Original Article

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Evaluation of human D-amino acid oxidase inhibition by antipsychotic drugs *in vitro*

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Summary **It is of importance to determine whether antipsychotic drugs currently prescribed for schizophrenia exert D-amino acid oxidase (DAO)-inhibitory effects. We first investigated whether human (h)DAO can metabolize D-kynurenine (D-KYN) to produce the fluorescent compound kynurenic acid (KYNA) by using high-performance liquid chromatography with mass spectrometry, and fluorescence spectrometry. After confirmation of KYNA production from D-KYN by hDAO, 8 first- and second-generation antipsychotic drugs, and 6 drugs often prescribed concomitantly, were assayed for hDAO-inhibitory effects by using *in vitro* fluorometric methods with D-KYN as the substrate. DAO inhibitors 3-methylpyrazole-5-carboxylic acid and 4*H*-thieno[3,2-b]pyrrole-5-carboxylic acid inhibited KYNA production in a dose-dependent manner. Similarly, the second-generation antipsychotics blonanserin and risperidone were found to possess relatively strong hDAO-inhibitory effects *in vitro* (5.29 ± 0.47 μM and 4.70 ± 0.17 μM, respectively). With regard to blonanserin and risperidone, DAO-inhibitory effects should be taken into consideration in the context of their *in vivo* pharmacotherapeutic efficacy.**

Keywords: Schizophrenia, D-kynurenine, D-serine, second-generation antipsychotic drug, risperidone, blonanserin

1. Introduction

Schizophrenia, a serious neuropsychiatric disease, affects 1% of the general population and consists of positive symptoms, negative symptoms, and cognitive impairments (*1*). Current therapeutics include first-generation (typical) antipsychotics (FGA) and second-generation (atypical) antipsychotics (SGA) (*2*), and selecting the most suitable antipsychotic is crucial for improving the quality of daily life for patients with schizophrenia.

Inhibition of D-amino acid oxidase (DAO) (EC 1.4.3.3.) (*3-5*) is considered to be an effective pharmacotherapy for schizophrenia. It has been reported that treatment of schizophrenia patients with D-serine, a co-agonist of the *N*-methyl-D-aspartate (NMDA) receptor, improved symptoms when administered in combination with antipsychotics (*6,7*). Therefore, much attention has recently been focused on the inhibition of DAO, which decomposes endogenous D-serine *in vivo*, and selective DAO inhibitors have been developed and investigated by pharmaceutical company research groups (*8-10*).

Despite this research focus on DAO inhibition, the primary pharmacological action of most institutionally prescribed antipsychotics is the blockade of dopamine D2 and serotonin receptors (*2*); however, these drugs might also inhibit DAO activity *in vivo*. Therefore, an evaluation of the DAO-inhibitory effects of antipsychotics presently prescribed in medical institutions is important because their efficacy might

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be partly due to increased endogenous D-serine levels. Recently, we reported a fluorescence assay method for the evaluation of pig kidney (pk) DAO inhibition by a drug using D-kynurenine (D-KYN) as a substrate. This assay has the advantage of simple and facile operation because it measures the fluorescence intensity of a fluorescent compound, kynurenic acid (KYNA), that is produced from D-KYN by DAO through a single enzymatic reaction (*11,12*). Thus, in the present study, this *in vitro* assay was applied to human DAO (hDAO) and was used to evaluate the DAO-inhibitory effects of currently prescribed antipsychotic drugs, as well as of concomitant drugs prescribed to schizophrenia patients.

2. Materials and Methods

2.1. *Materials*

Quetiapine fumarate (Seroquel® tablets) and aripiprazole (Abilify® tablets) were purchased from Astellas Pharma Inc. (Tokyo, Japan) and Otsuka Pharmaceutical Co., Ltd. (Tokyo, Japan), respectively. Other drugs were purchased from Sigma Co. Ltd. (St. Louis, MO, USA), Tokyo Chemical Industries Co. Ltd. (Tokyo, Japan), and Wako Pure Chemical Industries Co. Ltd. (Osaka, Japan). Recombinant hDAO was prepared according to previously described methods (*3, 13,14*), and the hDAO-inhibition assay was performed with D-KYN as a substrate, using methods similar to our previous paper (*12*).

