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The Antiviral Drug Acyclovir Is a Slow-Binding Inhibitor of D-Amino Acid Oxidase

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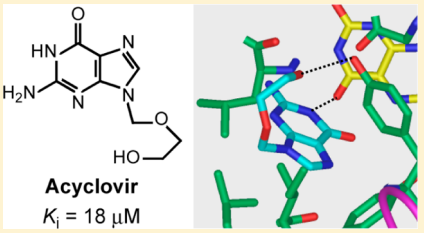
ABSTRACT: D-Amino acid oxidase (DAO) is a degradative enzyme that is stereospecific for D-amino acids, including D-serine and D-alanine, which are believed to be coagonists of the *N*-methyl-D-aspartate (NMDA) receptor. To identify a new class of DAO inhibitor(s) that can be used to elucidate the molecular details of the active site environment of DAO, manifold biologically active compounds of microbial origin and pre-existing drugs were screened for their ability to inhibit DAO activity, and several compounds were identified as candidates. One of these compounds, acyclovir (ACV), a well-known antiviral drug used for the treatment of herpesvirus infections, was characterized and evaluated as a novel DAO inhibitor *in vitro*. Analysis showed that ACV acts on DAO as a reversible slow-binding inhibitor, and interestingly, the time required to achieve equilibrium between DAO, ACV, and the DAO/ACV complex was highly dependent on temperature. The binding mechanism of ACV to DAO was investigated in detail by several approaches, including kinetic analysis, structural modeling of DAO complexed with ACV, and site-specific mutagenesis of an active site residue postulated to be involved in the binding of ACV. The results confirm that ACV is a novel, active site-directed inhibitor of DAO that can be a valuable tool for investigating the structure−function relationships of DAO, including the molecular details of the active site environment of DAO. In particular, it appears that ACV can serve as an active site probe to study the structural basis of temperature-induced conformational changes of DAO.



Among the free D-amino acids found in mammals, D-aspartate (D-Asp) and D-serine (D-Ser) have been studied most intensively. Substantial amounts of free D-Asp are present in a wide variety of mammalian tissues and cells, particularly in the central nervous, neuroendocrine, and endocrine systems. Several lines of evidence suggest that D-Asp plays an important role in regulating developmental processes, hormone secretion, and steroidogenesis.[1,2] Unlike the widespread expression of D-Asp, D-Ser is predominantly concentrated in the mammalian forebrain, where it is synthesized by Ser racemase (EC 5.1.1.16), a synthetic enzyme that produces D-Ser from L-Ser.[3] D-Ser persists at high concentrations throughout the life of the animal and is known to be a neuromodulator that binds to the glycine-binding site of the *N*-methyl-D-Asp (NMDA) receptor, a subtype of the L-glutamate (L-Glu) receptor family, and potentiates glutamatergic neurotransmission in the central nervous system.[4,5] In fact, astroglia-derived D-Ser has been shown to regulate NMDA receptor-dependent long-term potentiation and/or long-term depression in the hypothalamic and hippocampal excitatory synapses.[6,7] D-Ser is also found in the cerebellum during the early postnatal period, and it was recently reported that D-Ser derived from the Bergmann glia serves as an endogenous ligand for the δ2 Glu receptor in regulating long-term depression at synapses between parallel fibers and Purkinje cells in the immature cerebellum, but not in the mature cerebellum.[8] These lines of evidence suggest that D-Ser plays an important role in the regulation of higher brain functions through L-Glu receptors. Indeed, perturbation of D-Ser levels in the nervous system has recently been implicated in the pathophysiology of various neuropsychiatric disorders, including schizophrenia,[9−12] Alzheimer's disease,[13,14] and amyotrophic lateral sclerosis.[15,16]

In addition to D-Asp and D-Ser, D-alanine (D-Ala), another D-amino acid present in mammals at relatively low levels, has been of recent interest. Because D-Ala is expressed in the mammalian brain, peripheral tissues, and the anterior pituitary gland and pancreas, it has been postulated to be involved in endocrine activity.[17−19] Notably, D-Ala is known to stimulate the NMDA receptor by acting as a coagonist that binds to the glycine-binding site of the receptor.[20,21]

In mammalian tissues, two types of degradative enzymes that are stereospecific for D-amino acids have been identified: D-amino acid oxidase (DAO, also abbreviated DAAO, EC 1.4.3.3) and D-Asp oxidase (DDO, also abbreviated DASPO, EC

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1.4.3.1). DAO and DDO are flavin adenine dinucleotide (FAD)-containing flavoproteins that catalyze the oxidative deamination of D-amino acids with oxygen to generate the corresponding 2-oxo acids along with hydrogen peroxide and ammonia.[22,23] DAO displays broad substrate specificity and acts on several neutral and basic D-amino acids, such as D-Ser and D-Ala. On the other hand, DDO is highly specific for acidic D-amino acids, such as D-Asp and D-Glu, none of which are substrates of DAO. DAO and DDO have been identified in various organisms, and their physiological roles *in vivo* are being investigated extensively. Mammalian DAO and DDO are presumed to regulate the levels of several endogenous and exogenous D-amino acids, including D-Ser, D-Ala, and D-Asp, in various organs.[24−26] However, their physiological roles *in vivo* have yet to be fully clarified.

