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5β-Reduced Steroids and Human Δ⁴-3-Ketosteroid 5β-Reductase (AKR1D1)

Mo Chen[2] and **Trevor M. Penning**[1,2]

[1]Center of Excellence in Environmental Toxicology, University of Pennsylvania, Philadelphia, PA 19104-6160

[2]Department of Pharmacology Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA 19104-6160

Abstract

5β-Reduced steroids are non-planar steroids that have 90° bend in their structure to create an A/B *cis*-ring junction. This novel property is required for bile-acids to act as emulsifiers, but in addition 5β-reduced steroids have remarkable physiology and may act as potent tocolytic agents, endogenous cardiac glycosides, neurosteroids, and can act as ligands for orphan and membrane bound receptors. In humans there is only a single 5β-reductase gene *AKR1D1*, which encodes Δ^4-3-ketosteroid-5β-reductase (AKR1D1). This enzyme is a member of the aldoketo reductase superfamily, but possesses an altered catalytic tetrad, in which Glu120 replaces the conserved His residue. This predominant liver enzyme generates all 5β-dihydrosteroids in the C_{19}–C_{27} steroid series. Mutations exist in the *AKR1D1* gene, which result in loss of protein stability and are causative in bile-acid deficiency.

Keywords

bile acid biosynthesis; steroid metabolism; enzyme mechanism; genetics

1. Introduction

The C-4/C-5 double bond at the A-ring of Δ^4-3-ketosteroids is a characteristic structure of nearly all steroid hormones. This double bond can be saturated by either 5α- or 5β-reduction catalyzed by steroid 5α-reductases (SRD5A1-SRD5A3) or by steroid 5β-reductases (e.g. AKR1D1) [1]. 5α-Reduction generates planar steroids with an A/B *trans* ring-junction. This reaction produces the most potent androgen 5α-dihydrotestosterone and is essential for the development and regulation of male secondary sex-characteristics and the normal growth of the prostate [2]. By contrast, 5β-reduction introduces a 90° bend at the A/B ring junction forming a *cis*-configuration. In vertebrates, the most important and well-studied 5β-reduced steroids are bile acids. Bile acids are powerful emulsifying agents that facilitate

Corresponding Author: Trevor M. Penning, PhD, The Thelma Brown and Henry Charles Molinoff Professor of Pharmacology, Professor Biochemistry & Biophysics and OB/GYN, Director Center of Excellence in Environmental Toxicology, Department of Pharmacology, Perelman School of Medicine, University of Pennsylvania, 1315 BRBII/III, 421 Curie Blvd, Philadelphia, PA 19104-6160, Phone: 215-898-9445, FAX: 215-573-0200.

transportation and absorption of fat and fat-soluble vitamins. Bile acid biosynthesis also serves as a major pathway for cholesterol metabolism [3].

In addition to being an essential step in bile acid biosynthesis, 5β-reduction inactivates steroid hormones and initiates steroid hormone clearance. However, 5β-reduction may also be involved in the pre-receptor regulation of nuclear receptors by controlling ligand availability. For example, 5β-reduced steroids act as ligands for orphan nuclear receptors, the farnesoid X receptor (FXR) and the pregnane X receptor (PXR). 5β-Reduction also generates neuroactive 5β-pregnanes that modulate the activities of the $GABA_A$ and NMDA receptors. 5β-Reduced steroids play important roles in other species. In plants, 5β-reduction produces cardiac glycosides as an important defense mechanism against herbivores [4]. Cardiac glycosides such as digoxin obtained from *Digitalis lanata* are the mainstay therapy for congestive heart failure. In insects, 5β-reduction leads to the production of ecdysone and 20-hydroxyecdysone, which are essential for metamorphsis [5].

Steroid 5β-reductases in vertebrates and plants have been identified and characterized. Vertebrate 5β-reductases belong to the aldo-keto reductase (AKR) superfamily, 1D subfamily [1]. The plant 5β-reductase belongs to the short chain dehydrogenase/reductase family [6]. Even though both enzymes perform the same function and utilize NADPH as cofactor, the two enzyme families have evolved from different ancestors and do not share sequence or structure homology. Insect 5β-reductase has not been identified as yet. In this review, we focus on the function of 5β-steroids in humans and human 5β-reductase AKR1D1. As will be seen below 5β-reduced steroids are not inactive steroid metabolites and have their own unique physiology and pharmacology.

