Y-700 [1-[3-Cyano-4-(2,2-dimethylpropoxy)phenyl]-1H-pyrazole-4-carboxylic Acid]: A Potent Xanthine Oxidoreductase Inhibitor with Hepatic Excretion


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ABSTRACT

Y-700 (1-[3-Cyano-4-(2,2-dimethylpropoxy)phenyl]-1H-pyrazole-4-carboxylic acid) is a newly synthesized inhibitor of xanthine oxidoreductase (XOR). Steady-state kinetics with the bovine milk enzyme indicated a mixed type inhibition with $K_i$ and $K'_i$ values of 0.6 and 3.2 nM, respectively. Titration experiments showed that Y-700 bound tightly both to the active sulfo-form and to the inactive desulfo-form of the enzyme with $K_i$ values of 0.9 and 2.8 nM, respectively. X-ray crystallographic analysis of the enzyme-inhibitor complex revealed that Y-700 closely interacts with the channel leading to the molybdenum-pterin active site but does not directly coordinate to the molybdenum ion. In oxonate-treated rats, orally administered Y-700 (1–10 mg/kg) dose dependently lowered plasma urate levels. At a dose of 10 mg/kg, the hypouricemic action of Y-700 was more potent and of longer duration than that of 4-hydroxyxypyrrozolo(3,4-d)pyrimidine, whereas its action was approximately equivalent to that of 2-(3-cyano-4-isobutoxyphenyl)-4-methyl-5-thiazolecarboxylic acid, a nonpurine inhibitor of XOR. In normal rats, orally administered Y-700 (0.3–3 mg/kg) dose dependently reduced the urinary excretion of urate and allantoin, accompanied by an increase in the excretion of hypoxanthine and xanthine. Y-700 (1 mg/kg) was absorbed rapidly by the oral route with high bioavailability (84.1%). Y-700 was hardly excreted via the kidneys but was mainly cleared via the liver. These results suggest that Y-700 will be a promising candidate for the treatment of hyperuricemia and other diseases in which XOR may be involved.

XOR catalyzes the hydroxylation of hypoxanthine and xanthine, the last two steps in urate biosynthesis. The enzyme is found in a wide range of organisms from bacteria to man. It exists as a homodimer, each subunit of which contains one molybdo-pterin cofactor, two iron-sulfur clusters, and one FAD molecule (Bray, 1975; Hille, 1996). Mammalian XOR is synthesized as the dehydrogenase form, XDH (EC 1.1.1.204), and exists mostly as such in the cell, but can be readily converted to the oxidase form, XO (EC 1.1.3.22), by oxidation of sulphydryl residues or by proteolysis (Nishino, 1994; Hille and Nishino, 1995). XDH displays a preference for NAD+ reduction at the FAD site, whereas XO fails to react with NAD+ and exclusively uses molecular oxygen as its substrate, leading to the formation of superoxide anion and hydrogen peroxide (Hille and Nishino, 1995). XOR is a target of drugs against gout and hyperuricemia (Rundles et al., 1969), and the conversion of XDH to XO is of major interest because it has been implicated in diseases characterized by superoxide anion-induced tissue damage, such as postischemic-reperfusion injury (McCord, 1985). Allopurinol (Fig. 1), a purine analog, is a well-known inhibitor

ABBREVIATIONS: XOR, xanthine oxidoreductase; XDH, xanthine dehydrogenase; XO, xanthine oxidase; allopurinol, 4-hydroxyxypyrrozolo(3,4-d)pyrimidine; TEL-6720, 2-(3-cyano-4-isobutoxyphenyl)-4-methyl-5-thiazolecarboxylic acid; Y-700, 1-[3-cyano-4-(2,2-dimethylpropoxy)phenyl]-1H-pyrazole-4-carboxylic acid; AFR, activity/flavin ratio; HPLC, high-performance liquid chromatography; AUC, area under the curve; BOF-4272, sodium-8-(3-methoxy-4-phenylsulfanyl)phenylpyrazolo[1,5-a]1,3,5-triazine-4-olate monohydrate.
of XOR (Elion et al., 1963), and the drug has been applied for therapy of both the primary hyperuricemia of gout and that secondary to hematological disorders or antineoplastic therapy (Emmerson, 1996). The mechanism of inhibition of allopurinol has been studied in detail, indicating that oxipurinol, a product from allopurinol by XOR, binds stoichiometrically to Mo(IV) of the reduced form of the enzyme (Massey et al., 1970). Because the desulfo-form of XOR cannot be reduced with allopurinol, oxipurinol does not bind to the molybdenum of this form of the enzyme (Massey and Edmondson, 1970). The mechanism of inhibition of allopurinol by XOR is also expected to differ from that of allopurinol. Thus, we investigated the mechanism of inhibition of XOR by Y-700 on the basis of steady-state kinetic measurements and the 2.0-Å resolution crystallographic coordinates of the enzyme-inhibitor complex. Furthermore, the present article describes the pharmacokinetic properties of Y-700, particularly focusing on its elimination route in rats.