2.2. *DAO assay with D-KYN as a substrate*

A detailed procedure for the measurement of the DAO-inhibitory effects of test drugs was described in our previous papers (*12*). Briefly, we mixed 20 μL of 0.1 mg/mL DAO in 0.4 M Tris buffer solution (pH 8.3), 50 μL of 200 μM FAD solution, 20 μL of 2.0 mg/mL BSA in H_2O, 370 μL of 0.4 M Tris buffer solution (pH 8.3), and 20 μL of tested drugs or compounds dissolved in DMSO, and incubated this mixture at 37°C for 20 min. Next, 7.0 mM D-KYN (20 μL) was added, and the mixture was incubated at 37°C for 60 min. In the case of determination of Km, varying concentrations (0.1, 0.2, 0.4, 0.8, 1.75 mM) of D-KYN (20 μL) were added in the presence or absence of the tested drugs, and incubated at 37°C for 60 min. The reaction mixture was dissolved in H_2O with 1,500 μL of 0.4 M Tris buffer solution (pH 8.3) and 50 μL of 300 mM $ZnSO_4$. The final solution was subjected to vortex mixing, and the fluorescence of the solution was measured by a HITACHI F-7000 fluorescence spectrometer (Hitachi Co. Ltd., Tokyo, Japan). The fluorescence intensity of each solution was measured at an excitation wavelength of 250 nm and emission wavelength of 394 nm. According to the following equation (1), the ΔF value was determined and used to calculate kinetic parameters (K_m) using the Lineweaver-Burk plot:

$$\Delta F = F - F_0 \quad (1)$$

where F and F_0 are fluorescence intensities of the sample and blank sample (a sample treated without D-KYN), respectively. For the inhibition curve, the final concentrations of the test compounds were plotted on the X-axis, and the ΔF value in the absence of the inhibitor was set as 100% on the Y-axis. The degree of inhibition for each test compound was expressed as a percentage according to the following equation (2):

$$\text{Remaining activity of hDAO (\%)} = \Delta F' / \Delta F \times 100 \quad (2)$$

where $\Delta F'$ is the ΔF value at each concentration of the inhibitor. IC_{50} values of the tested drugs were calculated using the following equation (3):

$$IC_{50} = 10\ [\log (A/B) \times (50-C)/(D-C) + \log B] \quad (3)$$

where A and B are the higher and lower concentrations near 50% inhibition, respectively, and C and D are the inhibition percentages at B and A, respectively.

2.3. *LC-MS analysis*

After enzymatic reaction with DAO for 60 min, the reactant was subjected to ultra-filtration with a Centrifree® (EMD Millipore Corporation, Billerica, MA, USA), without the addition of Tris-buffer and $ZnSO_4$. The ultra-filtration was carried out at 2,000 g for 15 min at room temperature. For a blank sample, 0.4 M Tris buffer (pH 8.3) was added to the mixture instead of DAO solution. The filtrate was vortex-mixed with the mobile phase, and 20 μL of the filtrate was injected into the LC-MS apparatus, comprising an Agilent 1200 series HPLC system (Agilent Technologies, Santa Clara, CA, U.S.A.) and a time-of-flight (TOF)-MS (JMS-T100LP AccuTOF LC-Plus) equipped with an electrospray ionization (ESI) source (JEOL Co. Ltd., Tokyo, Japan). The separation column was a TSKgel ODS-100V (150 mm × 2.0 mm; *i.d.*: 5 μm; Tosoh Corporation, Tokyo, Japan) equipped with a guard column, and the mobile phase was 10 mM HCO_2NH_4 in H_2O (pH 3.5)/MeOH (80:20). The mobile phase was constantly pumped at 0.20 mL/min, and the column temperature was maintained at 45°C. The conditions for ESI-MS detection were as follows: positive ion mode, needle voltage set at 2,000 V, and ring lens and orifice 1 and 2 voltages set at 10, 70, and 10 V, respectively. Nitrogen was used as the nebulizing and desolvation gas, and the pressure was maintained at 0.608 MPa. The desolvation chamber and orifice 1 temperatures were set to 250°C and 80°C, respectively. Data were obtained using Mass Center software, MS-56010MP (JEOL).

2.4. *Fluorescence spectra*

Excitation and emission spectra of the reactant solution were measured by a HITACHI F-7000 fluorescence spectrometer at 15, 30, 45, and 60 min after onset of the enzymatic reaction of D-KYN with hDAO. Each sample was prepared and treated in a similar manner as described in section 2.2.

