observed in C_{max} and AUC values. The terminal half lives of D-serine in wild type mice (without or with CBIO) were 1.2 ± 0.1 hr and approximately 1.5 hr, respectively. In contrast, DAAO KO mice exhibited the highest C_{max} value among the three treatment groups with little sign of D-serine elimination during the period of the measurement (up to 4.0 hr). The half life of D-serine could not be estimated accurately due to insufficient data available for the DAAO knockout mice and lack of terminal elimination phase as depicted in Figure 2.

Systemic exposure of D-serine in the three cohorts was evaluated using the AUC_{last} values. The highest plasma exposure to D-serine was achieved in DAAO KO mice with AUC_{0-last} value of 95768 ± 6234 hr*ng/mL followed by wild type mice co-administered with CBIO (65778 hr*ng/mL) and wild type mice treated with D-serine alone (40648 ± 6602 hr*ng/mL) without

DMD#46482

CBIO. The DAAO-KO mice depicted about 2.4 fold higher exposures as compared to wild type mice and 1.4 fold higher exposures compared to wild type mice treated with CBIO, all of which were statistically significant differences ($P<0.05$). Additionally, wild type mice co-administered with CBIO exhibited 1.6 fold higher systemic exposures compared to wild type mice treated with D-serine alone ($P<0.05$). AUC extrapolated from AUC last is greater than 25% for DAAO KO mice and hence the $AUC_{0-\infty}$ value is not reported.

***in vitro* Metabolic stability of CBIO**

In mouse and human plasma CBIO was found to be stable over a period of 60 min (data not shown). In mouse and human liver microsomal incubations in the presence or absence of NADPH (Figure 3A and B), no metabolism of CBIO was observed (~100% remaining at the end of a 60 min incubation), suggesting that CBIO is not a substrate of cytochrome P450 enzymes. In microsomes fortified with UDPGA, however, CBIO was rapidly metabolized with 10% (mouse) and 22% (human) of the parent compound remaining after 60 min of incubation (Figure 3A and B). Subsequently, possible formation of CBIO-glucuronide (exact mass: 345.03) was examined in the microsome mixtures (Figure 4). A new peak with a distinct mass of m/z 344.02 (M-1) was detected by LC/MS analysis in both mouse and human microsomes fortified with UDPGA (data not shown).

Brain levels of D-serine

Levels of D-serine were measured in two regions of the brain: the cerebellum and the cortex, of wild-type and DAAO KO mice. For the D-serine treated groups, tissues were taken at 1 hr post oral administration of D-serine. As shown in Figure 5A, negligible basal levels of D-serine were detected in the cerebellum of wild-type mice. D-serine levels did not increase significantly in the

DMD#46482

cerebellum even in D-serine (no CBIO) treated wild-type mice 60 min after oral administration. Substantially higher basal quantities of D-serine were found in the cerebellum of DAAO KO mice (Figure 5B). D-serine levels were even higher in DAAO KO mice treated with D-serine though the difference was not statistically significant. In the cortex, the basal D-serine levels were nearly identical between wild-type and DAAO KO mice. Although both wild-type and DAAO KO mice showed higher levels of D-serine after D-serine treatment, neither of them achieved statistically significant increases in D-serine.

DISCUSSION

The present study examined the significance of DAAO-mediated metabolism (Figure 1) in the pharmacokinetics of orally administered D-serine. Plasma levels of D-serine prior to D-serine treatment were 3.5 and 10.8 μM in wild-type and DAAO KO mice, respectively. While genetic deletion of DAAO appears to result in increased plasma levels of D-serine, the origin of plasma D-serine is not well understood and could be attributed to either or both diet (Friedman, 1999) and that formed in the brain by serine racemase (Ohide et al., 2011). When D-serine was given to wild-type 129/SvEv mice, D-serine was immediately absorbed followed by rapid clearance. Co-administration of CBIO, a potent DAAO inhibitor, resulted in delayed T_{max} , longer half-life, and increased AUC. Since DAAO is highly expressed in kidneys of rodents, the enhanced plasma D-serine exposure can be attributed to the diminished DAAO-mediated metabolism of D-serine caused by CBIO. Interestingly, the extent of the increase in plasma D-serine levels was even more profound in DAAO KO mice treated with D-serine alone. Plasma D-serine levels increased slowly over a period of 2 hr with a C_{max} value of 27,264 ng/mL. Strikingly, the high levels of D-serine were sustained during the period of the measurement with little indication of plasma clearance. These findings clearly demonstrate that DAAO plays a predominant role in the overall