Materials and Methods

Chemicals

Y-700, 14C-labeled Y-700 (Fig. 1), and TEI-6720 were synthesized at Mitsubishi Pharma Corporation (Kanagawa, Japan). The specific activity of the 14C-Y-700 was 504.9 kBq/mg (13.6 μCi/mg). The radiochemical purity was 99% or more based on thin-layer chromatography, as determined by Daiichi Pure Chemicals Co., Ltd. (Ibaragi, Japan). Allopurinol was purchased from Sigma-Aldrich (St. Louis, MO). Oxipurinol was purchased from Wako Pure Chemicals (Osaka, Japan). Potassium oxonate, a uricase inhibitor, was purchased from Sigma-Aldrich. All other agents used were of the highest grade commercially available. For in vitro studies, Y-700 and allopurinol were dissolved in 0.1 M pyrophosphate buffer (pH 8.5), and oxipurinol was dissolved in 0.1 M NaOH. For oral administration to animals, Y-700, allopurinol, and TEI-6720 were ultrasonically suspended in 0.5% (w/v) hydroxypropylmethylcellulose aqueous solution. For intravenous administration, Y-700 was dissolved in saturated sodium bicarbonate aqueous solution containing 10% (w/v) polyethylene glycol 400. Potassium oxonate was ultrasonically suspended in physiological saline.

Enzyme Preparation

XO and XDH forms of the bovine milk enzyme were prepared according to the methods of Eger et al. (2000) and Okamoto et al. (2000). Both the fully active forms, as defined by their AFR, were obtained by further purification applying folate affinity chromatography as described by Nishino et al. (1981). Purified enzyme was stored on ice without freezing in a solution containing 20 mM pyro-
phosphate buffer (pH 8.5), 40 mM Tris-HCl buffer (pH 7.8), 1 mM salicylate, and 0.2 mM EDTA. The enzyme used was more than 85% active, i.e., it exhibited an AFR of more than 182. AFR means the enzyme activity defined as the absorbance change per minute at 295 nm (monitoring conversion of xanthine to uric acid), divided by the enzyme absorbance at 450 nm in the standard assay condition (Massey and Edmondson, 1970; Massey et al., 1970). The inactive desulfoform of XO was prepared by incubating the enzyme in 10 mM KCN for 1 h at 25°C followed by gel filtration on a PD-10 column (Amersham Biosciences AB, Upsala, Sweden) to remove KCN (Massey and Edmondson, 1970). Oxipurinol-bound XO was prepared by the method of Nishino et al. (1981) with minor modifications. Briefly, 2 ml of 6 μM enzyme was placed in the body of a Thunberg tube. The sidearm contained 20 μl of 10 mM oxipurinol and 50 μl of 10 mM hypoxanthine. The reagents were mixed with the enzyme anaerobically and incubated at room temperature for 1 h. Under aerobic conditions, oxipurinol-bound enzyme was purified by passage through a PD-10 column followed by application to a Sepharose-4B/folate affinity column. The XO concentration was determined spectrophotometrically using a molar coefficient absorbance of 37,800 M/cm at 450 nm (Massey et al., 1969). Spectrophotometric measurements were conducted on a spectrophotometer (U-3300; Hitachi Ltd., Tokyo, Japan) equipped with a thermostated cell holder.

**Enzyme Assay**

The molybdenum-pterin sites of both XDH and XO are structurally equivalent (Enroth et al., 2000). Thus, steady-state kinetics of the product inhibition-free xanthine-oxygen reductase activity of XO were measured spectrophotometrically by following the increase in absorbance of uric acid at 295 nm in 0.1 M pyrophosphate buffer (pH 8.5) containing 0.2 mM EDTA and various concentrations of xanthine and Y-700 under air-saturated conditions. All activity measurements were performed at 25°C.

**Determination of Dissociation Constant by Titration of XO with Y-700**

The $K_d$ values for the enzyme were determined by titrating XO with Y-700. The mixture of Y-700 and the enzyme was incubated for 5 min in the dark at 25°C to allow equilibration before the fluorescence (excitation at 310 nm and emission at 355 nm) of the solution was measured. The $K_d$ values were calculated from the plots of fluorescence versus the total concentrations of added inhibitor, as previously described (Okamoto and Nishino, 1995; Okamoto et al., 2003). The measurements of fluorescence intensity of Y-700 were conducted on a fluorescence spectrophotometer (FP-777; Jasco, Tokyo, Japan) equipped with a thermostated cell holder.