The use of specific enzyme inhibitor(s), X-ray crystallographic analysis, and *in vitro* mutagenesis are effective methods for the detailed investigation of structure−function relationships of the enzyme proteins. Benzoate, a classical and competitive DAO inhibitor,[27] was one of the first inhibitors to be used in these studies. Until recently, benzoate was the most extensively used DAO inhibitor in biochemical, biological, and pharmacological studies on DAO, although its inhibitory potency is limited. Indeed, the first three-dimensional (3D) X-ray crystallographic structure of mammalian DAO was determined in a complex with benzoate,[28,29] providing useful insights into the structural details of the active site environment of DAO. Meanwhile, decreased D-Ser levels in the central nervous system and a resultant dysfunction in NMDA receptor-mediated neurotransmission have now been postulated to occur during the onset of various mental disorders, including schizophrenia.[9−12] Hence, a substance(s) able to increase D-Ser and/or D-Ala levels and activate NMDA receptor function may present a novel foundation for the development of antipsychotic drug(s). One possible way to increase the levels of D-Ser and/or D-Ala is to prevent the metabolic degradation of these D-amino acids by DAO. Therefore, increased attention has been focused on DAO as a therapeutic target, and several compounds have already been identified as novel DAO inhibitors that may potentially lead to the development of a clinically useful DAO inhibitor, including 6-chlorobenzo[*d*]-isoxazol-3-ol, 4*H*-furo[3,2-*b*]pyrrole-5-carboxylic acid, 3-hydroxyquinolin-2(1*H*)-one, and 3-hydroxychromen-2-one.[30−33]

The main purpose of the present study was to identify a new class of DAO inhibitor(s) that can be used to elucidate the molecular details of the active site environment of DAO. Previously, we screened manifold biologically active compounds of microbial origin and pre-existing drugs[34] for their ability to inhibit the activity of DDO. This screening identified thiolactomycin as a novel DDO inhibitor whose mechanism of action is unique in that it inhibits the activity of DDO by competition with both the substrate and coenzyme, FAD.[35] In the present study, a similar approach was used to search for novel DAO inhibitor(s). The same compounds as in the previous study[35] were screened for their ability to inhibit the activity of DAO, and several were identified as candidate inhibitors. One of these compounds, acyclovir (ACV), which is a well-known antiviral drug for the treatment of herpesvirus infections,[36] was selected and characterized *in vitro* as a novel DAO inhibitor. ACV was found to act on DAO as a reversible slow-binding inhibitor, and the time required to attain equilibrium between DAO, ACV, and the DAO/ACV complex was highly dependent on temperature. Furthermore, the binding mechanism of ACV to DAO was investigated in detail by several approaches, including kinetic analysis, structural modeling of DAO complexed with ACV, and site-specific mutagenesis of an active site residue postulated to be involved in the binding of ACV. This work shows that ACV is a novel, active site-directed DAO inhibitor that will be a valuable tool for investigating the structure−function relationships of DAO, including the molecular details of the active site environment of DAO. In particular, ACV can serve as an active site probe to study the structural basis for temperature-induced changes in the conformation of DAO.

MATERIALS AND METHODS

Chemicals. D-Amino acids, ampicillin, bovine serum albumin, DAO from pig kidney, and catalase from *Aspergillus niger* were purchased from Sigma-Aldrich (St Louis, MO, USA). ACV, FAD, isopropyl-*β*-D-thiogalactopyranoside, imidazole, and benzoate were purchased from Wako Pure Chemical (Osaka, Japan). All other chemicals were of the highest grade available and purchased from commercial sources.

Screening of Compounds Inhibiting the Activity of DAO. Using a colorimetric assay,[37] manifold biologically active compounds of microbial origin and pre-existing drugs in the Kitasato Institute for Life Sciences Chemical Library[34] were screened for their ability to inhibit DAO. This assay was performed in 96-well plates, as described previously,[35] with the following modifications. DAO from pig kidney (0.2 μg) was added to a reaction mixture consisting of air-saturated 40 mM sodium pyrophosphate buffer (pH 8.3), 11 U *A. niger* catalase, 50 μM FAD, 20 mM D-Ala, and 3 μg of each compound in a final volume of 50 μL, and incubated at 37 °C for 30 min. Subsequently, 25 μL of 0.1% (weight/vol) 2,4-dinitrophenylhydrazine in 2 M HCl was added and incubated at 37 °C for 15 min, followed by the addition of 175 μL of 3.75 M NaOH. A plate reader (PowerWave XS, Bio-Tek Instruments, Winooski, VT, USA) was used to measure the A_{445} of the mixture against a blank mixture lacking D-Ala and compound. The relative inhibitory activity of each compound was determined by regarding the absorbance of the reaction mixture without compound as 100%.

Expression and Purification of Recombinant Proteins. The construction of expression plasmids for N-terminally His-tagged human DAO, human DDO, the Tyr-224-to-Phe (Y224F) variant of human DAO, and the Tyr-224-to-Ala (Y224A) variant of human DAO (pRSET-His-hDAO, pRSET-His-hDDO, pRSET-His-hDAO-Y224F, and pRSET-His-hDAO-Y224A, respectively) has been described previously.[32,35,38]

Escherichia coli strain BL21(DE3)pLysS cells were transformed with expression plasmids and cultured at 37 °C with shaking in Luria−Bertani medium containing ampicillin (100 μg/mL). When the culture reached A_{620} = 0.5, the incubation temperature was decreased to 26 °C and the cells were grown for an additional 30 min. After adding 0.5 mM isopropyl-*β*-D-thiogalactopyranoside, the culture was further incubated at 26 °C for 16 h. The cells were then centrifuged at 10,000*g* for 10 min at 4 °C, and crude extract was prepared using BugBuster Protein Extraction Reagent and Lysonase Bioprocessing Reagent (Novagen, Madison, WI, USA) in the presence of 50 μM FAD and protease inhibitors (Nacalai Tesque, Kyoto, Japan), according to the manufacturer's instructions.

All recombinant proteins were purified by affinity chromatography using a chelating column, as described below. Crude

extracts were applied to a His GraviTrap column (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA) equilibrated with 20 mM sodium phosphate buffer (pH 7.4) containing 0.5 M NaCl and 10 mM imidazole. The column was then washed with the same buffer, and bound proteins were eluted using a stepwise gradient of 50−500 mM imidazole. Each fraction (2.0 mL) containing recombinant protein was dialyzed twice for 3 h at 4 °C against 1 L of 10 mM sodium pyrophosphate buffer (pH 8.3) containing 2 mM EDTA, 5 mM 2-mercaptoethanol, and 10% (v/v) glycerol. The dialyzed fractions were recovered and centrifuged at 10,000*g* for 10 min at 4 °C to pellet the proteins denatured during dialysis. The supernatants were pooled as purified enzyme and used immediately for enzyme assays or stored frozen at −80 °C until use. All recombinant proteins were purified to near-homogeneity when examined by SDS-polyacrylamide gel electrophoresis. The protein concentrations of the purified enzyme preparations were determined using the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA) with bovine serum albumin as a standard.