DMD#46482

plasma clearance of D-serine. While sufficient data are not available to estimate $AUC_{0-\infty}$ of D-serine in DAAO KO mice, extrapolation of the given data clearly suggests that the enhancement of D-serine plasma exposures will be much greater than the 2-fold AUC_{last} increase seen over the first two hours. The much lower clearance value for D-serine in DAAO KO mice suggests that filtered D-serine is being reabsorbed from the renal proximal tubule where DAAO is highly expressed (Koibuchi et al., 1995). Similar reabsorption pathway has been proposed for rats (Silbernagl et al., 1999) and it has been reported that the urinary recovery of orally administered D-serine in rats is only 1.2% (Huang et al., 1998), indicating that D-serine exhibits similar renal pharmacokinetics in mouse and rat kidneys. Thus, it remains unclear why D-serine is nephrotoxic only in rats despite the presence of DAAO in the proximal tubule of both rats (Chan et al., 1979; Le Hir and Dubach, 1981; Usuda et al., 1986) and mice (Koibuchi et al., 1995). The urinary basal levels of D-serine in mice lacking DAAO activity was reported to be significantly higher than those of wild-type mice (Miyoshi et al., 2009). While these findings appear to suggest a high degree of renal excretion of D-serine, given the low clearance of D-serine in DAAO KO mice, it is more likely a result of shift in steady state concentrations of basal D-serine in serum and kidney due to the loss of DAAO activity.

In contrast to the marked increase in plasma D-serine half-life in DAAO KO mice, there was little difference in C_{max} values between wild-type and DAAO KO mice. This can be attributed to the significantly lower degree of DAAO activity in the liver compared to that of the kidney in rodents (Burch et al., 1958; Nagata et al., 1988). It is worth noting that humans express DAAO in both the liver and kidney (Holme and Goldberg, 1982) and that inhibition of DAAO in human is expected to increase not only plasma half-life as seen in mice but also C_{max} of oral D-serine by suppressing DAAO-mediated first pass metabolism in the liver.

DMD#46482

The pharmaco-enhancing effect of CBIO on D-serine was not nearly as drastic as that of genetic DAAO deletion, particularly at the later time points. This is presumably due to the rapid clearance of CBIO from the circulation. In vivo pharmacokinetic profile of CBIO was recently reported for rodent species (Lange et al., 2011). As expected from previous studies in which CBIO demonstrated in vivo pharmaco-enhancing effect on D-serine by oral administration (Ferraris et al., 2008), CBIO was reported to be orally available in mice ($F = 29\%$). While plasma half-life in mice was not reported, terminal elimination half-life in the brain was determined to be 1 hr, likely suggesting similar short half-life of CBIO in plasma. This explains CBIO's inability to maintain high levels of D-serine plasma levels for a sustained period of time. As expected, CBIO had no effects on plasma levels of co-administered D-serine in DAAO KO mice (data not shown), confirming that CBIO's ability to enhance plasma D-serine levels is predominantly associated with its inhibition of DAAO.

In an attempt to elucidate the mechanism by which CBIO is cleared from the circulation system, in vitro metabolic stability was measured in plasma and liver microsomes from mouse and human. CBIO showed high stability in mouse and human plasma (>95% remaining after 2 hr incubation; data not shown). In liver microsomes from mouse (Figure 3A) and human (Figure 3B), no significant loss of CBIO was detected in the absence or presence of NADPH. The presence of the chloro group appears to make CBIO resistant to CYP450-mediated oxidation. In liver microsomes containing UDPGA, however, CBIO was metabolized substantially with only 10% (mouse) and 21% (human) of the parent compound remaining at the end of a 60 min incubation (Figure 3A and B). The results clearly show that CBIO undergoes phase II glucuronidation in liver. Since CBIO possesses only a single moiety possibly subject to glucuronidation (3-hydroxyl group), the most likely metabolite is CBIO-3-glucuronide (Figure 4).

DMD#46482

Indeed, we have detected a new peak corresponding to a mass of CBIO-3-glucuronide with an increasing intensity over time in LC/MS (data not shown).