2. Functions of 5β-steroids

Bile acids

Bile acids are the most abundant 5β-reduced steroids. About 500 mg of cholesterol is converted to bile acids in adult human liver each day (~2 g total bile acid pool) [3, 7]. Bile acids solubilize dietary cholesterol, lipids, and fat soluble vitamins (A, D, E, and K) by forming mixed micelles and facilitate absorption of nutrients [8]. Compared to the 5α-reduced *allo*-bile acids, the 5β-configuration augments the facial amphipathicity of bile acids by providing a larger surface area of the hydrophobic β-face. This is greatly favored for micelle formation and makes the 5β-reduced bile acids superior emulsifiers [9, 10]. The mixed micelles enhance anchorage of pancreatic lipases to the micelle surface, where bile acids also act at activators for lipases to facilitate fat hydrolysis [11–14].

The introduction of the A/B *cis*-ring configuration into the bile acid structure can be traced back very early in vertebrate evolution, as C_{27} bile alcohols are found in cartilaginous fish [15]. In human, two primary bile acids are C_{24} chenodeoxycholic acid and cholic acid (Figure 1), which account for ~70% of total bile acids [16]. The other 30% are composed of secondary bile acids produced mainly by 7α-dehydroxylation of the primary bile acids by intestinal bacteria. Bile acids are synthesized in liver and released into the small intestine in conjugated forms with glycine or taurine [3, 7]. Bile acids are passively or actively reabsorbed in the small intestine and recycled in the liver [8].

For a long time, bile acids were considered solely as steroidal detergents and emulsifying agents. But now their roles as regulatory/signaling molecules have been recognized. They are ligands for the orphan nuclear receptors FXR and PXR. Through activating FXR, bile acids regulate many genes in the liver and intestine, which modulate the biosynthesis and metabolism of bile acids and lipoproteins and determine the composition of the intestinal flora and fuana [3, 7, 8, 17, 18]. Recently, δ-aminolevulinate synthase, the rate limiting

enzyme of porphyrin-heme synthesis, has also been identified as a gene regulated by FXR, indicating a role of bile acids and their precursors in regulating hepatic heme biosynthesis [19]. The secondary bile acid, lithocholic acid activates PXR [20] and the vitamin D receptor [21, 22]. PXR in turn regulates the expression of *CYP3A4*, which encodes the major drug and xenobiotic catabolizing P450 isozyme in human liver. The secondary bile acid, ursodeoxycholic acid activates the glucocorticoid receptor and exerts immunomodulatory effects [23–25]. Bile acids also activate several signaling pathways including the c-Jun NH_2-terminal kinase (JNK) 1/2 pathway (to feedback inhibit bile acid biosynthesis) [8], the protein kinase B (AKT) pathway (to regulate glucose metabolism) [8, 26], FXR, short heterodimer partner (SHP), liver X receptor (LXR), and the sterol regulatory element-binding protein (SREBP)-1c pathway (to regulate lipid metabolism) [8, 27], the extracellular-signal-regulated kinases (ERK) pathway (to prevent apoptosis) [28, 29], and the epidermal growth factor receptor (to modulate intestinal permeability) [30]. A recently identified G protein-coupled bile acid receptor (TGR5) [31] further expands the function of bile acids and their roles in energy metabolism [32], inflammation [33, 34], and gallbladder contractility [35]. Bile acids also affect cardiac function by regulating vascular tone and myocardial contractility, but the underlying mechanism for this remains largely unknown [36].

With their fat emulsification properties and regulatory functions, bile acid analogs and agents that control their levels have potential as therapeutic agents for many diseases [10]. Owning to their structural rigidity and chirality, bile acids/bile acid derivatives also represent chiral auxiliaries and building blocks in supramolecular and materials chemistry [37].

5β-Androstanes

Androgens have been used as a mainstay treatment for anemia until the introduction of recombinant human erythropoietin and are still used today as adjuvant therapy to enhance the effectiveness of recombinant human erythropoietin [38]. The mechanism, by which androgens stimulate erythropoiesis has not been completely elucidated but involves increased synthesis of erythropoetin in the kidney. 5β-Androstanes are more potent stimulators than 5α-androstanes to enhance growth and survival of colony forming unit-erythroid [39]. 5β-Androstanes function similarly to 5β-pregnanes in heme biosynthesis by inducing δ-aminolevulinate synthase expression [39–41]. Since 5β-androstanes are devoid of androgenic effects that are responsible for the major side effects associated with androgen replacement therapy, 5β-androstanes have been recommended as a substitute for testosterone in the treatment of anemia, especially for female and young patients.