Enzymatic Activity Assays. The activities of DAO and DDO were determined using a colorimetric assay for 2-oxo acid production, as previously described.[37] Briefly, appropriate amounts (0.30−8.0 μg) of the purified enzymes were mixed in a 1.5 mL microtube that contained a reaction mixture consisting of air-saturated 40 mM sodium pyrophosphate buffer (pH 8.3), 23 U *A. niger* catalase, 200 μM FAD, and 5 mM amino acids in a final volume of 150 μL. For reactions with the Y224F and Y224A variants of human DAO, 20 mM and 75 mM D-Ala, respectively, were used as the substrate. Unless otherwise noted, the reaction mixture was incubated at 37 °C for 10 min, and then 10 μL of 100% (weight/vol) trichloroacetic acid was added to stop the reaction. The 2-oxo acid product was reacted with 2,4-dinitrophenylhydrazine and quantitated by measuring the A_{445} against a blank mixture lacking amino acids. One unit of enzyme activity was defined as the production of 1 μmol of 2-oxo acid per min under the assay conditions described above.

For testing the inhibitory activities of ACV and benzoate, each compound was added to the reaction mixture. The relative inhibitory activities of the compounds were determined by regarding the activity of the enzyme in the absence of compound as 100%. The IC_{50} values of the tested compounds were determined using the following equation (eq 1), as described previously.[32]

$$IC_{50} = 10^{(\log[A/B]\times[50-C]/[D-C]+\log B)} \quad (1)$$

In eq 1, A and B are the higher and lower concentrations nearest to the middle of the curve, respectively, while C and D are the inhibition percentages at the concentrations of B and A, respectively. To examine the time- and temperature-dependent mechanisms of inhibition, enzymes were preincubated with inhibitors for 0, 1, 2, 4, 5, or 6 h at 0, 5, 10, 20, or 37 °C before the addition of substrate to initiate the enzymatic reaction. After the preincubation, the substrate was added to the mixture and the enzymatic activity was measured as described above. For determination of the K_m value for D-Ala, different final concentrations (0.5−40 mM) of D-Ala were used as the substrate, and the enzymatic reaction was conducted under conditions in which the production of 2-oxo acids was linear with incubation time and exhibited Michaelis−Menten-type properties. The data obtained were fitted to the Michaelis−Menten equation, and the K_m value was estimated using the nonlinear least-squares fitting algorithm in pro Fit 6.1 software (Quantum Soft, Zürich, Switzerland; http://www.quansoft.com). For the determination of several kinetic parameters associated with slow-binding inhibition, the enzymatic reaction was conducted at 0 °C for up to 6 h. The amount of 2-oxo acid produced at several time points was determined as described above and fitted to the following equation (eq 2) described by Morrison and Walsh (1988).[39]

$$P = v_s t + (v_0 - v_s)(1 - e^{-k_{obs}t})/k_{obs} \quad (2)$$

In eq 2, P is the amount of product, v_s is the final steady-state velocity, t is the time, v_0 is the initial velocity, and k_{obs} is the apparent first-order rate constant for equilibration. The k_{obs} values were estimated using the nonlinear least-squares fitting algorithm in the pro Fit 6.1 software (Quantum Soft) and plotted against the inhibitor concentration. Since this plot displayed linear dependence (see Figure 3C), which indicates that the binding of the inhibitor to the enzyme is a single-step process,[39] the data obtained were subsequently fitted to the following equation (eq 3), where k_{off} is the dissociation rate constant, k'_{on} is the uncorrected association rate constant, and [I] is the inhibitor concentration.

$$k_{obs} = k_{off} + k'_{on}[I] \quad (3)$$

The k'_{on} was then corrected for the substrate competition using the following equation (eq 4), in which [S] is the substrate concentration.

$$k_{on} = k'_{on}(1 + [S]/K_m) \quad (4)$$

In some cases, the inhibitory effects of ACV on the enzymatic activity of DAO on D-Ala were confirmed by high-performance liquid chromatography (HPLC) analysis. Briefly, the enzymatic reaction was conducted as described above, and then 600 μL of 100% (v/v) methanol was added to stop the reaction. Subsequently, the mixture was incubated at −80 °C for 1 h and centrifuged at 20,000*g* for 10 min at 4 °C to remove precipitated proteins. The supernatant (600 μL) was then filtered through a 0.45 μm filter (Millex-LH; Millipore, Bedford, MA, USA), and the filtered solution was appropriately diluted with H_2O. The concentrations of D-Ala were determined in the diluent by reversed-phase HPLC using the *o*-phthalaldehyde precolumn derivatization technique, as described previously.[32]

Spectral Analysis of DAO. A plate reader (PowerWave XS, Bio-Tek Instruments) was used to measure the absorption spectra of human DAO. Wavelength scans were carried out at 21 °C with purified recombinant human DAO (3.9 μg/μL) in 10 mM sodium pyrophosphate buffer (pH 8.3) containing 2 mM EDTA, 5 mM 2-mercaptoethanol, and 10% (v/v) glycerol, before and after the addition of compounds to a final concentration of 1000 μM. The spectra were recorded at 0, 2, 4, and 5 h after compound addition. The scan range was 300−600 nm in the presence of benzoate and 310−600 nm in the presence of ACV. In these ranges, the absorption spectrum of each compound did not interfere with the measurement of spectral changes of DAO.