Unfortunately, the 3-hydroxyl group is essential for the high affinity binding of CBIO to the DAAO active site (Ferraris et al., 2008). Therefore, removal or masking of this functional group is expected to result in a complete loss of inhibitory potency even though it may circumvent glucuronidation. Further structural optimization of the benzo[d]isoxazol-3-ol scaffold requires careful modulation of steric and electronic environment surrounding the hydroxyl group in a way that does not compromise the inhibitory potency while minimizing the degree of glucuronidation. It is also important to point out that glucuronidation is only one of many possible metabolic reactions that could take place at this site. Other phase II metabolic reactions, particularly methylation and sulfation, are also common at a free OH-group and much attention needs to be paid when structural optimization of CBIO is conducted for the enhancement of metabolic stability. If such improvements do not result in prolonged plasma half-life, the possibility of metabolism by other organs needs to be explored to determine the optimal strategy for further structural optimization.

In the brain, DAAO activity is known to be highest in the cerebellum while relatively low levels of DAAO activity are detected in the cortex. Not surprisingly, D-serine levels in the brain are inversely correlated with the DAAO activity. As shown in Figure 5A, only negligible basal levels of D-serine were detected in the cerebellum of wild-type mice while substantially higher quantities of D-serine were found in the cortex. This is consistent with the previously reported findings in other strains of mice (Morikawa et al., 2001; Labrie et al., 2009).

Oral administration of D-serine in wild-type mice did not result in increased levels of D-serine in the cerebellum 1 hr after administration, presumably due to rapid metabolism by DAAO. A

DMD#46482

slight increase in D-serine was observed in the cortex of D-serine-treated wild-type mice although the difference was not statistically significant. It was previously reported that, in rats, statistically significant increase in the cortical D-serine levels are only achieved when 320 mg/kg or higher doses of D-serine was given by subcutaneous injection (Smith et al., 2009). In contrast, CSF D-serine levels showed statistically significant increases at 160 mg/kg and achieved nearly 80-fold increases over the basal level at the highest dose tested (1280 mg/kg). These findings suggest that endogenous D-serine in the brain is mainly confined to the intracellular compartment probably by the action of alanine-serine-cysteine (Asc-1) transporter. Added D-serine has much less impact on its levels in the homogenized brain tissues containing substantial quantity of endogenous cytosolic D-serine. Meanwhile, extracellular D-serine concentrations such as those detected in CSF are more drastically boosted by the added D-serine due to the lower basal level of endogenous D-serine.

As shown in Figure 5B, basal D-serine levels in the cerebellum of DAAO KO mice were substantially higher than those of wild-type mice. Previous studies using other strains of mice lacking DAAO activity also showed similar results (Morikawa et al., 2001; Labrie et al., 2009). In the cortex, the basal D-serine levels were nearly identical between wild-type and DAAO KO mice. The results are in good agreement with the negligible DAAO activity detected in the cortex of wild-type rodents.

Although both cerebellum and cortex of DAAO KO mice showed higher levels of D-serine at 1 hr after D-serine treatment, neither of them achieved statistically significant increase. We have previously shown by in vivo microdialysis in the mouse frontal cortex that oral D-serine (30 mg/kg) can increase cortical D-serine levels of wild-type ddY mice by up to 4-fold when co-administered with CBIO (Hashimoto et al., 2009). The higher relative increase seen by

DMD#46482

microdialysis indicates that the impact of added D-serine is more robust in the extracellular compartment of the brain, where the therapeutic target (NMDA receptor) for D-serine is located.

CONCLUSIONS

Oral D-serine represents one of the most promising therapeutic agents to treat negative symptoms and cognitive deficits of schizophrenia, which have been poorly addressed by the existing antipsychotics. D-Serine therapy, however, remains impractical for clinical application because of the high dose required for the robust efficacy and the risk of nephrotoxicity. Co-administration of D-serine with a small molecule DAAO inhibitor may address both of these issues and lead to the development of a clinically viable therapeutic approach based on D-serine. Furthermore, the pharmaco-enhancing effects of DAAO inhibition could be more profound in human since substantial amount of DAAO is present in human liver and first-pass metabolism of D-serine can be blocked by DAAO inhibitors. DAAO in human liver, however, may play a crucial role in metabolism and/or detoxification of endogenous/exogenous substances. Therefore, it is critical to assess the potential consequences of liver DAAO inhibition in species known to express DAAO in not only kidney but also in liver. It is also worth noting that a cross species comparison of urinary basal D-serine concentrations revealed that much higher levels of D-serine were found in humans compared to rats and mice (Huang et al., 1998; Miyoshi et al., 2009). This could be at least partially due to higher rates of renal D-serine excretion in human. DAAO inhibitors, in theory, are incapable of minimizing the loss of D-serine by renal excretion. Hence, DAAO inhibitors may be less effective in enhancing plasma D-serine levels in human, if the renal excretion plays a larger role in human D-serine pharmacokinetics.