**Preparation of Y-700-Bound Active XDH Crystals**

Before crystallization, active XDH was passed through a Sephadex G-25 column (Amersham Biosciences AB) to remove salicylate. The eluate was then brought to a concentration of ca. 75 mg/ml using YM-100 concentrators (Millicron Corporation, Bedford, MA). Crystals could be grown under conditions very similar to the ones described by Okamoto et al. (2003). Thus, crystallization was carried out at 20°C employing an enzyme concentration of 7.5 mg/ml in a solution containing 50 mM potassium phosphate buffer (pH 6.5), 5 mM dithiothreitol, 1 mM salicylate, 0.2 mM EDTA, 30% glycerol, 0.5 mM Y-700, as well as 6 to 10% (w/v) polyethylene glycol 4000 as a precipitant.

**Data Collection for X-Ray Crystallography**

Crystals of the enzyme inhibitor complex were flash-frozen with their mother liquor as a cryoprotectant and mounted in cryoloops. Diffraction data were collected at beam line BL40B2 (Spring8, Harima Garden City, Japan); a temperature of 100K, radiation of 1.00 Å wavelength, and a Q4 area detector (ACSD) were used. Data were reduced with the help of the program package DENZO and scaled using SCALEPACK (Otwinowski and Minor, 1997).

**Crystal Structure Determination**

The program package EPMR (Kissinger et al., 1999) established the correct solutions of the rotational and translational components of the molecular replacement function (20.0–4.0-Å resolution range). Salicylate-bound bovine milk XDH (Protein Data Bank code 1F4Q) was employed as a search model. Molecular models were built with the help of the program package O (Jones et al., 1991). Subsequent refinement, including rigid body, simulated annealing, grouped B factors, and least squares minimization were carried out with CNS, version 1.0 (Brunger et al., 1998). No NCS restraints were used in the final round of the refinements. Figures were generated with MOLSCRIPT (Kraulis, 1991) and RASTER3D (Merritt and Bacon, 1997).

**Animals**

Male 6-week-old Sprague-Dawley rats were purchased from Charles River Japan, Inc. (Kanagawa, Japan). Animals were used after adaptation to their environment and were housed under a 12-h light/dark cycle (light period 6:30 AM to 6:30 PM). All animal experiments were in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and were approved in advance by the Committee of Animal Experiments in Research Laboratories of Mitsubishi Pharma Corporation.

**Pharmacodynamic Study**

**Hypouricemic Effects in Oxonate-Treated Rats.** Hypouricemic action of orally administered Y-700 was examined and compared with those of allopurinol and TEI-6720 in potassium oxonate (4,6-dihydroxy-1,3,5-triazine-2-carboxylic acid potassium salt)-treated rats. The doses (1, 3, and 10 mg/kg) of Y-700 were chosen because our previous study (Ishibuchi et al., 2001) showed that the compound has a hypouricemic effect in the same rat model of hyperuricemia with an ED$_{50}$ value of 1.4 mg/kg p.o. Eight rats were allocated to each group. As previously described (Ishibuchi et al., 2001), animals were repeatedly treated with the uricase inhibitor, potassium oxonate (250 mg/kg s.c.), 1 h before drug administration, and 3, 7, and 23 h afterward. Under halothane anesthesia, blood samples (100 μl each) were taken into a heparin-coated tube, immediately before potassium oxonate treatment and at 2, 4, 6, 10, and 24 h after drug administration, by cutting the tail tip. Plasma was obtained by centrifugation (3000/min, 10 min) at room temperature. Plasma urate levels were determined by a phosphotungstic acid colorimetric method using a commercially obtained kit (Wako Pure Chemicals) and a spectrophotometer (Ube50, Jasco).

**Effect on Urinary Excretion of Purine Metabolites in Normal Rats.** The suppressive effect of Y-700 on urate biosynthesis was examined in normal rats by analyzing the changes in amounts of purine metabolites (hypoxanthine, xanthine, urate, and allantoin) excreted in urine. Six animals were allocated to each group and were fasted for 18 h before being given oral doses of the drugs. The animals were placed in individual metabolic cages, and the urine was collected from the time of drug administration to 2 h postadministration. Animals were allowed free access to tap water during urine collection. At the end of collection, any urine remaining in the bladder was forcibly discharged. Urinary volume was calculated from its weight and specific gravity.