RESULTS AND DISCUSSION

Screening of Compounds Inhibiting the Activity of DAO. A total of 257 types of materials, including biologically active compounds of microbial origin and pre-existing drugs,[34] were screened for their ability to inhibit the enzymatic activity

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of pig DAO on D-Ala using the multiwell-plate method, as described in the Materials and Methods. From this screening, ACV,[36] manzamine A,[40] and aristeromycin[41] were identified as candidates; namely, these three compounds inhibited approximately 37, 46, and 20% of the activity of pig DAO, respectively. One of these compounds, ACV (Figure 1), a well-known

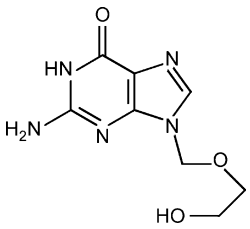

Figure 1. Chemical structure of ACV. ACV is a guanine derivative containing an acyclic side chain at the 9-position. Its chemical name is 9-[(2-hydroxyethoxy)methyl]guanine. The formula of ACV is $C_8H_{11}N_5O_3$, and it has a molecular weight of 225.2.

antiviral drug for the treatment of herpesvirus infections,[36] was subsequently characterized and evaluated as a novel DAO inhibitor, since it is a compound of relatively low molecular weight and therefore computational structural modeling of DAO/ACV complexes can be performed with high precision, as described in the following sections.

Selectivity Profile of ACV. The effect of 1000 μM ACV on the enzymatic activity of pig DAO was examined using D-Ala as a substrate. As expected, ACV inhibited the activity of pig DAO, which was reduced to 84.3 ± 2.3% of control in the presence of ACV (Table 1). The effects of 1000 μM ACV on

Table 1. Effect of Preincubation with 1000 μM ACV on Enzymatic Activities

	relative activities (%)[a]		
1 h preincubation	pig DAO	human DAO	human DDO
none	84.3 ± 2.3	99.5 ± 0.3	101.4 ± 2.4
0 °C	0 ± 0	0 ± 0	72.8 ± 0.9
5 °C	70.7 ± 1.3	99.7 ± 4.2	97.6 ± 1.1
10 °C	75.0 ± 0.5	95.6 ± 2.6	96.8 ± 2.1
20 °C	75.1 ± 2.6	94.2 ± 1.3	98.7 ± 3.0
37 °C	28.6 ± 4.1	46.6 ± 7.9	93.1 ± 3.1

[a]Enzyme activity is presented as a percentage relative to the activity obtained in the absence of ACV. Each value shown is the mean ± standard deviation (n = 3).

the enzymatic activities of recombinant human DAO and DDO on D-Ala and D-Asp, respectively, were also examined. Human DAO shares a high amino acid sequence identity with pig DAO (84.4%), while human DDO shows only moderate sequence identity with pig DAO (39.2%). Unexpectedly, no inhibition of human DAO was observed in the presence of ACV (Table 1). ACV also failed to inhibit the activity of human DDO.

Next, the effect of preincubation with ACV on the activities of DAO and DDO was examined. Pig and human DAO and human DDO were preincubated with 1000 μM ACV for 1 h at several temperatures (0, 5, 10, 20, and 37 °C) before substrate addition, and the activities of the enzymes were measured. Interestingly, inhibition of pig DAO by ACV was strongly enhanced by preincubation (Table 1). Moreover, the effect of preincubation was temperature-dependent and was most prominent at 0 °C, followed by 37 °C and then the other temperatures (5, 10, and 20 °C). Similar results were obtained with human DAO, demonstrating a slow onset of inhibition. Furthermore, the activity of human DDO was also decreased after preincubation. However, in contrast to the effect of preincubation on DAO activity, 72.8 ± 0.9% of the control activity remained even when this enzyme was preincubated with ACV at 0 °C. These results suggest that ACV acts as a slow-binding inhibitor of DAO and that ACV is selective for DAO but not for DDO.

Time and Temperature Dependence of the Binding of ACV to DAO. To confirm that ACV acts as a slow-binding inhibitor of DAO, the effect of preincubation with ACV on the enzymatic activity of DAO on D-Ala was further examined with respect to its time dependency. Human DAO was preincubated with various concentrations (0.01−1000 μM) of ACV for 0, 2, 4, 5, or 6 h at 0 °C before substrate addition, and DAO activity was measured. As expected, without preincubation (0 h preincubation), ACV had no effect, even at a concentration of 1000 μM (Figure 2A). On the other hand, after preincubation for 2 h, ACV inhibited DAO activity in a dose-dependent manner, and the 50% inhibitory concentration (IC_{50}) of ACV was determined to be 29.0 ± 5.8 μM. After preincubation of the enzyme for 4, 5, or 6 h, ACV displayed increasing potency, with the IC_{50} values decreasing to 10.7 ± 0.2, 4.7 ± 1.2, and 3.8 ± 0.6 μM, respectively (Figure 2A). These results suggest that the time required to establish equilibrium between DAO, ACV, and the DAO/ACV complex at 0 °C is approximately 5 h. The effect of benzoate, a known competitive inhibitor of mammalian DAO,[27] was tested as control inhibitor. In contrast to ACV, benzoate inhibited DAO activity in a dose-dependent manner, regardless of the preincubation time (Figure 2B). The IC_{50} value for benzoate determined with preincubation for 5 h (80.5 ± 7.5 μM) was equivalent to the IC_{50} without preincubation (0 h preincubation) (71.8 ± 12.5 μM).

The effect of preincubation with ACV on the enzymatic activity of DAO on D-Ala was then further examined with respect to its temperature dependency. Specifically, human DAO was preincubated with various concentrations (0.01−1000 μM) of ACV for 5 h at 0, 5, 10, 20, or 37 °C before substrate addition, and DAO activity was then measured. As shown in Figure 2C, ACV inhibited DAO activity in a dose-dependent manner, regardless of the preincubation temperature. The magnitude of the inhibition, however, differed with the preincubation temperature and was the greatest at 0 °C, followed by 37 °C, and then the other temperatures. These results indicate that the time required to establish equilibrium between DAO, ACV, and the DAO/ACV complex is highly temperature-dependent.

