An Extremely Potent Inhibitor of Xanthine Oxidoreductase

CRYSTAL STRUCTURE OF THE ENZYME-INHIBITOR COMPLEX AND MECHANISM OF INHIBITION

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TEI-6720 (2-(3-cyano-4-isobutoxyphenyl)-4-methyl-5-thiazolecarboxylic acid) is an extremely potent inhibitor of xanthine oxidoreductase. Steady state kinetics measurements exhibit mixed type inhibition with $K_i$ and $K_i'$ values of 1.2 ± 0.05 × 10^{-10}$ M and 9 ± 0.05 × 10^{-10}$ M, respectively. Fluorescence-monitored titration experiments showed that TEI-6720 bound very tightly to both the active and the inactive desulfo-form of the enzyme. The dissociation constant determined for the desulfo-form was 2 ± 0.03 × 10^{-10}$ M; for the active form, the corresponding number was too low to allow accurate measurements. The crystal structure of the active sulfo-form of milk xanthine dehydrogenase complexed with TEI-6720 and determined at 2.8-A resolution revealed the inhibitor molecule bound in a long, narrow channel leading to the molybdenum-pterin active site of the enzyme. It filled up most of the channel and the immediate environment of the cofactor, very effectively inhibiting the activity of the enzyme through the prevention of substrate binding. Although the inhibitor did not directly coordinate to the molybdenum ion, numerous hydrogen bonds as well as hydrophobic interactions with the protein matrix were observed, most of which are also used in substrate recognition.

Xanthine oxidoreductase (XOR) enzymes have been isolated from a wide range of organisms, from bacteria to man, and they accelerate the hydroxylation of a wide variety of purine, pyrimidine, pterin, and aldehyde substrates. All of these proteins have similar molecular weights and composition of redox centers (1, 2). In humans, the enzyme catalyzes the last two steps of purine catabolism, the oxidation of hypoxanthine to xanthine and of xanthine to uric acid. This reaction occurs at a molybdenum-pterin center and from there the electrons are transferred via two Fe$_2$S$_2$ clusters to the isoalloxazine ring of FAD, which then passes them on to the second substrate NAD$^+$ (1–5).

XOR is synthesized as xanthine dehydrogenase (XDH; EC 1.1.1.204) with very low reactivity toward molecular oxygen but high reactivity toward NAD$^+$ (6, 7). In mammals, however, XDH can easily be converted to xanthine oxidase (XO; EC 1.1.3.22), which does not interact with NAD$^+$ but is very efficient in producing superoxide anion (O$_2^-$) and H$_2$O$_2$ instead. The conversion is initiated either by formation of intramolecular disulfide bonds or by proteolytic cleavage of a loop region connecting the FAD-binding domain and the molybdenum-binding domain (8). This conversion has been implicated in O$_2^-$-mediated stress phenomena such as postischemic reperfusion injury (9). In an effort to elucidate the structural basis for these effects, we recently determined the crystal structures of both the XDH and XO forms of the bovine milk enzyme, a very close homologue of the human enzyme, at 2.1- and 2.5-A resolutions, respectively. These analyses showed that structural rearrangements next to the FAD cause this remarkable change of reactivity (10).

Allopurinol (Fig. 1A), an analogue of hypoxanthine, was developed by Elion et al. (11) as an XDH inhibitor 30 years ago and has been widely prescribed as a treatment of hyperuricemia and gout since (12). In addition, administration of allopurinol has been reported to prevent postischemic tissue damage by inhibiting XO activity (9). In some cases, however, severe life-threatening side effects have been reported, such as a toxicity syndrome dramatized by eosinophilia, vasculitis, rash hepatitis, and progressive renal failure (12). The intrinsic radical-scavenging features of allopurinol (13) make it difficult to distinguish between the effects it causes directly and the effects produced by XOR inhibition, e.g. in the production of radical species in reperfusion injury.

As oxypurinol, the oxidation product of allopurinol, is the actual inhibiting species (it coordinates to the reduced molybdenum center of XOR), a lag phase for complete inhibition is to be expected. Once inhibited, the enzyme can also be reactivated by spontaneous reoxidation of the metal cofactor ($t_{1/2} = 300$ min at 25 °C) (14). This feature requires the administration of at
least three relatively high doses of the drug per day to keep the plasma level of the drug at an effective concentration. Because of these shortcomings, new potent inhibitors, preferentially those with well defined inhibition mechanisms, are still most useful both under clinical and scientific experimental aspects. A potential single-dose, low-concentration regiment would be useful both under clinical and scientific experimental aspects.