**Measurement of Urinary Hypoxanthine, Xanthine, Urate, and Allantoin.** The urine was centrifuged (3000/min, 10 min) at room temperature for analysis of the supernatant. Hypoxanthine, xanthine, urate, and allantoin were determined by HPLC (System D-6100, Hitachi Ltd.) using a separation column (YM-C-Pack ODS-A, 150×6.0-mm i.d.; YMC, Inc., Wilmington, NC) at 30°C. Ammonium dihydrogenphosphate buffer (50 mM, pH 4.5) was used as the mobile phase at a flow rate of 1.0 ml/min. Hypoxanthine and xanthine were...
detected spectrophotometrically at 250 nm. Urate and allantoin were detected in the same way at 285 and 210 nm, respectively.

**Pharmacokinetic Study**

**Determination of Y-700 Concentrations in Plasma and Urine.** Rats received Y-700 at doses of 0.3, 1, and 3 mg/kg p.o. and 1 mg/kg i.v. Under halothane anesthesia, blood (200–300 μl) was collected into a heparin-coated tube by cutting the tail at the following sampling times: 0.08 (i.v. only), 0.25, 0.5, 1, 2, 4, 8, 12, and 24 h. Plasma was obtained by centrifugation (3000/min, 10 min) at room temperature and stored at −20°C until HPLC analysis.

Concentrations of Y-700 in rat plasma were determined by HPLC with fluorescence detection. Briefly, an aliquot of plasma (100 μl) was added to 10 μl of 1 M hydrochloric acid and 2 ml of toluene, and the mixture was vigorously shaken for 10 min. After centrifugation (3000/min, 10 min), the organic layer (1.6 ml) was separated and evaporated to dryness at 40°C for 30 min with a centrifugal concentrator. The residue was reconstituted in 200 μl of mobile phase as described below. A 10-μl aliquot was injected into the HPLC for analysis. The chromatographic system consisted of a system controller SCL-10A, an auto-injector SIL-10A, a pump LC-10AD, a computing integrator C-R6A, a spectrofluorometric detector RF-10A (Shimadzu, Kyoto, Japan), and a degasser KT-16. The residue was reconstituted in 200 ml of mobile phase as described below. A 10-μl aliquot was injected into the HPLC for analysis. The chromatographic system consisted of a system controller SCL-10A, an auto-injector SIL-10A (Shimadzu Co.), a UV detector UV-8000 (Tosoh Co., Tokyo, Japan) and a radiochemical detector LB506C (Berthold Technologies, Bad Wildbad, Germany). Chromatography was performed on an L-column ODS (250 × 4.6 mm, Shimadzu) and was maintained at 40°C.

Concentrations of Y-700 were determined spectrophotometrically at 295 nm. Urate and allantoin were detected spectrophotometrically at 295 nm. Urate and allantoin were detected spectrophotometrically at 295 nm. Urate and allantoin were detected spectrophotometrically at 295 nm. Urate and allantoin were detected spectrophotometrically at 295 nm. Urate and allantoin were detected spectrophotometrically at 295 nm. Urate and allantoin were detected spectrophotometrically at 295 nm. Urate and allantoin were detected spectrophotometrically at 295 nm. Urate and allantoin were detected spectrophotometrically at 295 nm.
mm., Chemicals Evaluation and Research Institute, Tokyo, Japan) at ambient temperature eluted at 1 ml/min. Eluents were 0.1 M ammonium acetate solution, pH 4.5 (A)/acetonitrile (B) (93/7) for 2 min, followed by a linear gradient to A/B (55/45) at 15 min, a linear gradient to A/B (48/52) at 25 min, and a linear gradient to A/B (0/100) at 27 min, and finally isocratic elution for 3 min. The UV detector monitored at 268 nm. Unchanged drug was identified from the retention time, compared with that of a synthetic standard, Y-700.