The molecular mechanism responsible for the temperature dependence of the binding of ACV to DAO is currently unclear. However, it has been reported that a temperature-induced conformational change may occur in the mammalian DAO molecule.[42−46] The Arrhenius plot of the DAO activity was reported to be nonlinear and best expressed by two straight lines with different activation energies. Thus, it was concluded that DAO can exist at least in two forms: a low-temperature form and a high-temperature form, in equilibrium with each other.[43] Moreover, from the results of fluorescence and ultraviolet spectral analyses, it was concluded that while substrate D-Ala has relatively equal affinities for both of these forms, D-methionine, which is also a good substrate of DAO *in vitro*, has a greater affinity for the low-temperature form than

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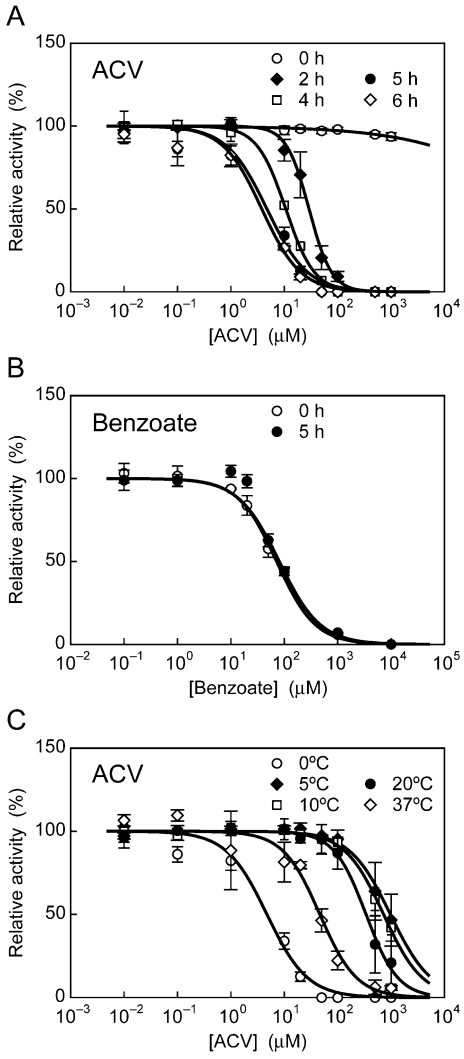

Figure 2. Dose-dependent effects of inhibitors on the enzymatic activity of human DAO. To examine the effect of preincubation with ACV and benzoate on DAO activity, human DAO was preincubated with ACV at the indicated concentrations for 0, 2, 4, 5, or 6 h at 0 °C before substrate addition, and DAO activity was measured using D-Ala as a substrate (A). Similarly, human DAO was preincubated with benzoate at the indicated concentrations for 0 or 5 h at 0 °C before substrate addition, and DAO activity was measured using D-Ala as a substrate (B). The effect of preincubation with ACV on DAO activity with respect to its temperature dependency was also examined (C). Human DAO was preincubated with the indicated concentrations of ACV for 5 h at 0, 5, 10, 20, or 37 °C before substrate addition, and DAO activity was measured using D-Ala as a substrate. Enzyme activity in the presence of inhibitors is presented as a percentage of its activity in the absence of inhibitors. Data are presented as the mean ± standard deviation (n = 3). Where not shown, the error bars are smaller than the symbols used.

the high-temperature form. On the basis of the results of molecular dynamic simulation, it was recently proposed that DAO adopts different structures at different temperatures, including the conformation of the active site.[44] As described in the following sections, the present study indicates that ACV acts on DAO as an active site-directed inhibitor and that the FAD bound to the enzyme and the active site residue Tyr-224 are important regulators of the interaction of DAO with ACV. Thus, it is likely that the binding of ACV to the active site in DAO is influenced by temperature-induced structural changes, especially in the vicinity of the bound FAD and Tyr-224 regions. On the other hand, when DAO was preincubated with ACV at several different temperatures, the magnitude of the inhibition of DAO by ACV was the largest at 0 °C, followed by 37 °C and then the other temperatures (5, 10, and 20 °C) (Figure 2C). Thus, the effect of the temperature on the binding of ACV to DAO appears to be biphasic. Therefore, it is possible that the molecular mechanisms that underlie the binding of ACV to DAO at low and high temperatures are different. Through one of the mechanisms, the binding affinity of ACV for the DAO active site increases as the temperature decreases, which is evident at 0 °C compared to 5 °C, while, by the other mechanism, the binding affinity increases as the temperature increases, which is evident at 37 °C compared to 20 °C. Determination of the crystal structure of DAO complexed with ACV will enhance our understanding of the mechanics of ACV binding to DAO.

Reversibility of the Inhibition of DAO by ACV. To determine whether ACV is a reversible slow-binding inhibitor or an irreversible inhibitor of DAO, enzyme dilution experiments were performed to examine the kinetics of inhibitory activity. In the control experiment, human DAO was first exposed to 1000 μM benzoate for 5 h at 0 °C, which inhibits approximately 90% of the enzyme activity (Figure 2B), and the benzoate concentration was then diluted to 10 μM. Following dilution, DAO activity recovered rapidly to a value similar to that of the enzyme after continuous exposure to 10 μM benzoate (Table 2). To examine the kinetics of ACV inhibition, human DAO was first exposed to 100 μM ACV for 5 h at 0 °C, which inhibits enzyme activity almost completely (Figure 2A), after which the ACV concentration was diluted to 1 μM. Following dilution, the reaction mixture was incubated for an additional 0, 15, 30, or 60 min at 0 °C before substrate addition, at which point DAO activity was then measured. After dilution of ACV, DAO activity slowly recovered to a value approximately equal to that of the enzyme when continually exposed to 1 μM ACV (Table 2). This gradual recovery of

Table 2. Reversibility of Inhibition of DAO by ACV

inhibitor conc before and after dilution (μM)	preincubation time after the dilution (min)	relative activity (%)[a]
Benzoate		
0 → 0	0	100 ± 2.0
10 → 10	0	90.5 ± 4.9
1000 → 1000	0	10.6 ± 2.1
1000 → 10	0	91.8 ± 1.0
1000 → 10	15	87.3 ± 0.8
1000 → 10	30	87.6 ± 7.1
1000 → 10	60	88.2 ± 0.8
ACV		
0 → 0	0	100 ± 1.2
1 → 1	0	96.1 ± 1.1
100 → 100	0	0 ± 0
100 → 1	0	29.3 ± 2.7
100 → 1	15	37.9 ± 1.0
100 → 1	30	44.8 ± 1.8
100 → 1	60	68.6 ± 1.1

[a]Enzymatic activity of DAO is presented as a percentage relative to the activity obtained in the absence of inhibitor (benzoate or ACV) and without preincubation after the dilution process. Each value shown is the mean ± standard deviation (n = 3).