We have undertaken a series of investigations aimed at providing more information about the kinetic and structural properties of recently developed XOR inhibitors. As a first step into this direction, we described the inhibition mechanism of a newly introduced inhibitor, BOF-4272 (sodium-8-(3-methoxy-4-phenylsulfinyl-phenyl) pyrazolo[1,5-a]pyrimidine) (16), which has been tested in animal studies. We have undertaken a series of investigations aimed at providing more information about the kinetic and structural properties of recently developed XOR inhibitors. As a first step into this direction, we described the inhibition mechanism of a newly introduced inhibitor, BOF-4272 (sodium-8-(3-methoxy-4-phenylsulfinyl-phenyl) pyrazolo[1,5-a]pyrimidine) (16), which has been tested in animal studies. We have undertaken a series of investigations aimed at providing more information about the kinetic and structural properties of recently developed XOR inhibitors. As a first step into this direction, we described the inhibition mechanism of a newly introduced inhibitor, BOF-4272 (sodium-8-(3-methoxy-4-phenylsulfinyl-phenyl) pyrazolo[1,5-a]pyrimidine) (16), which has been tested in animal studies.

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Tight-binding Inhibitor Complexes of Xanthine Oxidoreductase

Fig. 2. Time course of inhibition of XO activity by TEI-6720 and allopurinol. Reactions were performed under standard conditions and started by adding 2 nM XO (AFR = 160). Closed circles, no inhibitor; closed squares, in the presence of 53 nM TEI-6720; open triangles, in the presence of 3.3 M allopurinol.

RESULTS AND DISCUSSION

Time Course of TEI-6720 Inhibition—As is commonly observed in tight-binding inhibitors (36), both allopurinol and TEI-6720 show time-dependent inhibition (Fig. 2). The underlying reasons for their time dependence, however, are quite different. A relatively large excess of 3.3 M allopurinol reduced the rate gradually until complete inactivation was achieved; in contrast, a slight excess of 33 nM TEI-6720 caused a progressive promotion of the active enzyme to the oxidase state which indicated that the formation of the inhib-

TEI-6720, as recovered from its XOR complex, was chemically unchanged, no hydroxylation had occurred (data not shown). Therefore, we interpret the time dependence of the inhibition as caused by multiple steps for the finally settled enzyme-inhibitor complex after initial binding of the inhibitor, a process rather commonly observed in the formation of such tight binding. No hydroxylation during binding is consistent with the fact that no coordination to the metal cofactor was observed in the crystal structure of the XDH/TEI-6720 complex as described below.

Steady State Kinetics—The coexistence of inhibitory effects caused by TEI-6720 and product inhibition by NADH (38) conspired to prevent a meaningful steady state analysis of XOR activity. As the Mo-pterin sites of both XDH and XO are structurally equivalent (10), steady state kinetics of the product-inhibition-free oxidase reaction were measured instead.

These analyses were performed varying the concentrations of xanthine and TEI-6720 under air-saturated conditions. A representative Lineweaver-Burk plot is given in Fig. 3. As described for BOF-4272 (16), TEI-6720 exhibits mixed-type inhibition. Binding of TEI-6720 to the active enzyme was too tight to allow concentrations of free inhibitor (I_{inhib}) to be set equal to the initial concentrations of TEI-6720 (I_0). In the inset, concentrations of free TEI-6720 were corrected according to Equations 1 and 2 (16, 36).

\[ [I_{inhib}] = [I] - [E]I \quad \text{(Eq. 1)} \]

\[ [E]I = 1/2(K + I_0 + E_0 - \sqrt{(K + I_0 + E_0)^2 - 4I_0E_0}) \quad \text{(Eq. 2)} \]

Analysis of the kinetic results indicates a K_i value of 1.2 ± 0.05 × 10^{-10} M for TEI-6720, 1 order of magnitude smaller than that of BOF-4272 (16). Plotting the apparent V_{max} versus the inhibitor concentrations gives a K_i' value of 9 ± 0.05 × 10^{-10} M. In contrast, when PMS was used as an electron donor, TEI-6720 showed a competitive inhibition pattern (Fig. 4), with a K_i value of 1.2 ± 0.03 × 10^{-10} M. Again, I_{inhib} was calculated as described above.