### Analysis of Pharmacokinetics

The maximum plasma concentration ($C_{\text{max}}$) and the time of the maximum plasma concentration ($t_{\text{max}}$) were obtained from the measured values. Linear regression was performed on the linear part of the terminal phase (p.o. groups, from 4–24 h after administration; i.v. groups, from 2–12 h after administration) of the plasma concentration-time curve, in which the concentration was expressed logarithmically by the least squares method. The elimination rate constant ($k_e$) was calculated from the slope of the linear regression curve. The elimination half-life ($t_{1/2}$) was calculated according to the equation $t_{1/2} = \ln 2/k_e$. The area under the plasma concentration time curve (AUC) from 0 (immediately after administration) to infinite time ($\text{AUC}_0-\infty$) was a summation of AUC from 0 to the last measurement time point (t) ($\text{AUC}_0-t$) and AUC from time t to infinite time (AUC$_{t-\infty}$) that was calculated according to $C_t/k_e$ ($C_t$, plasma concentration at time t). Here, AUC$_{0-t}$ was obtained by the trapezoidal method. Total body clearance (CL) was obtained as dose/AUC$_{0-\infty}$, and volume of distribution at the steady-state (Vss) was calculated according to the equation $V_{\text{ss}} = \text{MRT} \times \text{CL}$, where MRT was obtained by the moment analysis method.

### Statistical Analysis

Data were expressed as the means ± S.D. Differences in the means of plasma urate levels and amounts of urinary purine metabolites between the control group and groups that received test drugs were analyzed by analysis of variance using Dunnett’s multiple comparison test. Differences were assessed with two-sided tests, with an $\alpha$ level of 0.05. Values of $C_{\text{max}}$ and AUC$_{0-\infty}$ versus doses were logarithmically plotted, and the slopes of the plots were calculated by linear regression according to a least squares procedure. The criterion for linearity was for the slope of the regression curve to be within the range from 0.85 to 1.15.

### Results

#### Steady-State Kinetic Analysis of Inhibition of XO with Y-700

Steady-state kinetic analyses of Y-700 inhibition of the enzyme activity were performed with various concentrations of xanthine and the compound. The elimination rate constant ($k_e$) was calculated from the slope of the linear regression curve. The elimination half-life ($t_{1/2}$) was calculated according to the equation $t_{1/2} = \ln 2/k_e$. The area under the plasma concentration time curve (AUC) from 0 (immediately after administration) to infinite time ($\text{AUC}_0-\infty$) was a summation of AUC from 0 to the last measurement time point (t) ($\text{AUC}_0-t$) and AUC from time t to infinite time (AUC$_{t-\infty}$) that was calculated according to $C_t/k_e$ ($C_t$, plasma concentration at time t). Here, AUC$_{0-t}$ was obtained by the trapezoidal method. Total body clearance (CL) was obtained as dose/AUC$_{0-\infty}$, and volume of distribution at the steady-state (Vss) was calculated according to the equation $V_{\text{ss}} = \text{MRT} \times \text{CL}$, where MRT was obtained by the moment analysis method.

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### Table 1

<table>
<thead>
<tr>
<th>Crystallographic statistics</th>
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<tr>
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RMSD, root mean square deviation.

$R_{\text{cryst}} = \sum_{hkl} |F_{\text{calc}} - F_{\text{obs}}| / \sum_{hkl} F_{\text{obs}}$, where $F_{\text{calc}}$ and $F_{\text{obs}}$ are the observed and the calculated structure factors, respectively, and the summation is over the reflections used for model refinement.

#### Fig. 4

Electron density representing Y-700 bound to bovine XDH. Stereo representation of the $F_o-F_c$ electron density corresponding to Y-700 (purple), contoured at a 3.0 $\sigma$ cutoff, and the $2F_o-F_c$ electron density corresponding to adjacent amino acid residues and the molybdenum cofactor (brown), contoured at a 1.8 $\sigma$ cutoff. The $F_o-F_c$ density was calculated before introducing the Y-700 molecules into the crystallographic model to avoid model bias. The molybdenum-pterin cofactor (cyan) and the 700 molecule (yellow) are shown, as are adjacent side chains in ball and stick representation.

#### Fig. 5

Interaction of Y-700 with residues lining the access channel to the molybdenum-pterin cofactor. Hydrophilic (A) and hydrophobic residues (B) surrounding the Y-700 molecule are shown in stick representation and labeled with their residue numbers. Y-700 itself is shown in green.
Burk plots of the enzyme inhibition in the presence or absence of Y-700 indicated that inhibition by the compound was of the mixed type (Fig. 2). The result obtained from a plot of the slope from the primary plot versus the inhibitor concentration indicated a $K_i$ value of 0.6 ± 0.2 nM for Y-700, whereas a plot of the apparent $V_{\text{max}}$ versus the inhibitor concentrations indicated a $K_i'$ value of 3.2 ± 0.6 nM.

