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# Structure of aldehyde reductase in ternary complex with coenzyme and the potent 20α-hydroxysteroid dehydrogenase inhibitor 3,5-dichlorosalicylic acid: Implications for inhibitor binding and selectivity

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ABSTRACT

The structure of aldehyde reductase (ALR1) in ternary complex with the coenzyme NADPH and 3,5-dichlorosalicylic acid (DCL), a potent inhibitor of human 20α-hydroxysteroid dehydrogenase (AKR1C1), was determined at a resolution of 2.41 Å. The inhibitor formed a network of hydrogen bonds with the active site residues Trp22, Tyr50, His113, Trp114 and Arg312. Molecular modelling calculations together with inhibitory activity measurements indicated that DCL was a less potent inhibitor of ALR1 (256-fold) when compared to AKR1C1. In AKR1C1, the inhibitor formed a 10-fold stronger binding interaction with the catalytic residue (Tyr55), non-conserved hydrogen bonding interaction with His222, and additional van der Waals contacts with the non-conserved C-terminal residues Leu306, Leu308 and Phe311 that contribute to the inhibitor's selectivity advantage for AKR1C1 over ALR1.



The kinetic and pro-regulatory activities of the aldo-keto reductase (AKR[1]) superfamily of enzymes such as aldehyde reductase (ALR1, EC 1.1.1.2) have been studied widely and include the regulation of the pro-inflammatory response [1], the synthesis of metabolically vital compounds such as prostaglandins [2,3] and steroidal modification *in vivo* [4–7]. ALR1 is a monomeric protein of 325 residues (approximately 36 kDa) [8,9] comprising of an α/β-TIM barrel structure [10,11]. The diverse studies on ALR1 are due to the ubiquitous expression of the enzyme, which has been isolated and purified from a number of tissues including the brain, kidney, liver, lens and skeletal muscle [8,12–14]. ALR1 is involved in a wide variety of biological functions including NADPH-dependent reduction of endogenous and xenobiotic aldehydes and dicarbonyl compounds including methylglyoxal and 3-deoxyglucosone [8,15–19].

A group of AKRs known as hydroxysteroid dehydrogenases (HSDs), play a vital role in the modulation and regulation of steroid hormones [20,21] such as androgens, estrogens and progestins and as such are considered important targets for drug design [22,23]. These drug targets include three human HSDs belonging to the AKR1C subfamily, of which AKR1C1 (displaying both 20α- and 3α-HSD activity) has a major role in progesterone metabolism and the maintenance of pregnancy [24,25] *via* the formation of its inactive form progestin (20α-hydroxyprogesterone). The accumulation of excess progestin can ultimately lead to premature birth and infant mortality [26,27]. Moreover, the over expression of AKR1C1 has been linked to the development of tumours *via* the formation of tumour promoting metabolites in tissues such as the lung, endometrium, oesophagus, ovaries and breasts, and may also be linked to drug-resistance of several anti-cancer agents [28–32]. Thus the discovery of inhibitors for AKR1C1, in particular those targeted specifically towards the enzyme should provide a new therapeutic approach for its associated pathologies. However, potent AKR1C1 inhibitors may cause undesirable side effects due to their interaction with the conserved catalytic residues His113 and Tyr50 of ALR1 [33–36], an AKR enzyme that metabolizes 3-deoxyglucosone and methylglyoxal which induce apoptosis [37] and are intermediates in the formation of advanced glycation end products [38].

Salicylic acid-based analogues have been identified *via in silico* screening, kinetic and crystallographic analysis as potent and competitive inhibitors of AKR1C1 that bind in the active site [39,40]. Compounds such as 3,5-diiodosalicylic acid were potent inhibitors

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[1] *Abbreviations used*: AKR, aldo-keto reductase; ALR1, aldehyde reductase; ALR2, aldose reductase; HSD, hydroxysteroid dehydrogenase; AKR1C1, 20α-hydroxysteroid dehydrogenase; DCL, 3,5-dichlorosalicylic acid.

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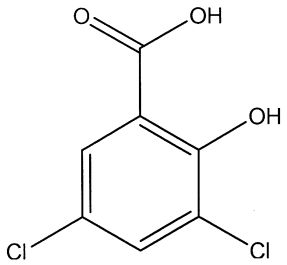


**Scheme 1.** Chemical structure of DCL.

with an $IC_{50}$ (concentration required for 50% inhibition) value of 99 nM [39]. We subsequently found a more potent competitive inhibitor, 3,5-dichlorosalicylic acid (DCL; Scheme 1) with a $K_i$ value of 5.9 nM, and determined the AKR1C1-coenzyme-DCL ternary complex at a resolution of 1.8 Å. The inhibitor is held in place by a network of tight hydrogen bonding interactions with the active site residues Tyr55, His117 and His222 [40]. In this study, we describe the ternary structure of ALR1 in complex with coenzyme and DCL determined at 2.41 Å resolution. To investigate inhibitor selectivity, the active sites of ALR1 and AKR1C1 were compared, along with molecular modelling calculations and inhibitory activity measurements. Both enzymes contain common characteristics in the manner by which they bind the inhibitor; however, our results clearly indicate that DCL is more selective towards AKR1C1 with both conserved and non-conserved residues accounting for the difference in potency between the two enzymes.