When catalyzing the transfer of electrons from xanthine to oxygen as the terminal acceptor, XO applies a ping-pong mechanism alternating the positive charge of molybdenum between Mo(VI) and Mo(IV) (27, 39). However, when PMS is used as an electron acceptor, it very rapidly oxidizes Mo(IV), not allowing the collection of meaningful information about this state during turnover. The same is true in the case of allopurinol and BOF-4272 inhibition (15, 16, 40), suggesting that the inhibitor-Mo(VI) complex is the main molecular species formed and represented in a competitive inhibition pattern in Fig. 4. As the K_i value determined for the xanthine oxidase activity (estimated by plotting the slopes in the secondary plot) is almost identical to the one found for xanthine-PMS activity, these values would be representative for the Mo(VI) state of the enzyme, whereas the K_i' value for the xanthine oxidase activity, the same as the one established for BOF-4272 (16), would refer to the Mo(IV) state.

Spectral Perturbation upon Inhibitor Binding—When fully active XO was titrated with TEI-6720 (Fig. 5A), significant spectral perturbations were observed in the UV and visible regions of the spectrum. The difference spectrum exhibited two negative peaks; the larger one at 400 nm (Δε = 1.3 × 10^4 M⁻¹ cm⁻¹) and a rather shallow, ill-defined one centered at 550 nm. Their amplitudes increased in proportion to the amount of inhibitor added until the inhibitor concentration reached that of the enzyme, which indicated that the formation of the inhibitor-enzyme complex was causing the spectral perturbation. However, both the spectral difference and the dissociation constant were too small to allow the direct determination of the K_i value from a spectral titration experiment.

If, however, desulfo-XO was used in the titration experiment, no absorption changes were recorded (Fig. 5B) despite the facile formation of a tight-inhibitor complex. The absorbance changes described above are different from those Ryan et al. (41) assigned to the change of the redox state of the molybdenum ion.

Quenching of TEI-6720 Fluorescence upon Enzyme Binding—Upon the formation of the enzyme complex, the fluorescence of TEI-6720 with a peak at 390 nm (excitation at 319 nm) is quenched. We used this property to measure the dissociation constants of the TEI-6720-XO complexes of both desulfo-XO (AFR = 3) and fully active XO (AFR = 190). A plot of fluorescence intensity against the TEI-6720 concentration is given in Fig. 6. The fluorescence signal increased markedly after equimolar amounts of TEI-6720 had been added to the enzyme solution, implying a 1:1 ratio of TEI-6720 to enzyme in the

³ B. T. Eger, K. Okamoto, T. Nishino, T. Nishino, and E. F. Pai, unpublished results.
complex and making a single, specific binding site of the inhibitor on the enzyme very plausible. The $K_d$ value for TEI-6720 binding to desulfo-XO was calculated as $2 \pm 0.03 \times 10^{-9}$ M.

Again, the corresponding value for the fully active enzyme could not be determined because the combination of very tight binding by the inhibitor and rather low fluorescence intensity prevented the sufficiently accurate determination of the concentration of free inhibitor (Fig. 6, inset). Given the very small structural difference between the sulfo- and desulfo-forms of the enzyme (replacement of a sulfur atom by an oxygen), one might expect the interactions between the inhibitor molecule and the protein matrix to stay the same in the two forms of the enzyme. The distinctive spectral changes together with the difference in $K_d$ values between the respective TEI-6720 complexes, however, seem to reflect subtle differences in the local electrostatic fields and/or minor structural rearrangements that occur around the molybdenum ion when the active sulfo-form of the enzyme is transformed into the inactive desulfo-form.