Some steroids with the 3α-hydroxy-5β configuration, especially etiocholanolone, are pyrogenic in humans [42]. When injected intramuscularly, etiocholanolone elicits a latent fever through a local inflammatory response by activating leukocytes [43]. The symptom is more prominent in men than in women [44]. Elevated plasma levels of unconjugated etiocholanolone have been associated with periodic disease manifested by recurrent episodes of fever and serious membrane inflammation [45]. This clinical syndrome is denoted etiocholanolone fever. Aberrant steroid metabolism and extrahepatic production of etiocholanolone, where steroid conjugation is limited, were suggested to be responsible for the symptom [45]. However, there were reports questioning the validity of the disease by showing high but similar levels of etiocholanolone were present during both febrile and afebrile periods in patients [46].

5β-Pregnanes

5β-Pregnanes are neurosteroids devoid of progestogenic effects but modulate $GABA_A$ receptor and *N*-methyl-D-aspartate (NMDA) receptor activity. Both $GABA_A$ and NMDA receptors are ligand-gated neurotransmitter receptors required for normal brain function and development. The $GABA_A$ receptor mediates fast synaptic inhibition and functions as a chloride ion conducting channel that opens upon γ-aminobutyric acid (GABA) binding [47]. Pregnanolone (3α-hydroxy-5β-pregnan-20-one) potentiates the GABA response by promoting the open state of the $GABA_A$ receptor, and at higher concentrations the steroid directly activates the $GABA_A$ receptor in the absence of GABA [48]. The 3β enantiomers of pregnanolone as well as 5β-pregnane sulfates inhibit potentiation and may also act as direct $GABA_A$ receptor antagonists [49]. The NMDA receptor regulates excitatory neurotransmission and functions as a cation channel that requires binding of both glutamate and the co-agonist glycine for full activation [50]. Pregnanolone sulfate inhibits NMDA receptors by binding to active receptors and shifting the active receptors into conformations that cause NMDA desensitization [51]. However, whether *de novo* synthesis of 5β-reduced pregnanes occurs in the central nervous system remains to be proven.

5β-Pregnanes, especially 5β-dihydroprogsterone, have also been reported as potent tocolytic agents and maybe responsible for the uterine quiescence maintained by progesterone during pregnancy [52, 53]. 5β-Pregnanes inhibit myometrial contractions *in vitro* [54, 55]. Interestingly, plasma 5β-pregnane concentrations or the 5β-pregnane/progesterone ratio decreases during late pregnancy until post-partum [53, 56, 57] with a concurrent decrease in 5β-reductase expression in the uterus [53]. The mechanism through which the 5β-pregnanes exert the tocolytic effect remains controversial, and possibly involves PXR [58], the $GABA_A$ receptor [59], calcium signaling [60, 61], and the oxytocin receptor [62, 63].

5β-Pregnanes are involved in erythropoiesis in birds [40], rodents [64], and primates [65] and have also been reported to stimulate the growth of erythroid progenitor cells in human [41]. 5β-Pregnanes promote iron uptake in human bone marrow culture [66] and experiments on avian liver suggest that the steroids exert stimulatory effects by inducing δ-aminolevulinate synthase, which augments heme synthesis [41].

5β-pregnanes are also agonists for PXR and human constitutive androstane receptor (CAR). PXR and CAR are orphan nuclear receptors most abundantly expressed in liver. The function of PXR solely depends on ligand binding, whereas CAR is active in the absence of ligand but can be further regulated by activators and repressors [67]. Both receptors can be activated by a variety of xenobiotics and exert a xenoprotective function by regulating phase I and II detoxification enzymes and transporters. 5β-Pregnanes are among the most potent agonists for PXR and CAR, indicating their important xenoprotective role and potential to mediate liver xenobiotic metabolism [68, 69].

5β-Dihydrocortisol

5β-Reduced cortisol was once thought inactive until studies revealed the abnormal accumulation of 5β-dihydrocortisol in patients with primary open angle glaucoma [70]. 5β-Reduced glucocorticoid metabolites do not bind to the glucocorticoid receptor [71]. But 5β-dihydrocortisol sensitizes ocular tissue to glucocorticoids by triggering glucocorticoid receptor nuclear translocation [72]. The hypersensitivity to glucocorticoids was proposed to be the basis of ocular hypertension associated with primary open angle glaucoma. By contrast, 3α,5β-tetrahydrocortisol, a metabolite of 5β-dihydrocortisol, reduces intraocular pressure. Mechanisms for both actions have not been elucidated. However, the reduction of intraocular pressure by tetrahydrocortisol may be related to its ability to modify the cytoskeleton through actin organizing proteins, which causes drainage through the trabecular

meshwork [73]. 3α,5β-Tetrahydrocortisol also exhibits antagonist properties on the $GABA_A$ receptor [74].

