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X-ray structure of the V301L aldo–keto reductase 1B10 complexed with NADP$^+$ and the potent aldose reductase inhibitor fidarestat: Implications for inhibitor binding and selectivity

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ABSTRACT

Only one crystal structure is currently available for tumor marker AKR1B10, complexed with NADP$^+$ and tolrestat, which is an aldose reductase inhibitor (ARI) of the carboxylic acid type. Here, the X-ray structure of the complex of the V301L substituted AKR1B10 holoenzyme with fidarestat, an ARI of the cyclic imide type, was obtained at 1.60 Å resolution by replacement soaking of crystals containing tolrestat. Previously, fidarestat was found to be safe in phase III trials for diabetic neuropathy and, consistent with its low *in vivo* side effects, was highly selective for aldose reductase (AR or AKR1B1) *versus* aldehyde reductase (AKR1A1). Now, inhibition studies showed that fidarestat was indeed 1300-fold more selective for AR as compared to AKR1B10, while the change of Val to Leu (found in AR) caused a 20-fold decrease in the IC_{50} value with fidarestat. Structural analysis of the V301L AKR1B10-fidarestat complex displayed enzyme-inhibitor interactions similar to those of the AR-fidarestat complex. However, a close inspection of both the new crystal structure and a computer model of the wild-type AKR1B10 complex with fidarestat revealed subtle changes that could affect fidarestat binding. In the crystal structure, a significant motion of loop A was observed between AR and V301L AKR1B10, linked to a Phe-122/Phe-123 side chain displacement. This was due to the presence of the more voluminous Gln-303 side chain (Ser-302 in AR) and of a water molecule buried in a subpocket located at the base of flexible loop A. In the wild-type AKR1B10 model, a short contact was predicted between the Val-301 side chain and fidarestat, but would not be present in AR or in V301L AKR1B10. Overall, these changes could contribute to the difference in inhibitory potency of fidarestat between AR and AKR1B10.

1. Introduction

Aldo–keto reductases (AKR) are cytosolic monomeric NADP(H)-dependent enzymes which catalyze the reduction of carbonyl groups using a variety of physiological and xenobiotic substrates, including lipids, steroids, catecholamines, prostaglandins and retinoids. Structurally, AKRs fold into a typical $(\alpha/\beta)_8$-barrel structure [1–3].

Human AKR1B subfamily members aldose reductase (AR, also named AKR1B1) and aldose reductase-*like* or small intestine reductase (AKR1B10) have been extensively studied because of their involvement in human pathologies: AR is upregulated in hyperglycemia, reducing glucose to sorbitol, which is related to secondary diabetic complications [4,5] and recently with cancer, notably colon cancer metastasis [6]. AKR1B10 displays high activity with the retinoic acid (RA) precursor all-*trans*-retinaldehyde [7] and is induced in different types of cancer, notably in non-small cell lung and hepatocellular carcinomas [8–13].

The simple paradigm configured by the "Osmotic Hypothesis", relating secondary diabetic complications with overexpression of AR, sorbitol and fructose accumulation, and the subsequent osmotic swelling and cell dysfunction, motivated researchers to design aldose reductase inhibitors (ARIs) [4,5]. In summary, ARIs contain a carboxylic acid moiety or a hydantoin group, which binds to the anion-binding pocket, formed by Tyr-48, His-110 and Trp-111 along with the positively charged nicotinamide moiety of the cofactor NADP$^+$. Also, most of them include another key pharmacophore, an aromatic moiety, which opens a hydrophobic pocket bordered by Trp-111, Phe-122 and Leu-300, known as the specificity pocket [14]. Regarding the selectivity of ARIs *versus* other enzymes, the main cross-inhibition target systematically analyzed has been aldehyde reductase (AKR1A1) [1,14,15]. The

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growing interest in the more closely related AKR1B10 (71% amino acid sequence identity) has motivated studies on the selectivity of ARIs for AKR1B10 [16–28]. However, in spite of the large number of publications where ARIs and AKR1B10 inhibitors are directly compared, to date only one structure has been obtained with AKR1B10, complexed with $NADP^+$ and tolrestat (PDB ID: 1ZUA) [38]. Further structural comparisons between AR:ARI and AKR1B10:inhibitor complexes are therefore necessary.

Fidarestat is a spirohydantoin-containing compound, widely studied both *in vitro* and *in vivo* [6,15,29–33]. In fact, our laboratory obtained an ultrahigh resolution structure (0.92 Å) of the AR holoenzyme in complex with fidarestat [31] and identified a hydrogen bond between the amide N of Leu-300 of AR and the exocyclic amide group of fidarestat as the key determinant for the higher selectivity of this compound for AR over AKR1A1 [34]. It is noteworthy that fidarestat has undergone 52 weeks of phase III clinical trial for diabetic neuropathy and was found to have no irreversible side effects [33]. Thus, fidarestat is a promising compound as a therapeutic drug, taking into account that currently there are no pharmacologically accepted ARIs, except for epalrestat in Japan [35].

Here we have solved the three-dimensional X-ray structure of V301L AKR1B10 complexed with $NADP^+$ and fidarestat (the 2*S*,4*S* stereoisomer, which is the most potent one [29]), by replacement soaking with fidarestat of V301L AKR1B10:$NADP^+$:tolrestat crystals. A comparison of the fidarestat-binding sites of V301L AKR1B10 and the published AR complex (PDB ID: 1PWM) has allowed us to infer some of the structural determinants behind the different binding and selectivity properties of this promising drug. Finally, these new data provide a significant improvement in the Structure–Activity Relationship (SAR) of AR and AKR1B10, both of them being important pharmacological targets.