Crystal Structure of the Enzyme-Inhibitor Complex—Freshly purified XDH produced crystals in space group C2 with unit cell axes $a = 168.3$ Å, $b = 124.6$ Å, $c = 147.3$ Å, and $\beta = 91.0^\circ$. These parameters correspond closely to those of the free or salicylate-bound crystals of XDH (26). The crystals diffracted to

![Fig. 3. Kinetics of xanthine-oxygen transferase inhibition. A, Lineweaver-Burk plots of xanthine-oxygen transfer activity of XO in the presence of TEI-6720. Final concentration of XO (AFR = 200) was 0.5 nM. Final concentrations of TEI-6720: open circles, no inhibitor; closed circles, 0.5 nM; open squares, 1 nM; closed squares, 1.5 nM. B, secondary plots of A. The $K$ and $K'$ values were obtained from secondary plots of the slopes of the Lineweaver-Burk plots (square) and the $y$ axis intercepts (circle) versus the inhibitor concentrations, respectively.](image)

![Fig. 4. Kinetics of xanthine-PMS transferase inhibition. A, Lineweaver-Burk plot of xanthine-PMS activity of XO in the presence of TEI-6720. Reoxidation of XOR-reduced PMS was determined by monitoring the reduction of cytochrome $c$ at 550 nm. Final concentration of XO (AFR = 200) was 0.5 nM. Final concentrations of TEI-6720: circles, no inhibitor; squares, 0.5 nM; triangles, 1 nM. B, secondary plots of A. The $K_i$ value was obtained from the slopes of the Lineweaver-Burk plots.](image)
2.8-Å resolution and contained two subunits in the asymmetric unit.

The overall structure of the protein chain in the inhibitor complex was identical to the one found in the salicylate-bound enzyme (10). Clear electron density representing the bound TEI-6720 molecule was identified and easily interpreted (Fig. 7). TEI-6720 bound in the channel leading from bulk solvent to the buried Mo-pterin cofactor, closing it off like a plug. With a distance of 4.9 Å, the methyl carbon in the thiazole ring was the atom closest to the molybdenum ion. However, no electron density representing a potential covalent bond between TEI-6720 and molybdenum was observed. The torsion angle between the planes of the thiazole and benzonitrile rings was 30°.

Six hydrogen bonds and one charge-charge interaction were observed between TEI-6720 and the protein. The most tightly bound part of the inhibitor molecule was its carboxylate group. It was located at almost the same position as the carboxylate side chain. The hydrophobic character of large parts of the TEI-6720-binding channel would favor a shift of the pKa value of glutamate in the required direction.

The thiazole ring as a whole was sandwiched between two phenylalanine residues, Phe914 and Phe1009 (Fig. 8A). The aromatic ring of Phe914 lay parallel to the plane of the thiazole ring at a distance of 3.4 Å, whereas the side chain of Phe1009 pointed perpendicularly to the center of the thiazole ring, approaching it to 4.0 Å. This arrangement of energetically favorable aromatic/ aromatic interactions (42) had also been seen in the crystal structure of the salicylate complex and its conservation argues for an important role in stabilizing the binding positions of aromatic substrates; it might well represent one of the key features of substrate recognition.

Hydropathic interactions also contributed to the binding of TEI-6720 to XOR. The benzonitrile portion of TEI was inserted between Leu672 and Leu1014, keeping a distance of 3.4 and 3.7 Å from each side chains. Together with the Asn768-nitrite bond, this arrangement guides the orientation of the benzonitrile part of the inhibitor. The hydrophobic 4-isobutoxy tail of TEI-6720 was surrounded by amino acids Leu648, Phe649, Val1011, and Leu1013, with distances ranging from 3.7 to 4.2 Å (Fig. 8B).
Although some of these crystallographically determined values are too large to argue for direct van der Waals binding, they establish a pocket well suited to accommodate bulky hydrophobic moieties, which are often found as part of the molecular structures of good substrates or inhibitors of XOR (16, 43). The numerous interactions, which the TEI-6720 molecule displayed when assuming its position in the elongated access channel leading to the Mo-pterin group, are illustrated in Fig. 8A. The space filling representation conveys the generally very tight fit between the inhibitor molecule and surrounding residues.

Almost all amino acids, whose interactions with the TEI-6720 molecule are discussed above, are conserved among bovine (44) and human XOR (45). The only exceptions are Leu648 and Phe649 (bovine XOR), which are Ile and Cys in human XOR, respectively. This change still preserves the hydrophobic character of the side chains, the property important in their interaction with the inhibitor molecule. Therefore, the mecha-
nistic conclusions drawn in our discussion are fully applicable to the human enzyme as well.