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enzyme activity following dilution is a hallmark of slow-binding inhibitors[39] and was in marked contrast to the results obtained in the control experiment. Taken together, these results indicated that ACV is a reversible, slow-binding inhibitor of DAO.

Kinetics of ACV Mechanism of Action. To confirm that ACV acts on DAO as a slow-binding inhibitor, enzymatic reaction of DAO with D-Ala was assayed without preincubation of the enzyme with ACV, and progress curves in the presence of varying concentrations of ACV were examined. The enzymatic reaction was carried out at 0 °C to exclude inactivation of the enzyme during the reaction period (0–6 h). Progress curves in the presence of varying concentrations of benzoate at 0 °C were also generated as controls. As expected, in the presence of benzoate, the reaction rates of DAO activity remained linear during the reaction period (Figure 3A). In contrast, the progress curves generated in the presence of ACV showed a clear time-dependent approach to the steady state (Figure 3B), which is a characteristic of slow-binding inhibition kinetics,[39] thus confirming the slow-binding behavior of ACV.

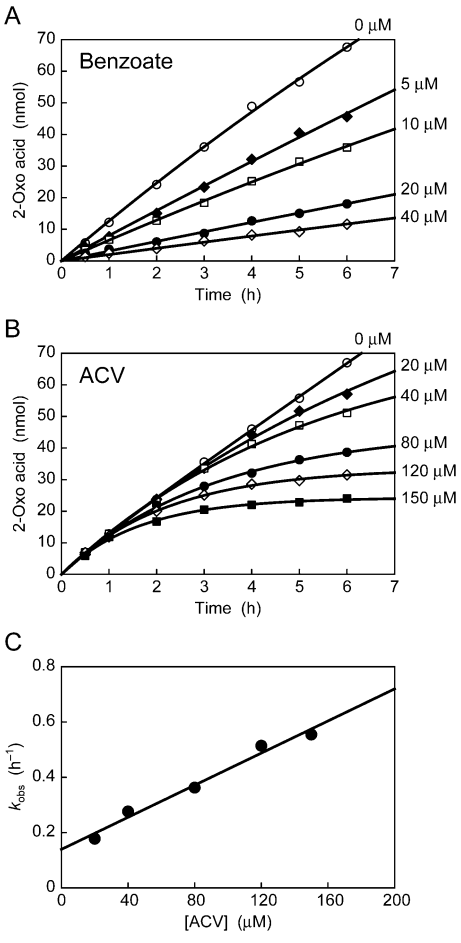
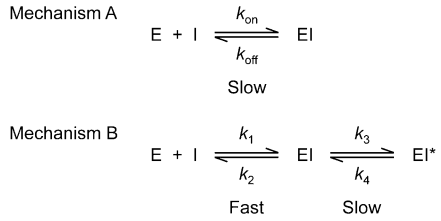


Figure 3. Slow-binding kinetics of inhibition of human DAO by ACV. Without preincubation of the enzyme with inhibitors, enzymatic reaction of human DAO with D-Ala was assayed at 0 °C for up to 6 h, and progress curves in the presence of the indicated concentrations of benzoate (A) and ACV (B) were generated. The data sets in panel B were fitted to eq 2 to estimate the k_{obs} values. Subsequently, the estimated k_{obs} values were plotted against the ACV concentration (C) to confirm the mechanism of action of ACV. For details, see the text.

Two basic mechanisms have been proposed to explain slow-binding enzyme inhibition: mechanism A and mechanism B.[39] As shown in Scheme 1, mechanism A assumes a single-step

Scheme 1



mechanism in which formation of the initial complex is the slow step relative to catalysis, while mechanism B is a two-step mechanism involving the formation of an initial inhibitory complex followed by slow equilibration to a more tightly bound complex. To determine the true mechanism of ACV inhibition of DAO, the apparent first-order rate constant for equilibration (k_{obs}) was estimated by regression analysis using eq 2, as described in Materials and Methods, and plotted against inhibitor concentration. As shown in Figure 3C, this plot displayed linear dependence, indicating that the binding of ACV to DAO occurs via a single-step mechanism, as depicted in mechanism A.[39] The association and dissociation rate constants (k_{on} and k_{off}) of 2.14 $M^{-1}\cdot s^{-1}$ and 3.87 × 10^{-5} s^{-1}, respectively, were then estimated by regression analysis using eqs 3 and 4, and accordingly, the K_i value (= k_{off}/k_{on}) of ACV against DAO was calculated to be 18 μM.

Structural Insight into the Binding of ACV to DAO. Next, the effect of ACV on the flavin absorbance spectrum of recombinant human DAO was examined using benzoate as control inhibitor. As expected, the addition of benzoate yielded obvious perturbations in the absorption spectrum of the enzyme (Figure 4A). The spectral changes upon benzoate binding were consistent with those reported for recombinant human DAO.[32,47] These spectral changes were also observed when the enzyme was mixed with ACV (Figure 4B), indicating that ACV binds to the DAO active site.