Cardiac glycosides

Cardiac glycosides are predominantly produced by plants and have also been identified in toads and insects [4]. Cardiac glycosides bind to the α-subunit of Na^+/K^+-ATPase and inhibit ion transport. In plants, these compounds are used as a defense mechanism against herbivores. In humans, cardiac glycosides increase myocardial contraction and exhibit natriuretic and vasoconstrictive effects. Exogenous cardiac glycosides are well known for their use in treatment of congestive heart failure. In the past two decades the presence of endogenous cardiac glycosides in human and other mammals has also been reported [75–79]. Though the origin of the endogenous glycosides remains controversial as these substances could also be obtained from diet, radiotracer studies in rats using both [^{14}C]-labeled acetic acid and cholesterol supports that the adrenal gland could at least generate some of the glycosides endogenously through cholesterol biosynthesis [80]. Endogenous cardiac glycosides function as regulatory molecules, which influence Na^+/H^+ and Na^+/Ca^{2+} exchange and affect various signaling pathways mediated by Na^+/K^+-ATPase. Increased plasma concentrations of endogenous cardiac glycosides were observed in patients with asymptomatic left ventricular dysfunction [81]. The steroids may have the potential to be developed into a biomarker for predicting heart failure.

Ecdysteroids

Ecdysteroids are nonvertebrate 5β-steroids produced by insects, crustaceans (zooecdysteroids), plants (phytoecdysteroids), and fungi (mycoecdysteroids). There are over 300 ecdysteroids identified to date [82]. Zooecdysteroids, especially ecdysone and 20-hydroxyecdysone, are crucial hormones for insect and crustaceans, which regulate molting and metamorphosis during arthropod development and also affect reproduction, behavior, and stress resistance in adults [83]. In plants and fungi, phytoecdysteroids and mycoecdysteroids function as protective agents against insect predators and soil nematodes. The ecdysteroidogenesis pathway has not been fully elucidated. A Δ^4-3-ketosteroid and a Δ^4-diketol, have been proposed as intermediates [5]. Ecdysteroids exert many beneficial effects on mammals, including anabolic, hepatoprotective, neuroprotective, and immunoprotective properties [82]. The steroids stimulate protein synthesis, increase muscle mass, influence cholesterol absorption, and exhibit anti-tumor effects. Ecdysteroids are devoid of androgenic, estrogenic, or glucocorticoid effects and are considered safe for human consumption. Ecdysteroids have been developed as food supplements for their anabolic properties but have not been thoroughly evaluated for therapeutic uses. Ecdysteroids are not ligands for mammalian steroid nuclear receptors due to their substantially higher dissociation constants compared to the natural ligands for these receptors. Ecdysteroids are proposed to act through membrane bound receptors that influence signal transduction pathways [82].

3. Human Δ^4-3-Ketosteroid 5β-Reductase (AKR1D1)

The metabolism of steroid hormones that contain the Δ^4-3-ketosteroid functionality proceeds in the liver by either 5α- or 5β-reduction. Subsequently the 3-ketone group is metabolized to yield four stereoisomeric tetrahydrosteroids, in which the alcohol can assume either a 3α-axial or 3β-equatorial configuration. This route of steroid metabolism was originally proposed by Tomkins for glucocorticoids even though the discrete enzymes involved had not yet been identified [84]. Remarkably, the introduction of the A/B *cis*-ring junction in 5β-reduced steroids is a reaction that is difficult to perform chemically, since common reductants produce the allylic-3β-alcohol. The enzyme, which catalyzes the 5β-reduction of

Δ^4-3-ketosteroids, was first purified from rat liver to homogeneity [85] and in 1991 its cDNA was cloned [86]. The rat enzyme not only exhibits activity towards Δ^4-steroid hormones like testosterone and progesterone, but also turns over glucocorticoids and bile acid precursors with lower catalytic efficiency. 5β-Reductases were later annotated as belonging to the 1D subfamily of the aldo-keto reductase superfamily. The rat enzyme is denoted as AKR1D2. In 1994, human steroid 5β-reductase AKR1D1 was cloned using a rat cDNA as a probe [87]. Initially it was postulated that AKR1D1 might not be the only human 5β-reductase due to discrepancies in its substrate preference reported by different studies using different assay conditions or transfection conditions [88]. This debate was settled when recombinant AKR1D1 was purified to homogeneity and shown to have activity towards all tested Δ^4-3-ketosteroids, including steroid hormones (testosterone, progesterone, glucocorticoids, and mineralcorticoids) and bile acid precursors [89]. In addition, genetic defects in AKR1D1 not only cause bile acid deficiency but also elicits a significant decrease in all the 5β-reduced steroid metabolites in humans, suggesting AKR1D1 is the only enzyme capable of performing 5β-reduction [90].