Correlation of Spectroscopic and Structural Results in the TEI-6720 Complex of XOR—TEI-6720 bound more tightly to the sulfo-form than to the desulfo-form of XOR (16) and only in the case of the catalytically competent sulfo-form were spectral perturbations observed. The display of biphasic inhibition kinetics was probably caused by two binding modes of the inhibitor (e.g., 1, attached to the channel entrance and 2, fully inserted) and not by structural rearrangements of the accommodating binding site, as there were no overall changes observed in the location and orientation of the protein matrix when TEI-6720 was bound to XOR. In the crystal structure, the methyl substituent of the thiazole ring represented the part of the inhibitor closest to the molybdenum complex approaching its hydroxy ligand to a distance of 3.5 Å. The oxygen atom of the latter has been proposed as the one incorporated into substrate molecules (46). However, an analogous compound, in which the methyl moiety of TEI-6720 has been removed, will also perturb the spectrum in a way very similar to TEI-6720,5 effectively ruling out this interaction as the source of spectral variation.

As the spectral changes are only observed in the sulfo-form, it is interesting to note that the shortest distance between the sulfur ligand of Mo-pterin and the TEI-6720 molecule was 5.0 Å, again making it very improbable that the reason for the modified absorption behavior would be a direct influence by the inhibitor on the sulfur ligand of the cofactor. Although a crystal structure of the desulfo-form of XOR is not yet available, the structure of the salicylate-bound form of bovine XDH (10) supported by the recent results of a 1.7-A crystal structure of the desulfo-form of XOR is not yet available, will also perturb the spectrum in a way very similar to TEI-6720,5 effectively ruling out this interaction as the source of spectral variation.

The only significant difference found was the movement of the side chain of Glu802. Above, we have discussed the potential of this side chain to undergo protonation and to engage in a hydrogen bond with the N-3 atom of the thiazole ring. On the other hand, in the salicylate-bound form, Glu802 is 3.3 Å apart from the sulfur ligand and likely to have a hydrogen bond to the sulfur (10). We believe that such a change in charge close to the sulfur ligand combined with an increase in distance between these groups, at the moment represents the best explanation of how inhibitor binding can influence the electronic structure of the Mo-pterin cofactor.

CONCLUSIONS

The crystal structure of the TEI-6720 complex of bovine XOR showed the inhibitor bound in a narrow channel leading to the molybdenum center of the enzyme. The potential drug molecule fills the entire pocket thereby inhibiting the activity of the enzyme simply by obstructing substrate binding. Although no direct coordination was observed between the molybdenum ion and the inhibitor, numerous hydrogen bonds and hydrophobic interactions are evident, some of them conserved in their contribution to substrate recognition. A slight reorientation of a glutamate side chain, probably accompanied by its protonation, is postulated to be the cause of the spectral changes observed upon binding of TEI-6720 to the sulfo-form of XOR.

In stark contrast to oxypurinol, the metabolite of the standard anti-gout drug allopurinol, which is the actual inhibitor of XOR activity in vivo and binds tightly only to the reduced form of the enzyme, TEI-6720 forms very strong complexes with both Mo(VI) ($K_f = 1.2 \pm 0.5 \times 10^{-10}$ M) and Mo(IV) forms ($K_f' = 9 \pm 0.5 \times 10^{-10}$ M) of XOR. In addition, oxypurinol-inhibited enzyme is reactivated relatively quickly ($t_{1/2} = 300$ min at 25 °C) by spontaneous reoxidation of the molybdenum cofactor (15). TEI-6720, however, would be expected to inhibit the enzyme in vivo for long periods of time, because the enzyme-inhibitor complex is very stable and not influenced by changes in the redox status of the cofactor. These differences between the two XOR inhibitors should be helpful in elucidating into obvious therapeutic advantages for TEI-6720 enabling treatment of patients with a single daily dose at significantly lower and more constant plasma concentration levels of the drug. As a pure research tool, an inhibitor like TEI-6720 will provide the opportunity to selectively evaluate the contribution of XOR activity to pathogenesis in effects such as postischemic reperfusion injury, independent of the metabolic state of the tissue investigated.

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