The 3D X-ray crystallographic structures of pig and human DAO have been solved.[28,29,48] In the structures complexed with benzoate, the carboxyl group of benzoate interacts with the side-chain guanidino group of Arg-283 and with the side-chain hydroxyl group of Tyr-228 (Figure 5A).[28,29,48] Importantly, a loop formed by residues 216–228 has been postulated to act as an "active-site lid" that opens and closes on substrate/product migration in and out of the active site.[49] Tyr-224 within the lid is presumably important in the hydrophobic environment of the active site in the closed conformation,[28,29,48] and the side chain of this residue potentially covers the bound ligand (Figure 5A). Accordingly, the side-chain aromatic ring of this residue also contributes to the binding to the ligand by means of its π–π interaction with the aromatic ring moiety of the bound ligand. On the basis of these experimentally determined structures, structural models of human DAO complexed with ACV were created in this study to gain insight into the mechanism of binding of ACV to DAO. Specifically, structural models of human DAO complexed with ACV were constructed using Glide software (Schrödinger Suite 2009; Schrödinger, LLC, New York, NY, USA) in the standard precision (SP) mode. The binding mode of ACV with the best Glide Score is shown in Figure 5B. In this model, ACV is situated in the

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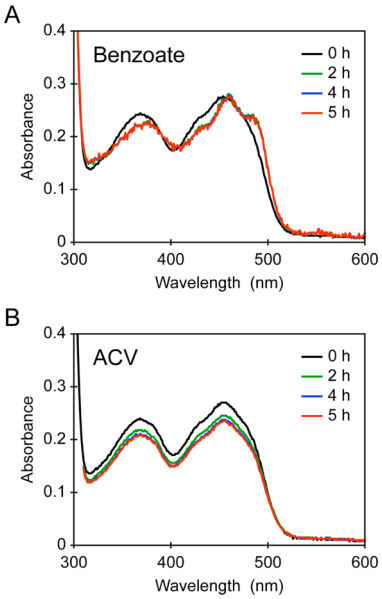

Figure 4. Absorption spectra of human DAO in the absence or presence of inhibitors. Wavelength scans were carried out at 21 °C with recombinant human DAO (3.9 μg/μL) in 10 mM sodium pyrophosphate buffer (pH 8.3) containing 2 mM EDTA, 5 mM 2-mercaptoethanol, and 10% (v/v) glycerol, before and after the addition of compounds to a final concentration of 1000 μM at the indicated times. The scan range was 300−600 nm in the presence of benzoate (A) and 310−600 nm in the presence of ACV (B).

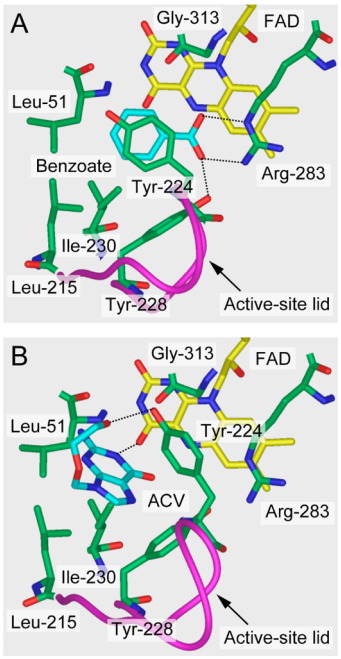

Figure 5. Structural model of human DAO complexed with ACV. The experimentally determined structure of human DAO complexed with benzoate (PDB ID: 2DU8)[48] is shown in panel A. The proposed structural model of human DAO complexed with ACV is shown in panel B. The carbon atoms in FAD, bound ligands, and side-chains of amino acid residues are colored yellow, cyan, and green, respectively. Other atoms are colored as follows: nitrogen, blue; and oxygen, red. A loop formed by residues 216−228 ("active-site lid") is colored magenta, while black dotted lines denote possible hydrogen bonds.

binding pocket of the active site of human DAO, and the hydroxyl and lactam NH groups of ACV interact with the side-chain hydroxyl group of Tyr-224 and the O4 atom of FAD, respectively. Thus, this model predicts that ACV binds to the active site of DAO through the above-mentioned interactions and thereby interferes with the proper orientation of the substrate in the active site, resulting in the inhibition of enzymatic activity.

Importance of the FAD Bound to the Enzyme and the Active Site Residue Tyr-224 for the Binding of ACV to DAO. To assess the validity of the proposed model, the issue of whether the FAD bound to DAO is involved in the interaction with ACV was addressed. For this, the dose-dependent effect of ACV on the enzymatic activity of DAO on D-Ala was examined in the presence of varying concentrations of FAD. Specifically, human DAO was preincubated with various concentrations (0.01−1000 μM) of ACV for 5 h at 0 °C in the presence of 2, 20, or 200 μM FAD before substrate addition, and DAO activity was then measured. The effect of benzoate was also tested as a control inhibitor. As expected, inhibition of DAO activity by benzoate in the presence of 2 μM FAD was relatively equal to that measured in the presence of 200 μM FAD (Figure 6A). By contrast, decreasing concentrations of FAD markedly reduced the inhibitory activity of ACV against DAO (Figure 6B). Thus, these results indicate that the FAD bound to DAO is important for its interaction with ACV.

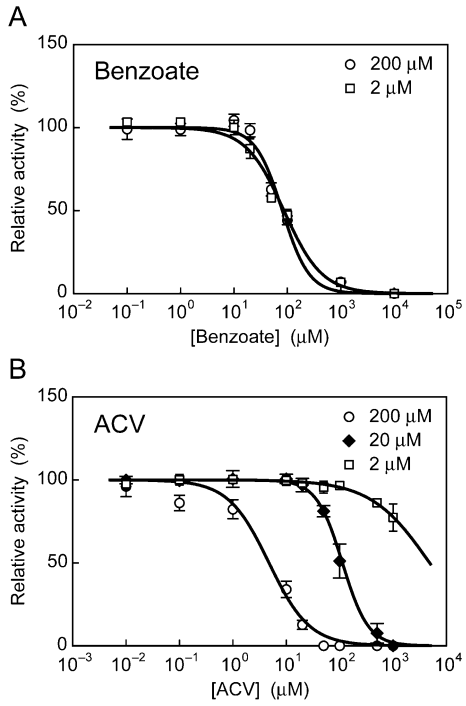

Figure 6. Sensitivity of human DAO to inhibitors in the presence of varying concentrations of FAD. Human DAO was preincubated with the indicated concentrations of benzoate for 5 h at 0 °C in the presence of 2 or 200 μM FAD before substrate addition, and then DAO activity was measured using D-Ala as a substrate (A). Similarly, human DAO was preincubated with the indicated concentrations of ACV for 5 h at 0 °C in the presence of 2, 20, or 200 μM FAD before substrate addition, and then DAO activity was measured using D-Ala as a substrate (B). Enzyme activity in the presence of inhibitors is presented as a percentage of its activity in the absence of inhibitors. Data are presented as the mean ± standard deviation (n = 3). Where not shown, error bars are smaller than the symbols used.