**Titration of XO with Y-700.** Y-700's fluorescence at 355 nm (excitation at 310 nm) was quenched upon the formation of the enzyme complex. Using this property of Y-700, we determined the dissociation constants of the enzyme-inhibitor complexes of both the fully active XO (AFR = 191) and the desulfo-form of the enzyme (AFR = 0.6). Figure 3 shows the titration pattern of each enzyme type with Y-700. The fluorescence quenching by XO was dependent on the enzyme concentration, and the fluorescence increased immediately after an equimolar amount of Y-700 with the enzyme was reached. The $K_i$ values of the active and desulfo-forms of XO for Y-700 were calculated as 0.9 ± 0.3 and 2.8 ± 1.1 nM, respectively, from each plot (three tests). The $K_i$ value of the active XO for Y-700 was in reasonable agreement with the $K_i$ value for the compound estimated from the steady-state kinetic studies. In contrast, the fluorescence increased almost linearly with the concentration of Y-700 when oxipurinol-bound XO was titrated with the compound (Fig. 3).

**X-Ray Crystal Structure Analysis of the Enzyme-Inhibitor Complex.** Y-700-bound XDH crystallized in space group C2 with unit cell axes $a = 166.8$ Å, $b = 123.9$ Å, $c = 148.9$ Å, and $\beta = 91.2^\circ$ with two subunits in the asymmetric unit. The molecular model was refined at 2.0-Å resolution to an $R_{\text{free}}$ value of 22%. For some residues (166–191, 532–536) in interdomain loops, no corresponding electron density was observed. Statistics of data collection and refinement are shown in Table 1. The atomic coordinates and structure factors (code 1VDV) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics (Rutgers University, New Brunswick, NJ; http://www.rcsb.org/).

Electron density that was not explained by the protein or its cofactors was found in the channel leading from the enzyme's surface to the active center molybdenum (Fig. 4). Its shape unequivocally indicated that it represented a bound Y-700 molecule. The interactions of Y-700 with several residues lining the access channel to molybdenum-pterin are shown in Fig. 5. The atom closest to the molybdenum-coordinated sphere was the C-3 of the pyrazole ring with a distance of 6.0 Å to the molybdenum atom and 4.2 Å to the catalytically exchangeable oxygen. No electron density between Y-700 and molybdenum was observed. However, the carboxyl oxygen of Y-700 and the catalytically exchangeable oxygen were both linked via hydrogen bonds to water W498. The carboxyl oxygen of Y-700 was also bridged to the side-chain carboxyl group of Glu1261 via waters W62 and W498. W62 was equidistant (2.6 Å) between the oxygen atoms of Y-700 and Glu1261. The distance from W498 to the atom of Y-700 and to that of Glu1261 was 2.8 Å and 2.6 Å, respectively. The second carboxyl oxygen of Y-700 and the side-chain guanidinium group of Arg880 were involved in an electrostatic interaction (minimum distance of 2.8 Å). The same carboxyl oxygen atom also interacted with the backbone amide of Thr1010 (2.9 Å). There is also a hydrogen bond formed between the side chain amide of Asn768 and the nitrile group of Y-700 (3.2 Å). Moreover, hydrogen bonding between the carboxyl group of Glu802 and the N-2 of the pyrazole ring of the inhibitor occurred (2.7 Å). The pyrazole ring of Y-700 was sandwiched between the aromatic side chains of Phe914 and Phe1009 with minimum distances of 3.5 and 3.7 Å, respectively. The ring of Phe914 was parallel to the plane of the pyrazole ring, whereas that of Phe1009 was perpendicular. The phenyl ring of Y-700 was inserted between Leu873 and Leu1014 with a distance of 3.6 Å to both residues. Surrounded by several hydrophobic residues, the 2,2-dimethylpropoxy moiety of Y-700 was positioned at the entrance of the active site-directed channel.

**Safety in Animal Experiments.** No abnormality definitely attributable to the test drugs was observed throughout...
the entire period of the present animal experiments (data not shown).

**Pharmacodynamics.** Fig. 6 shows the time course of changes in plasma urate levels in oxonate-treated rats. Repeated subcutaneous injection of the uricase inhibitor, potassium oxonate, caused a marked increase in rat plasma urate levels, and the increase was maintained throughout the experiment. In this condition, the hypouricemic effect of Y-700 was examined, in comparison with those of allopurinol and TEI-6720. Y-700 (1, 3, and 10 mg/kg p.o.) dose dependently decreased plasma urate levels, and the action was evident by 10 h after oral administration at doses over 3 mg/kg. Allopurinol (10 mg/kg p.o.) also displayed hypouricemic action, although the action was less potent and of shorter duration than that of Y-700. The hypouricemic effect of TEI-6720 (10 mg/kg p.o.) was very similar in potency and duration to that of Y-700.