Gene structure and regulation

The *AKR1D1* gene is located on chromosome 7, and is in close proximity to the aldose reductases, AKR1B1 and AKR1B10 [91]. AKR1D1 shares the highest sequence identity (>50%) with hydroxysteroid dehydrogenases, AKR1C1-AKR1C4. But the *AKR1C* genes are located on chromosome 10 [91]. The *AKR1D1* gene consists of nine exons and eight sizable introns that make the *AKR1D1* the largest gene in the AKR superfamily [92]. The mRNA of the gene possesses a long 3'-untranslated region containing AT-rich sequences signifying that it may undergo rapid degradation [92]. Transcriptional regulation of AKR1D1 has not been fully investigated. The FXR has been reported to upregulate rat 5β-reductase (AKR1D2) expression [93]. In the presence of the synthetic FXR agonist GW4064, *AKR1D2* gene transcription was greatly enhanced in rat primary hepatocytes. The fold induction was similar to that observed with the SHP, a well-known nuclear receptor corepressor induced upon FXR activation. However, expression of SHP inhibits bile acid biosynthesis by repressing *CYP7A1* gene transcription as part of the feedback inhibition of bile acid biosynthesis. Thus it is uncertain why FXR enhances 5β-reductase expression. In addition, the promoter region of the *AKR1D1* gene contains consensus sequences for *cis*-regulatory elements, including an osmotic response element, an estrogen response element, a phorbol ester response element, and an anti-oxidant response element [91]. Though there is still no direct evidence to prove that these consensus response elements regulate AKR1D1 expression in human, it has been reported in hamster, rat, and dove that 5β-reductase activity is induced by estrogens [94–96]. Rat 5β-reductase has been shown to be modestly induced by dietary antioxidants [97].

Tissue distribution

AKR1D1 is predominantly expressed in the liver where it is present at least 10-fold higher than in any other tissue [98, 99]. This is in agreement with the major biological function of AKR1D1 in bile acid biosynthesis and steroid hormone clearance, both of which take place in the liver. Expression of AKR1D1 in brain, uterus, and placenta has also been reported and this would be consistent with the function of active 5β-reduced metabolites acting as neurosteroids and tocolytic agents [53, 100]. In addition, AKR1D1 has also been detected in testis, colon, skeletal muscle, prostate, lymph node, breast, thyroid, adipose tissue, and blood cells [92, 101–103]. There is no sex-related differences in AKR1D1 expression in humans [104], but differences in 5β-reductase activity towards glucocorticoids between men and women has been reported [105]. Sexual dimorphism for 5β-reductase expression/activity has been reported in mouse, rat, fish, frog, and bird [1, 106–108].

Alternative splice variants, single-nucleotide polymorphisms, and genetic defects

Six transcripts of AKR1D1 have been reported in the Ensembl database, four of which can be translated into protein variants (Figure 2) [109]. Besides the transcript that encodes the full length protein of 326 residues (AKR1D1-002, ENSP00000242375, CCDS5846), the other three splice variants code for protein sequences that are of 290, 285, and 96 residues in length. None of the splice variants have been biochemically characterized. Based on structural analyses, the 290-residue-long variant AKR1D1-006 (ENSP00000389197, CCDS55169) lacks the last α helix and the *C*-terminal flexible loop. The variant may still be able to fold properly but since the missing loop is involved in steroid binding, the isoform would likely have low activity due to decreased affinity for steroid substrates. On the other hand, the 285-residue-long variant AKR1D1-001 (ENSP00000402374, CCDS55170) lacks residues 153–193 and the 96-residue-long variant AKR1D1-009 (ENSP00000397042) lacks major parts of the protein, both of which would compromise the structural integrity of the $(\alpha/\beta)_8$ barrel structure and would render the variants inactive.