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Subsequently, the effects of ACV on the enzymatic activities of two human DAO mutants carrying either Tyr-224-to-Phe or Tyr-224-to-Ala substitutions (Y224F and Y224A variants, respectively) were examined to determine whether the side-chain hydroxyl group of Tyr-224 is also involved in the interaction with ACV. These variants were preincubated with various concentrations (0.01−1000 μM) of ACV for 5 h at 0 °C before substrate addition, and DAO activity was then measured. The effect of benzoate was also tested as a control inhibitor. The Y224F and Y224A variants were recently shown to retain the enzymatic activity on D-Ala,[32] and in this study, the final concentrations of substrate D-Ala in the assay were set at approximately six times higher than the independently determined K_m values for D-Ala (data not shown), as in the assay using the wild-type enzyme (details are provided in the Materials and Methods section). As expected, inhibition of the Y224F and Y224A variants by benzoate was significantly lower than that of the wild-type enzyme (Figure 7A). Specifically, the

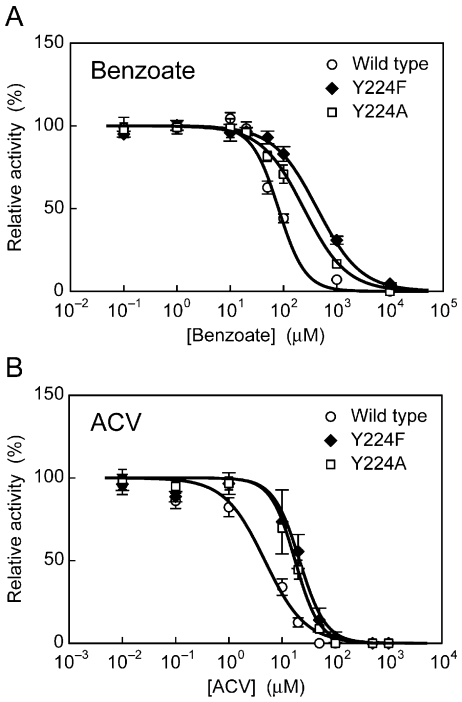

Figure 7. Sensitivity of human DAO and the Y224F and Y224A variants to inhibitors. Human DAO and its Y224F and Y224A variants were preincubated with the indicated concentrations of benzoate (A) and ACV (B) for 5 h at 0 °C before substrate addition, and DAO activity was then measured using D-Ala as a substrate. The activity of the enzyme in the presence of inhibitors is presented as a percentage of its activity in the absence of inhibitors. Data are presented as the mean ± standard deviation (n = 3). Where not shown, error bars are smaller than the symbols used.

IC_{50} values of benzoate for the Y224F and Y224A variants were 431 ± 55 and 242 ± 39 μM, respectively, which are approximately 3.0−5.4 times higher than the IC_{50} for the wild-type enzyme. Thus, introduction of Y224F or Y224A mutations into human DAO reduced its sensitivity to benzoate. These results indicated that the Tyr-224 residue of DAO is involved in the interaction with benzoate, which is consistent with the observations described above[28,29,48] (Figure 5A) and with our previous study.[32] Importantly, the Y224F and Y224A variants were also less sensitive to inhibition by ACV compared to the wild-type enzyme (Figure 7B). Specifically, the IC_{50} values of ACV for the Y224F and Y224A variants were 21.3 ± 6.8 and 17.5 ± 2.4 μM, respectively, which are approximately 3.7−4.5 times higher than the IC_{50} for the wild-type enzyme. Thus, the introduction of Y224F or Y224A mutations into human DAO reduced its sensitivity to ACV. Collectively, these results indicate that, in addition to the FAD bound to the enzyme, the side-chain hydroxyl group of the Tyr-224 residue of DAO is also an important determinant of ACV binding.

In the present study, manifold materials were screened for their ability to inhibit the enzymatic activity of DAO, and ACV was successfully identified as a novel DAO inhibitor. Notably, the materials screened in this study were primarily biologically active compounds of microbial origin and pre-existing drugs.[34] ACV is a well-known antiviral drug used for the treatment of herpesvirus infections.[36] It is of note that the mechanism of action underlying the antiviral activity of ACV[50,51] is not associated with its capacity to inhibit DAO. Thus, the inhibitory action of ACV on DAO is a novel function unveiled in the present study. The safety and pharmacokinetics of ACV have already been established. The mechanism of ACV action on DAO is unique in that it is a reversible slow-binding inhibitor. Therefore, it will be interesting to examine whether ACV is applicable as a candidate DAO inhibitor.

■ CONCLUSIONS

The present study demonstrates that ACV is a novel, active site-directed DAO inhibitor that can be used to investigate the structure−function relationships of DAO, including the molecular details of the active site environment of DAO. Interestingly, ACV acts on DAO as a reversible slow-binding inhibitor, and the time required to attain equilibrium between DAO, ACV, and the DAO/ACV complex is highly temperature-dependent. Since the temperature dependency of ACV binding to DAO is possibly due, at least in part, to differences between the structures of the DAO active site at different temperatures, ACV can also serve as an active site probe to study the structural basis for temperature-induced conformational changes of DAO.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

ACV, acyclovir; DAO, D-amino acid oxidase; DDO, D-aspartate oxidase; FAD, flavin adenine dinucleotide; HPLC, high-performance liquid chromatography; IC_{50}, 50% inhibitory concentration; NMDA, *N*-methyl-D-aspartate

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