DMD#46482

While CBIO has served as a useful pharmacological probe to elucidate the pharmaco-enhancing effects of DAAO inhibition on D-serine (Ferraris et al., 2008; Hashimoto et al., 2009), our studies revealed that a further improvement of DAAO inhibitors can be made by indentifying CBIO analogs resistant to glucuronidation. Given the favorable oral D-serine pharmacokinetics in DAAO knockout mice, a potent DAAO inhibitor with a longer half-life should be capable of maintaining high plasma D-serine levels over a sustained period of time and have therapeutic implication for the treatment of schizophrenia. In our studies, orally administered D-serine had little impact on brain tissue levels of D-serine in both wild-type and DAAO KO mice. It is conceivable that the added D-serine contributes only a minor portion of D-serine in brain tissues and that microdialysis studies are better suited to examine CNS distribution of orally given D-serine and its therapeutic effects.

DMD#46482

Authorship Contributions

Participated in research design: Rais, Thomas, Brandon, Rojas, Engle, Strick, Slusher, and Tsukamoto

Conducted experiments: Rais, Thomas, Wozniak, Wu, Jaaro-Peled, and Engle

Contributed new reagents or analytical tools: Sawa

Performed data analysis: Rais, Thomas, and Tsukamoto

Wrote or contributed to the writing of the manuscript: Rais, Thomas, Jaaro-Peled, Sawa, and Tsukamoto

DMD#46482

References

- Bailer AJ (1988) Testing for the equality of area under the curves when using destructive measurement techniques. *J Pharmacokinet Biopharm* **16**:303-309.
- Burch HB, Lowry OH, De Gubareff T and Lowry SR (1958) Flavin enzymes in liver and kidney of rats from birth to weaning. *J Cell Physiol* **52**:503-510.
- Burnet PW, Eastwood SL, Bristow GC, Godlewska BR, Sikka P, Walker M and Harrison PJ (2008) D-amino acid oxidase activity and expression are increased in schizophrenia. *Mol Psychiatry* **13**:658-660.
- Chan AW, Perry SG, Burch HB, Fagioli S, Alvey TR and Lowry OH (1979) Distribution of two aminotransferases and D-amino acid oxidase within the nephron of young and adult rats. *J Histochem Cytochem* **27**:751-755.
- Curti B, Ronchi S and Simonetta PM (1992) D- and L-Amino Acid Oxidases, in: *Chemistry and Biochemistry of Flavoenzyme* (Muller F ed), pp 69-94, CRC Press, Boca Raton, FL.
- Dixon M and Kleppe K (1965a) D-Amino Acid Oxidase I. Dissociation and Recombination of the Holoenzyme. *Biochim. Biophys. Acta* **96**:357-367.
- Dixon M and Kleppe K (1965b) D-Amino acid oxidase II. Specificity, competitive inhibition and reaction sequence. *Biochim. Biophys. Acta* **96**:368-382.
- Ferraris D, Duvall B, Ko YS, Thomas AG, Rojas C, Majer P, Hashimoto K and Tsukamoto T (2008) Synthesis and biological evaluation of D-amino acid oxidase inhibitors. *J Med Chem* **51**:3357-3359.
- Friedman M (1999) Chemistry, nutrition, and microbiology of D-amino acids. *J Agric Food Chem* **47**:3457-3479.

DMD#46482

Ganote CE, Peterson DR and Carone FA (1974) The nature of D-serine--induced nephrotoxicity.

Am J Pathol **77**:269-282.

Gong N, Gao ZY, Wang YC, Li XY, Huang JL, Hashimoto K and Wang YX (2011) A series of

D-amino acid oxidase inhibitors specifically prevents and reverses formalin-induced tonic pain in rats. *J Pharmacol Exp Ther* **336**:282-293.

Hashimoto A and Chiba Y (2004) Effect of systemic administration of D-serine on the levels of

D- and L-serine in several brain areas and periphery of rat. *Eur J Pharmacol* **495**:153-158.