3. Results and Discussion

3.1. *KYNA production by hDAO*

In a previous paper, we reported a fluorescence assay for the evaluation of pkDAO-inhibitory effects of a drug by utilizing an enzymatic reaction of D-KYN as a DAO substrate to produce KYNA (Figure 1) (*11,12*). The enzymatic product, KYNA, emits fluorescence at 398 nm (ex. 250 nm) in the presence of Zn (II), and the attenuation of fluorescence intensity originating from KYNA can be used for the evaluation of the DAO-inhibitory effect of a drug. However, results obtained using hDAO have not been reported. Some differences in physical properties and substrate specificity between pkDAO and hDAO have been reported by Molla *et al*. (*5*). In the present study, our previous assay was altered to use hDAO, and we evaluated the hDAO-inhibitory effects of currently prescribed antipsychotic drugs. Firstly, the enzymatic production of KYNA from D-KYN by hDAO was investigated using high-performance liquid chromatography with UV and mass spectrometric detection (LC-UV-MS), and measurement of the fluorescence spectrum.

Figure 2 shows enzymatic reactant chromatograms and MS spectra obtained by LC-UV-MS. In the chromatogram of enzymatic reactant (Figure 2c), a peak corresponding to the retention time of KYNA was found, and the mass spectrum was consistent with KYNA (Figures 2a and 2c).

Next, a time-dependent generation of fluorescence due to KYNA in the enzymatic reactant was investigated using fluorescence spectroscopy. Figure 3 shows the time-course of excitation and fluorescence spectra of the reaction mixture of D-KYN with hDAO. A fluorescence intensity at 398 nm, which is the wavelength for KYNA, increased with reaction time. The excitation and emission spectra of the product generated by the enzymatic reaction of hDAO with D-KYN were consistent with

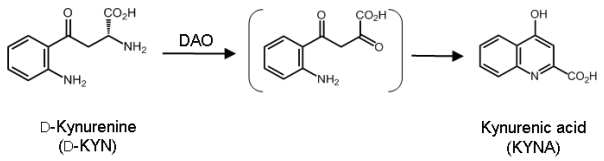

Figure 1. Transformation of D-kynurenine (D-KYN) to kynurenic acid (KYNA) by DAO.

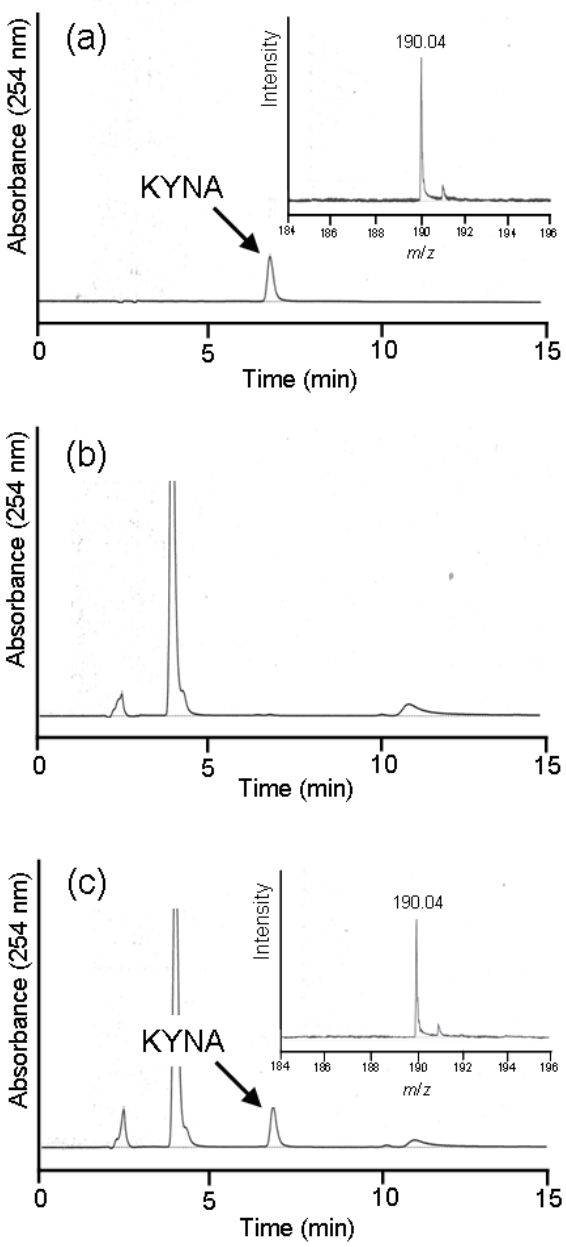

Figure 2. Chromatograms of standard KYNA (20 μM) (a), blank sample without hDAO (b), and enzymatic reactant of D-KYN with hDAO at 60 min (c). The insets in **(a)** and **(c)** are mass spectra of standard KYNA and the peak indicated by the arrow, respectively, obtained by LC-UV-MS.