## Materials and methods

### *Materials*

Coenzyme (NADPH) and chemicals of the highest purity were purchased from the Sigma–Aldrich Chemical Company, Fluka and MERCK.

### *Enzyme purification*

Porcine ALR1 was purified to homogeneity from porcine kidney following established methodologies [41]. For crystallization purposes, the purified enzyme solution in 5 mM Tris–HCl (pH 6.5) containing 5 mM 2-mercaptoethanol was concentrated to 15–17 mg/mL by ultrafiltration. Recombinant AKR1C1 was expressed in *Escherichia coli* JM109, and purified to homogeneity as previously described [42,43]. Protein concentrations were determined by a bicinchoninic acid protein assay reagent kit (Pierce) using bovine serum albumin as the standard.

### *Assay of enzyme activity*

The activity of ALR1 was spectophotometrically determined at 298 K by monitoring the decrease in NADPH absorbance at 340 nm in a reaction mixture consisting of 0.1 M potassium phosphate (pH 6.7), 5 mM dl-glyceraldehyde, 0.1 mM NADPH and enzyme. The activity of AKR1C1 was fluorometrically assayed by measuring the rate of NADPH formation coupled with the oxidation of 1 mM *S*-(+)-1,2,3,4-tetrahydro-1-naphthol (*S*-tetralol) [39]. In the determination of inhibitor potency of DCL for ALR1, the inhibitor was dissolved in dimethyl sulphoxide (DMSO) and added to the reaction mixture, in which the concentration of DMSO was less than 1.5%. The $IC_{50}$ values were estimated from non-linear regression analyses of percent inhibition versus inhibitor concentration graphs and expressed as the means of at least three determinations. To estimate the inhibition pattern of DCL for ALR1, the initial velocities were determined over a range of four concentrations of dl-glyceraldehyde at a saturating concentration of coenzyme in the absence or presence of DCL, and were analysed by a Lineweaver–Burk plot.

### *Crystallization*

Crystals of the ALR1 holoenzyme were grown at 295 K by vapor-diffusion using the hanging drop method [44]. The enzyme (16.38 mg/mL) was first mixed with NADPH to give a 1:20 ratio of enzyme to coenzyme (final concentration; 16.07 mg/mL). Each droplet consisted of 3 μL of enzyme/NADPH mixture, mixed with 2.5 μL solution containing the mother liquor (2.0 M ammonium sulphate and 0.1 M Tris–HCl buffer, pH 8.5) and 0.5 mM DCL dissolved in DMSO. Each well was seeded with 0.5 μL of the mother liquor containing crushed binary crystals to initiate crystal growth of the ternary complex. Crystals grew to their maximum dimensions (0.40 × 0.10 × 0.10 mm) within 1 week. The ternary complex crystals were mounted onto a nylon loop (Hampton), and then briefly immersed into a cryo-protecting solution (20% v/v glycerol in 2.0 M ammonium sulphate and 0.1 M Tris–HCl buffer, pH 8.5) before being flash-frozen in liquid nitrogen for X-ray diffraction studies.

### *X-ray data collection and processing*

Diffraction data was collected at a temperature of 100 K on a MAR-345 image plate system mounted on a Rigaku RU300 rotating anode generator operating at 50 kV and 90 mA. A near complete data set was obtained and processed with *HKL2000* and *SCALEPACK* [45]. The ternary complex crystallized in the hexagonal space group $P6_522$, with unit cell parameters $a = b = 67.385$ Å, $c = 244.106$ Å, $\alpha = \beta = 90.0°$ and $\gamma = 120.0°$. There was one monomer per asymmetric unit, consisting of 322 amino acid residues. The solvent content was estimated to occupy 44.14% of the unit cell volume [46]. Data collection and processing statistics are shown in Table 1.

### *Structural refinement*

The coordinates for porcine ALR1 holoenzyme structure (PDB code 2AO0) [41] were used to solve the ternary structure by molecular replacement utilizing *MOLREP* in the *CCP4* suite of crystallographic programs [47]. Structural refinement was carried out using *REFMAC* [48] with the initial model subjected to multiple cycles of manual fitting into $2F_o–F_c$ and $F_o–F_c$ electron density maps using *Coot* [49]. The inhibitor molecule, waters and sulphate molecules were added towards the end of the refinement. The electron density in the $F_o–F_c$ map corresponding to the bound inhibitor DCL is shown in Fig. 1. The statistics of stereochemistry and geometry of the final model are shown in Table 1. The atomic coordinates for the ternary complexes of ALR1 and AKR1C1 were deposited in the Protein Data Bank (PDB codes 3CV7 and 3C3 U, respectively) and will be released immediately upon the publication of the structures.

### *Molecular modelling calculations*

Energy minimization calculations were carried out on the structures of the ternary complexes of ALR1 and AKR1C1 using the Discover 2.7 package (Biosym Technologies, San Diego, CA, USA), on a Linux workstation following established procedures found to be effective in examining conformational space with a protein–ligand complex [50,51]. Briefly, the constant valence force field incorporating the simple harmonic function for bond stretching and excluding all non-diagonal terms was used (cut-off distance of