Hashimoto A, Nishikawa T, Oka T, Takahashi K and Hayashi T (1992) Determination of free

amino acid enantiomers in rat brain and serum by high-performance liquid chromatography after derivatization with N-tert.-butyloxycarbonyl-L-cysteine and o-phthaldialdehyde. *J Chromatogr* **582**:41-48.

Hashimoto K, Fujita Y, Horio M, Kunitachi S, Iyo M, Ferraris D and Tsukamoto T (2009) Co-

administration of a D-amino acid oxidase inhibitor potentiates the efficacy of D-serine in attenuating prepulse inhibition deficits after administration of dizocilpine. *Biol Psychiatry* **65**:1103-1106.

Holme DJ and Goldberg DM (1982) Development of a fluorometric assay for amino-acid

oxidase activity and its application to the study of human tissues. *Biochem Med* **28**:51-61.

Huang Y, Nishikawa T, Satoh K, Iwata T, Fukushima T, Santa T, Homma H and Imai K (1998)

Urinary excretion of D-serine in human: comparison of different ages and species. *Biol. Pharm. Bull.* **21**:156-162.

Javitt DC (2010) Glutamatergic theories of schizophrenia. *Isr J Psychiatry Relat Sci* **47**:4-16.

Joyner AL (2000) *Gene targeting: a practical approach*. Oxford University Press, Oxford.

DMD#46482

- Kantrowitz JT, Malhotra AK, Cornblatt B, Silipo G, Balla A, Suckow RF, D'Souza C, Saksa J, Woods SW and Javitt DC (2010) High dose D-serine in the treatment of schizophrenia. *Schizophr Res* **121**:125-130.
- Koibuchi N, Konno R, Matsuzaki S, Ohtake H, Niwa A and Yamaoka S (1995) Localization of D-amino acid oxidase mRNA in the mouse kidney and the effect of testosterone treatment. *Histochem Cell Biol* **104**:349-355.
- Labrie V, Duffy S, Wang W, Barger SW, Baker GB and Roder JC (2009) Genetic inactivation of D-amino acid oxidase enhances extinction and reversal learning in mice. *Learn Mem* **16**:28-37.
- Labrie V and Roder JC (2010) The involvement of the NMDA receptor D-serine/glycine site in the pathophysiology and treatment of schizophrenia. *Neurosci Biobehav Rev* **34**:351-372.
- Lange JH, Venhorst J, van Dongen MJ, Frankena J, Bassissi F, de Bruin NM, Besten C, de Beer SB, Oostenbrink C, Markova N and Kruse CG (2011) Biophysical and physicochemical methods differentiate highly ligand-efficient human D-amino acid oxidase inhibitors. *Eur J Med Chem* **46**:4808-4819.
- Le Hir M and Dubach UC (1981) The activity pattern of two peroxisomal oxidases in the rat nephron. *FEBS Lett* **127**:250-252.
- Liu P, Jenkins NA and Copeland NG (2003) A highly efficient recombineering-based method for generating conditional knockout mutations. *Genome Res* **13**:476-484.
- Lu JM, Gong N, Wang YC and Wang YX (2011) D-Amino acid oxidase-mediated increase in spinal hydrogen peroxide is mainly responsible for formalin-induced tonic pain. *Br J Pharmacol* **165**:1941-1955.

DMD#46482

- Madeira C, Freitas ME, Vargas-Lopes C, Wolosker H and Panizzutti R (2008) Increased brain D-amino acid oxidase (DAAO) activity in schizophrenia. *Schizophr Res* **101**:76-83.
- Maekawa M, Okamura T, Kasai N, Hori Y, Summer KH and Konno R (2005) D-amino-acid oxidase is involved in D-serine-induced nephrotoxicity. *Chem Res Toxicol* **18**:1678-1682.
- Matsui T, Sekiguchi M, Hashimoto A, Tomita U, Nishikawa T and Wada K (1995) Functional comparison of D-serine and glycine in rodents: the effect on cloned NMDA receptors and the extracellular concentration. *J Neurochem* **65**:454-458.
- Miyoshi Y, Hamase K, Tojo Y, Mita M, Konno R and Zaitzu K (2009) Determination of D-serine and D-alanine in the tissues and physiological fluids of mice with various D-amino-acid oxidase activities using two-dimensional high-performance liquid chromatography with fluorescence detection. *J Chromatogr B Analyt Technol Biomed Life Sci* **877**:2506-2512.
- Morikawa A, Hamase K, Inoue T, Konno R, Niwa A and Zaitzu K (2001) Determination of free D-aspartic acid, D-serine and D-alanine in the brain of mutant mice lacking D-amino acid oxidase activity. *J Chromatogr B Biomed Sci Appl* **757**:119-125.
- Nagata Y, Shimojo T and Akino T (1988) D-amino acid oxidase in mouse liver—II. *Comp Biochem Physiol B* **91**:503-504.
- Nagy A, Gertsenstein M, Vintersten K and Behringer R (2003) *Manipulating the mouse embryo : a laboratory manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
- Ohide H, Miyoshi Y, Maruyama R, Hamase K and Konno R (2011) D-Amino acid metabolism in mammals: biosynthesis, degradation and analytical aspects of the metabolic study. *J Chromatogr B Analyt Technol Biomed Life Sci* **879**:3162-3168.