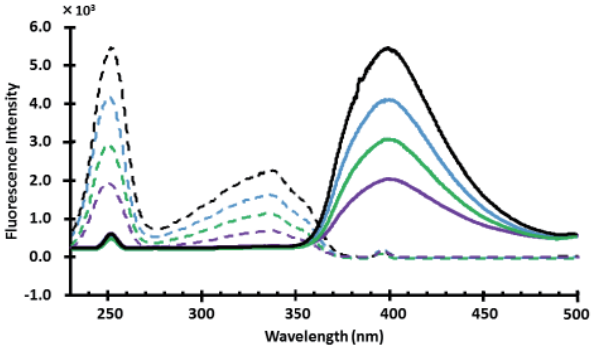

Figure 3. Excitation (dotted line) and emission spectra (solid line) of the reaction solution of D-KYN with hDAO at 15 (purple), 30 (green), 45 (blue), and 60 min (black), respectively.

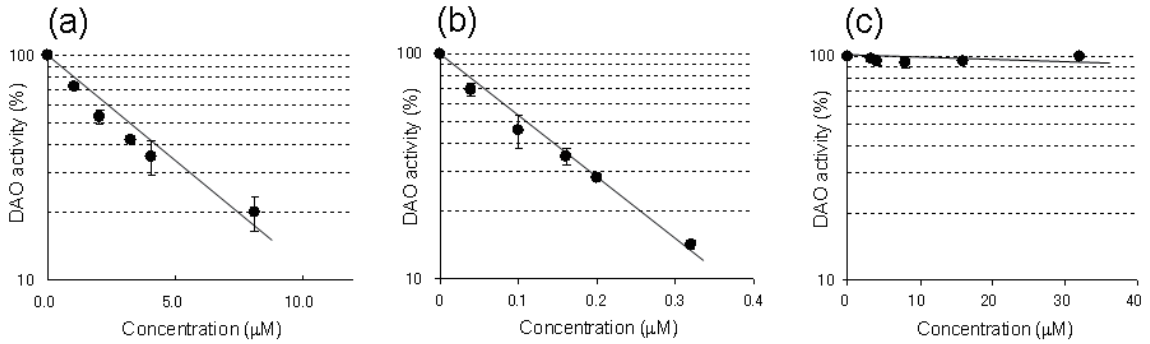

Figure 4. Dose-inhibition curves for 3-methylpyrazole-5-carboxyl acid (MPC) (circle), 4*H*-thieno[3,2-b]pyrrole-5-carboxylic acid (compound 8) (triangle), and 3-methylpyrazole-4-carboxyl acid (3,4-MPC) (square). Human D-amino acid oxidase (DAO) inhibition by MPC and compound 8 was clearly observed.

those of KYNA (*15*), and the increase in fluorescence intensity was dependent on reaction time. This result indicated that, like pkDAO, hDAO can metabolize D-KYN to KYNA (*11,12*).

Based on these results, it was concluded that KYNA was produced from D-KYN by hDAO. The K_m and V_{max} values of D-KYN determined using the Lineweaver-Burk plots were 1.78 mM and 5.88 μmol/(min·mg), respectively. Considering that the reported Km values of D-Ser and D-Ala were 41 and 3.1 mM, respectively (*5*), it may be considered that D-KYN has a moderate affinity to hDAO.

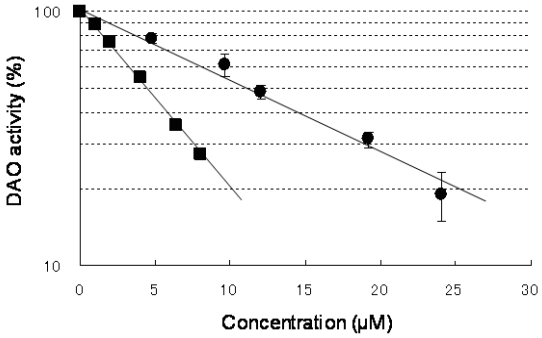

Figure 5. Dose- inhibition curves for chlorpromazine-(circle) and risperidone (square)-mediated human D-amino acid oxidase (DAO) inhibition.

3.2. *Inhibition of DAO activity*

Under the enzymatic reaction conditions described earlier, two DAO inhibitors reported on previously, 3-methylpyrazole-5-carboxylic acid (MPC, AS057278) (*16*) and 4*H*-thieno[3,2-b]pyrrole-5-carboxylic acid (Compound 8) (*17*) were tested for enzyme inhibition. As shown in Figures 4a and 4b, both inhibitors inhibited KYNA production in a dose-dependent manner, indicating that the proposed *in vitro* assay can be used for the evaluation of hDAO activity in a similar manner to that of pkDAO. The IC_{50} values for MPC and compound 8 were 2.41 ± 0.23 μM and 90.3 ± 18.1 nM, respectively. Compound 8 exhibited a more intense DAO-inhibitory effect than MPC, which was consistent with previously reported results (*9*).