Figure 7 shows the effects of Y-700 and allopurinol on urinary purine metabolites, which were determined within 6 h after oral administration to normal rats. Both Y-700 and allopurinol dose dependently decreased amounts of urinary allantoin but not urate, increasing the amounts of oxypurines (hypoxanthine and xanthine), the substrates of XOR. In terms of the observed effect, Y-700 appeared to be 10 to 30 times more potent than allopurinol. Both compounds had no effect on the urinary volume at any of the doses applied (data not shown).

**Pharmacokinetics.** The plasma concentration-time curve of Y-700 (unchanged drug) and pharmacokinetic parameters are shown in Fig. 8 and Table 2. After oral administration of Y-700 to rats (0.3, 1, and 3 mg/kg), the concentrations of unchanged drug in plasma reached $C_{\text{max}}$ at 0.3 to 0.5 h. The $t_{1/2}$ was 2.7 to 5.0 h. $C_{\text{max}}$ and AUC$_{0-\infty}$ increased nearly in parallel with the increasing Y-700 doses. CL and V$_{ss}$ calculated from the concentrations after intravenous administration were 0.12 ±...
Ketry. This finding indicates that Y-700 binds tightly not only the binding of inhibitor to enzyme followed a 1:1 stoichiometric equimolar amount of Y-700 had been added, evidence that predominantly to the oxidized form of XOR.

... explanation for the mixed-type inhibition of Y-700 is that the reoxidizes Mo(IV) very rapidly (Okamoto and Nishino, 1995; BOF-4272 and TEI-6720 show a competitive inhibitory effect methosulfate (PMS) is used as an electron acceptor, both (Massey et al., 1969; Olson et al., 1974). When phenazine methosulfate for xanthine, XOR acts via a ping-pong mechanism, altering between oxidized and reduced forms... 6720 (Okamoto et al., 2003). When oxygen is the terminal electron acceptor for XOR, the mechanism of inhibition against the enzyme activity by obstructing access of substrate to the active site. We also clarified that Y-700 is absorbed with high... active desulfo-form (Fig. 1), a phenyl 4-isobutoxy analog, when each... is positioned closest to the molybdenum but might overlap the product from the molybdenum coordination sphere. In the structure of the Y-700 complex, we found two water molecules (W62 and W498) near the molybdenum ion (Fig. 5A). The structural basis of enzyme inhibition by Y-700 is very... and hydrophobic interactions.

**Discussion**

In the present study, we demonstrated that the mechanism of inhibition of Y-700 is distinctly different from that of allopurinol. X-ray crystallographic analysis showed that a Y-700 molecule filled the entire pocket, thereby inhibiting the enzyme activity by obstructing access of substrate to the active site. We also clarified that Y-700 is absorbed with high oral bioavailability and is predominantly eliminated via the liver in normal rats.

The steady-state kinetic analysis under air-saturated conditions showed that the mechanism of inhibition against bovine milk XO by Y-700 was of a mixed type, and the $K_I$ and $K_{II}$ values for this compound were 0.6 and 3.2 nM, respectively. Similar findings have been reported with two other inhibitors, BOF-4272 (Okamoto and Nishino, 1995) and TEI-6720 (Okamoto et al., 2003). When oxygen is the terminal electron acceptor for xanthine, XOR acts via a ping-pong mechanism, altering between oxidized and reduced forms (Massey et al., 1969; Olson et al., 1974). When phenazine methosulfate (PMS) is used as an electron acceptor, both BOF-4272 and TEI-6720 show a competitive inhibitory effect on the bovine milk enzyme because phenazine methosulfate reoxidizes Mo(V) very rapidly (Okamoto and Nishino, 1995; Okamoto et al., 2003). Referring to the data, an appropriate explanation for the mixed-type inhibition of Y-700 is that the compound binds to both oxidized and reduced forms but predominantly to the oxidized form of XOR.

In the enzyme titration, fluorescence increased only after the enzyme-inhibitor complex showed no covalent bond between Y-700 and molybdenum. Instead, Y-700 was bound in a narrow channel leading to the molybdenum center of the enzyme, through a variety of hydrogen bonds and hydrophobic interactions.

The structural basis of enzyme inhibition by Y-700 is very similar to that of TEI-6720, which has been described in detail by Okamoto et al. (2003). However, the potency of Y-700 to inhibit XOR was slightly weaker than that of TEI-6720; the $K_I$ and $K_{II}$ values of TEI-6720 are 0.12 and 0.9 nM, respectively. The difference in the steric bulk of the heterocyclic rings is a plausible explanation for the potency difference. First, the sulfur atom of TEI-6720 is nearly twice as large as the corresponding C-5 of Y-700. Second, the methyl group of TEI-6720 adds additional bulk and hydrophobicity; Y-700 lacks such a substituent. Thus, the thiazole of TEI-6720 more completely fills the binding pocket.