DMD#46482

Oldendorf WH (1971) Brain uptake of radiolabeled amino acids, amines, and hexoses after arterial injection. *Am J Physiol* **221**:1629-1639.

Silbernagl S, Volker K and Dantzler WH (1999) D-Serine is reabsorbed in rat renal pars recta. *Am J Physiol* **276**:F857-863.

Smith SM, Uslander JM, Yao L, Mullins CM, Surles NO, Huszar SL, McNaughton CH, Pascarella DM, Kandebo M, Hinchliffe RM, Sparey T, Brandon NJ, Jones B, Venkatraman S, Young MB, Sachs N, Jacobson MA and Hutson PH (2009) The behavioral and neurochemical effects of a novel D-amino acid oxidase inhibitor compound 8 [4H-thieno [3,2-b]pyrrole-5-carboxylic acid] and D-serine. *J Pharmacol Exp Ther* **328**:921-930.

Usuda N, Yokota S, Hashimoto T and Nagata T (1986) Immunocytochemical localization of D-amino acid oxidase in the central clear matrix of rat kidney peroxisomes. *J Histochem Cytochem* **34**:1709-1718.

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

Yuan J (1993) Estimation of variance for AUC in animal studies. *J Pharm Sci* **82**:761-763.

DMD#46482

Footnote

This work was in part supported by National Institutes of Health [R01MH091387] to T.T., and the Johns Hopkins Brain Science Institute NeuroTranslational Drug Discovery program.

DMD#46482

Figure Legends

Fig. 1. DAAO-mediated metabolism of D-serine. Small molecule DAAO inhibitors such as CBIO have the potential to provide a significant improvement to D-serine therapy by increasing D-serine bioavailability and minimizing the formation of hydrogen peroxide, a potential cause of nephrotoxicity.

Fig. 2. Plasma concentrations of D-serine as a function of time in wild-type and DAAO KO mice following oral administration of D-serine.

Fig. 3. Metabolic stability of CBIO in (A) mouse and (B) human liver microsomes.

Fig. 4. Putative metabolic pathway of CBIO in liver microsomes in the presence of UDPGA.

Fig. 5. D-Serine concentrations in the cerebellum and cortex of wild-type (A) and DAAO KO mice (B). For the D-serine treated groups, tissues were taken at 1 hr post oral administration.

DMD#46482

Table 1. Pharmacokinetic parameters of D-Serine administered P.O. to mice at 30mg/kg

Cohorts	Dose (mg/kg)	Tmax (hr)	Cmax (ng/mL)	T 1/2 (hr)	AUC last (ng*hr/mL)	AUC _{0-∞} (ng*hr/mL)
WT	30	0.7 ± 0.3	19609 ± 1338	1.2 ± 0.1	40648 ± 6602	46023 ± 8976
WT + CBIO ^a	30	1.0	24277	1.5	65778	80899
KO	30	2.7 ± 0.9	27791 ± 2019	>10 ^b	95768 ± 6234	- ^c

^aStandard deviation of the data cannot be determined due to insufficient number (n = 2) of data points at 120 and 240 min.

^bInsufficient data for accurate approximation.

^cAUC extrapolated from AUC last is greater than 25% and hence the AUC_{0-∞} value is not reported.