In contrast, little inhibition of human DAO was observed due to addition of 3-methylpyrazole-4-carboxylic acid (3, 4-MPC), a structural isomer of MPC (Figure 4c). This result was similar to previously published results using pkDAO (*12*).

Next, the *in vitro* fluorescence assay using D-KYN as a substrate was used to determine the hDAO-inhibitory effects of FGAs (chlorpromazine, haloperidol, and sulpiride) and SGAs (risperidone, olanzapine, aripiprazole, quetiapine, and blonanserin). In addition, other drugs given to schizophrenia patients were also tested, including anti-parkinson drugs (trihexyphenidyl, biperiden), anti-depressant drugs (sertraline, escitalopram, duloxetine), and the mood stabilizer valproate (unpublished data). Figure 5 shows dose-inhibition curves representative of chlorpromazine and risperidone that were obtained using the proposed fluorescence assay. Attenuation of hDAO activity in a concentration-dependent manner was observed with both drugs, indicating that considerable inhibition of hDAO activity was caused, and that more intense inhibition of hDAO was produced by risperidone than by chlorpromazine. The 50% inhibitory concentration (IC_{50}) values of test drugs obtained using the proposed assay are summarized in Table 1. Several drugs exhibited IC_{50} values for hDAO-inhibitory effects below 50 μM. Among them, the second-generation antipsychotics blonanserin and risperidone were found to possess relatively strong hDAO-inhibitory effects *in vitro* (5.29 ± 0.47 and 4.70 ± 0.17 μM, respectively). These IC_{50} values were comparable to that (2.41 ± 0.23 μM, $n = 3$) of MPC, a compound with established DAO-inhibitory activity. The precise mechanism responsible for hDAO-inhibition is not clear, but chlorpromazine and risperidone have been reported to inhibit hDAO (*13,18,19*). The common features of the tested drugs were a heterocyclic moiety and a tertiary amino group. In the case of chlorpromazine, replacement of flavin adenine dinucleotide, a co-factor of DAO, occurred that might have resulted in attenuated DAO activity (*19-*

Table 1. IC_{50} values (μM) of FGAs, SGAs, and concomitant drugs for the inhibition of hDAO activity by the proposed assay (mean ± S.D., *n* = 3-4)

Drug	IC_{50}
FGA	
Chlorpromazine	11.6 ± 0.58
Haloperidol	13.4 ± 1.87
Sulpiride	14.3 ± 0.81
SGA	
Aripiprazole	12.4 ± 1.38
Blonanserin	5.29 ± 0.47
Olanzapine	> 50.0
Quetiapine	43.9 ± 9.27
Risperidone	4.70 ± 0.17
Anti-parkinson	
Trihexyphenidyl	> 50.0
Biperiden	> 50.0
Anti-depressant	
Sertraline	> 50.0
Escitalopram	> 50.0
Duloxetine	> 50.0
Mood stabilizer	
Valproate	> 50.0

21). Thus, it may be reasonable to conclude that the hDAO-inhibitory effects of drugs in this study were at least partially due to the presence of a heterocyclic moiety and a tertiary amino group, similar to those in chlorpromazine. However, it is conceivable that the structure of a compound can be strictly discriminated by the active site of DAO, because various IC_{50} values for each drug were revealed (Table 1). To clarify the precise mechanism by which each drug inhibited DAO activity, co-crystallographic analyses of DAO with each compound or drug will be necessary.

The present data suggest that prescribed anti-psychotic drugs might inhibit hDAO activity *in vivo*. From surveillance data collected by the Japanese Psychiatric Clinical Pharmacy Research Group (*22*), poly-pharmacy of anti-psychotic drugs, particularly SGAs, occurs in medical institutions in Japan. Therefore, drug combination therapies consisting of SGAs might enhance inhibition of DAO in the central nervous system. In the future, the DAO-inhibitory effect of SGAs should be taken into consideration with regard to their prescription.

In summary, by using the assay proposed herein, we revealed that SGAs blonanserin and risperidone possess hDAO-inhibitory effects *in vitro*. In addition to primary pharmacological actions such as blockade of dopamine D_2 and serotonin receptors, the DAO-inhibitory effects of these drugs may contribute to their pharmacotherapeutic efficacy.

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