Recently, we solved the crystal structure of a reaction intermediate of XDH (Okamoto et al., 2004). Based on this result, we propose that the breakdown of the enzyme-product intermediate proceeds via hydroxide displacement of the product from the molybdenum coordination sphere. In the structure of the Y-700 complex, we found two water molecules (W62 and W498) near the molybdenum ion (Fig. 5A). W62 is located near the side chain of Glu1261, 5.2 Å from the closest atom of the molybdenum coordination sphere. W498 is positioned closest to the molybdenum but might overlap the location of the purine substrate. One of these waters should be the one to be incorporated as the replacement ligand for the released product, after which it will undergo base-assisted nucleophilic attack on the next substrate molecule.

Even though TEI-6720 was more potent in vitro, the hypouricemic effect of Y-700 was equivalent in vivo. We previously demonstrated that the AUC$_{0-24}$ (25.63 µg/h/ml) of Y-700 is remarkably higher than that (5.38 µg/h/ml) of compound 5d (Fig. 1), a phenyl 4-isobutoxy analog, when each compound is orally administered (3 mg/kg) to oxonate-treated rats (Ishibuchi et al., 2001). This finding suggests that the 2,2-dimethylpropoxy moiety of Y-700 contributes to its excellent bioavailability in vivo rather than to its inhibitory efficacy against XOR in vitro. The present pharmacokinetic assessment also showed that Y-700 has an excellent bioavailability (84.1%) at a dose of 1 mg/kg, displaying no evidence of any major circulating metabolites. Comprehensive studies on the metabolism of Y-700 are now in progress, and the structures of the major metabolites in urine and feces will be reported elsewhere. To our knowledge, there is no report on the pharmacokinetics of TEI-6720. Nevertheless, it can be stated that the in vivo suppressive efficacy of Y-700 on urate biosynthesis is no less potent than that of TEI-6720.
Almost all of the amino acids that interact with the Y-700 molecule are conserved in the bovine (Berglund et al., 1996) and human enzymes (Ichida et al., 1993). The only exceptions are Leu648 and Phe649 (bovine XOR), which are changed to Ile and Cys in human XOR, respectively. However, these changes still preserve the hydrophobic character of the side chains, and this is important in the interaction with the Y-700 molecule. Therefore, the mechanism of the enzyme inhibition by Y-700 seems applicable to human XOR as well.

In oxonate-treated rats, the hypouricemic effect of allopurinol (10 mg/kg) was only evident by 6 h after oral administration. Other investigators have also reported that the hypouricemic effect of allopurinol is rapid in onset and short in duration in rodents (Osada et al., 1993; Naito et al., 2000). By contrast, in patients with normal renal function, allopurinol decreases serum urate levels within a few days (Fox, 1993). This discrepancy might be explained by differences in the clearance of allopurinol between humans and animals. Indeed, both allopurinol and oxipurinol are cleared rapidly via the kidneys in mice and dogs (Elion, 1966). In humans, allopurinol is also cleared rapidly, but oxipurinol resembles urate in having a slow clearance (Elion, 1966). Although we must be prudent when extrapolating the efficacy of XOR inhibitors in animals, a potent and long-lasting hypouricemic action of Y-700 can be explained, in part, by its distinctive mechanism of enzyme inhibition compared with that of allopurinol. The oxipurinol-inhibited enzyme can be reactivated with a half-time of 300 min at 25°C by spontaneous reoxidation of the molybdenum cofactor (Massey et al., 1970). On the other hand, because Y-700 binds tightly to both the oxidized and reduced forms of XOR, it can be expected to inhibit the enzyme for longer periods than allopurinol.

In most mammals except for humans and certain other primates, the end product of purine metabolism is allantoin rather than urate because a large fraction of the extracellular urate is converted to inosine-5′-monophosphate (Rundles et al., 1969). This is due to the fact that mammalian XOR contains a carboxyl group within its structure, which is possible that this compound might be a substrate for organic anion transporters in the liver, although the exact mechanism of elimination of this drug remains to be examined in more detail.

In conclusion, we have demonstrated that Y-700 is a potent and orally effective XOR inhibitor with hepatic excretion. It is expected that Y-700 can provide stable and safe management of hyperuricemia, even in patients with renal disorder. Moreover, Y-700 will be an alternative probe to investigate pathogenic processes, such as postischemic-reperfusion injury, in which XOR may be involved.

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References


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