2. Materials and methods

2.1. Expression and purification of AKR1B enzymes

Wild-type and V301L AKR1B10 enzymes were recombinantly expressed and purified based on the procedure described previously [36]. Briefly, *Escherichia coli* BL21(DE3) strain (Novagen), transformed with the pET16b plasmid for AKR1B10 or pET30-Xa/LIC for the V301L enzyme, both encoding the protein with an N-terminal His-tag, was grown in 2xYT medium at 24 °C overnight. Protein expression was induced by the addition of 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG, Euromedex). Proteins were purified using a HisTrap FF crude column (GE Healthcare). The enzymes were eluted by a 0.015–1.0 M imidazole gradient in 50 mM Tris–HCl, 100 mM NaCl, pH 8.0. Purified proteins were incubated overnight with Factor Xa at room temperature; then the His-tag and nondigested proteins were removed by 30-min incubation with a nickel-charged chelating Sepharose Fast Flow resin at room temperature. The proteins were further purified with a Red-Sepharose (Sigma–Aldrich) resin and eluted with 2 M NaCl, 50 mM Tris–HCl, pH 8.0.

AR was expressed and purified as described previously [37]. AR was cloned into the pET15b vector (Novagen). Expression of the $(His)_6$-AR in the *E. coli* BL21(DE3) strain (Novagen) was induced by IPTG (Euromedex) during a 3-h incubation at 37 °C. Protein was purified on a TALON® metal affinity column (Clontech). The enzyme was eluted by a 0.01–0.5 M imidazole gradient in 20 mM Tris–HCl, 500 mM NaCl, pH 8.0. Purified protein was incubated overnight with thrombin; then His-tag and nondigested proteins were removed by 30-min incubation with TALON® metal affinity resin. The untagged protein was then loaded on a DEAE Sephadex column (Pharmacia) and eluted with a NaCl gradient.

2.2. Cocrystallization, soaking and structure determination of AKR1B10

Crystals of the complexes with AKR1B10 wild-type:$NADP^+$:fidarestat or with the V301L enzyme could not be obtained after robotic and manual screenings. Thus, in order to perform replacement soaking, initially crystals of wild-type or V301L AKR1B10 were obtained with inhibitor tolrestat as described previously [38]. Cocrystallization was performed at 24 °C by vapor diffusion using the hanging-drop method. An aliquot (1 μl) of protein solution (18 mg/ml) containing 2 mM tolrestat was mixed with 1 μl of reservoir solution (100 mM sodium cacodylate, pH 9.0, and 30% v/v, polyethylene glycol (PEG), 6000). The tolrestat was replaced by soaking for 14 days the V301L AKR1B10:$NADP^+$:tolrestat crystals with 25 mM fidarestat dissolved in the reservoir solution. Crystals were cryocooled in liquid nitrogen by using a cryoprotecting solution containing 40% PEG 6000. The X-ray data set was collected at 1.6 Å resolution on the laboratory source and processed with the program HKL2000 [39].

2.3. Structural refinement and structure analysis

The atomic coordinates of the AKR1B10:$NADP^+$:tolrestat complex (PDB ID: 1ZUA) were used to solve the structure of the V301L AKR1B10:$NADP^+$:fidarestat complex. Crystallographic refinement involved repeated cycles of conjugate gradient energy minimization and temperature factor refinement, performed with the CCP4 suite [40]. Amino acid side-chains were fitted into 2Fo – Fc and Fo – Fc electron density maps. The final Fo – Fc map indicated clear electron density for the different inhibitors. Water molecules were fitted into difference maps and in the final cycles riding H-atoms were introduced. The programs Phenix [41] and Coot [42] were used for refinement and fitting the models to the electron density. The atomic coordinates have been deposited in the PDB (PDB ID: 4GAB). The fidarestat-binding site in the V301L AKR1B10 ternary complex was analyzed with Coot [42] and LigPlot+ [43], and figures were built with the PyMOL Molecular Graphics System (Schrödinger, LLC). For interpretation of the references to color in the figure captions, the reader is referred to the web version of the article.

2.4. IC_{50} determination

The IC_{50} activity assays were carried out according to a previously described method [44], based on measuring NADPH consumption when the enzyme catalyzes the conversion of glyceraldehyde into glycerol. The assays were performed at 25 °C in 100 mM sodium phosphate, pH 7.0, 0.2 mM NADPH and glyceraldehyde concentrations (1 mM for AR, and 60 mM for wild-type and V301L AKR1B10) that are ten times the K_m values. The previous studies showed that the steady-state kinetic constants for wild-type and V301L AKR1B10 acting on glyceraldehyde were essentially the same, with a K_m value of 6 mM [38]. Thus, the IC_{50} values are not equivalent to the K_d values. Fidarestat was dissolved in dimethyl sulfoxide, and the corresponding solution was added to the cell and incubated for 5 min at 25 °C prior to addition of the substrate. The reaction was initiated by addition of glyceraldehyde and the decrease in optical density at 340 nm was monitored for 3 min at 25 °C in a UV–VIS spectrophotometer (UV-1700 PharmaSpec, Shimadzu). The IC_{50} value was determined as the concentration that inhibits enzymatic activity by 50%. IC_{50} was calculated using the Grafit program (version 5.0; Erithacus Software), and values were given as the mean of three experiments ± standard deviation.