Figure 1

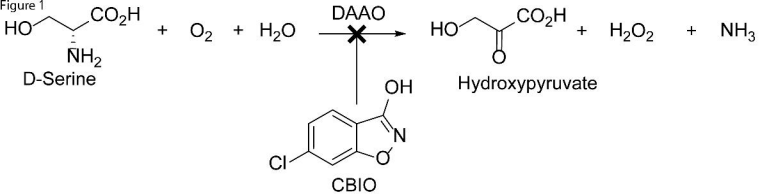


Figure 2

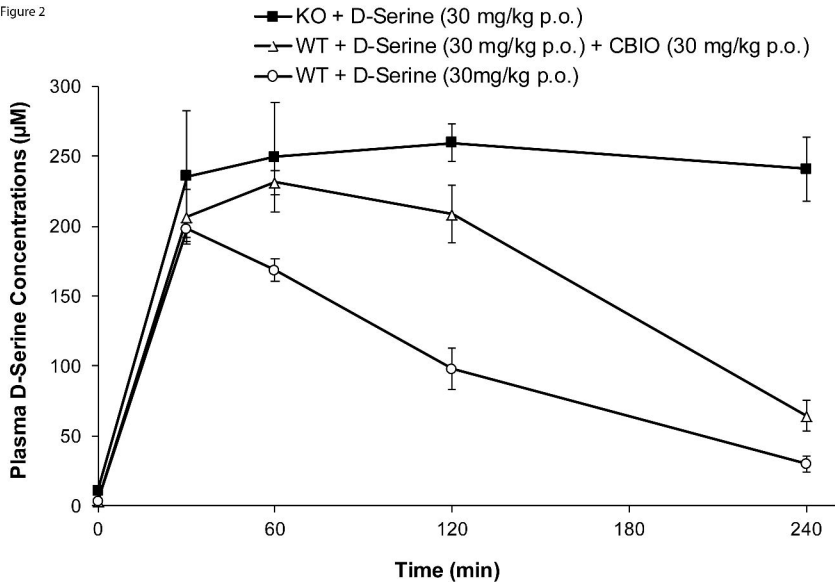


Figure 3

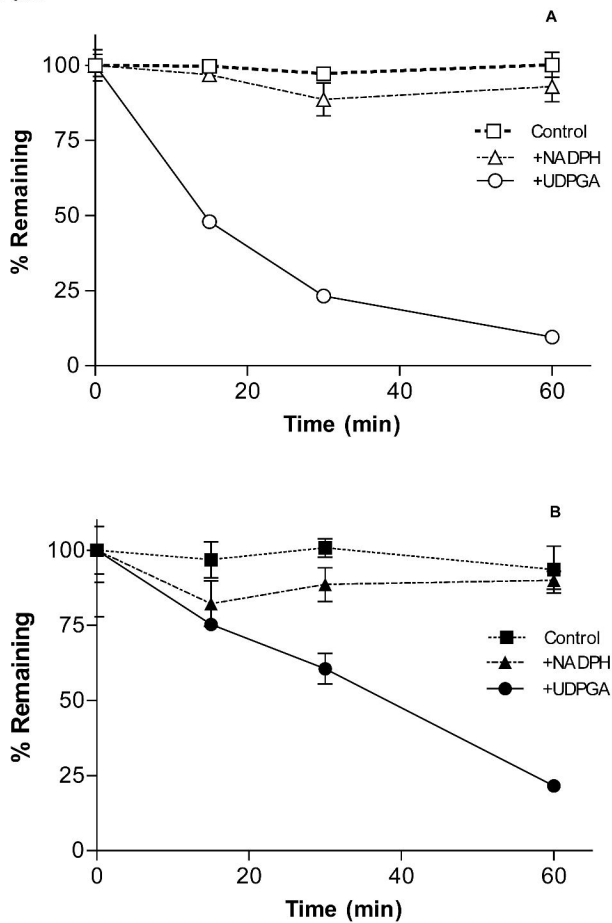
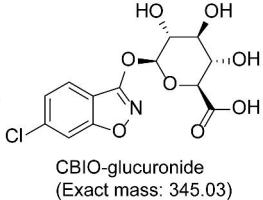
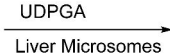
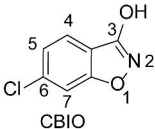
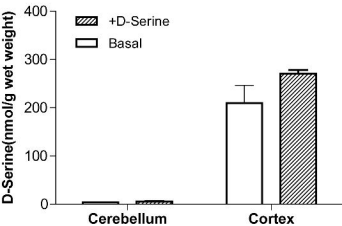


Figure 4



A

Figure 5



B