In addition to the splice variants, there are also numerous single-nucleotide polymorphisms (SNPs) reported for AKR1D1. The majority of the SNPs are located in the 5'- and 3'-untranslated regions and introns. There are 42 non-synonymous mutations in the protein coding region. None of these non-synonymous mutations have a reported minor allelic frequency so their frequency in the population is unknown. There are studies associating several of these polymorphic variants with serious genetic defects, which could lead to neonatal lethal bile acid deficiency as described below. But most of the SNPs have not been functionally characterized.

AKR1D1 associated bile acid deficiency is an autosomal recessive condition first recognized in 1988 [110]. It is now considered one of the most common genetic defects in bile acid biosynthesis. Clinical representation of the defect includes neonatal cholestatic jaundice and fat soluble vitamin malabsorption, which may rapidly progress to cirrhosis, hemochromatosis, and neonatal liver failure [111, 112]. But these manifestations are not necessarily specific to 5β-reductase deficiency. Diagnosis of the defect needs to be confirmed with steroid urine analysis by mass spectrometry. Patients with inborn errors in AKR1D1 exhibit low or undetectable levels of plasma and urine primary bile acids (chenodeoxycholic acid and cholic acid) but significantly elevated concentrations and higher ratios of unsaturated bile acids that retain the Δ^4-3-oxo-functionality (>70 %) and 5α-reduced *allo*-bile acids [113, 114]. These abnormal bile acids are hepatotoxic and less soluble than the primary bile acids, which could be responsible for their lack of canalicular secretion [3]. In addition, the lack of primary bile acids prevents the feed-back inhibition of cholesterol 7α-hydroxylase (*CYP7A1*) and sterol 12α-hydroxylase (*CYP8B1*), which will exacerbate the built up of Δ^4-3-oxo- and *allo*-bile-acids (Figure 3).

Genetic analyses have revealed three obvious inborn errors in AKR1D1. Two caused nonsense mutations in exon 2 and 3 [115, 116], the other caused a frameshift mutation in exon 5, which produces a non-functional AKR1D1 with a premature stop-codon [117]. In addition, a growing list of missense mutations have also been associated with inherited 5β-reductase deficiency, including Leu106Phe, Pro133Arg, Pro198Leu, Gly223Glu, Asp241Val, Arg261Cys, and Arg266Gln [115–118]. These residues are evolutionary conserved among all AKR1 family members [119] and a review of the AKR1D1 structure shows that none of these residues are involved in catalysis, substrate binding, or cofactor binding [120]. However, when expressed in human embryonic kidney cells, these AKR1D1 mutants showed moderate (Pro133Arg) to severe (Leu106Phe, Pro198Leu, Gly223Glu, Arg261Cys) decreases in expression and enzyme activity, suggesting that the mutations impair protein folding and stability [121]. Thus far the Asp241Val and the Arg266Gln mutants have not been characterized. Of the natural mutations that cause bile acid

deficiency, only the Pro133Arg mutant could be expressed in *E. coli* and obtained in homogeneous form for biochemical characterization [121]. The Pro133Arg mutant exhibited a much higher K_d value for the cofactor NADPH, and transient kinetic studies showed that the mutant was compromised in its ability to carry out the chemical reaction (M. Chen and Y. Jin, unpublished data). The bile acid deficiency caused by the missense AKR1D1 mutants may result from diminishment of both enzyme expression and activity. Clinical observations also suggest that AKR1D1 is a labile enzyme since hepatocyte damage causes a loss in 5β-reductase activity (secondary 5β-reductase deficiency), which could be due to either low expression or rapid degradation of AKR1D1 [122]. Fortunately, bile-acid deficiency can be treated by supplementation with primary bile-acids [117].

AKR1D1 Enzymology

a) Kinetic mechanism—The detailed kinetic mechanism of AKR1D1 has not been fully elucidated. Based on the previous studies on the AKR1C enzymes and the similarity between AKR1C enzymes and AKR1D1, AKR1D1 likely utilizes the same ordered bi-bi reaction mechanism, in which the cofactor is the first to be bound and the last to be released (Figure 4) [119]. For AKR1C enzymes, the release of oxidized cofactor product is characteristically slow, whereas the rates of hydride transfer and release of reduced steroid product vary greatly and are vulnerable to perturbation/mutation in the steroid binding site [123–125]. Depending on the steroid substrate used, the rate-determining step in AKR1C catalyzed ketosteroid reduction can be chemistry, steroid release, or cofactor release [123]. AKR1D1 is anticipated to utilize a similar kinetic mechanism for 5β-reduction. AKR1D1 binds NADPH tightly with a K_d value of 320 nM (100 mM potassium phosphate buffer, pH 7.0, 37 °C, unpublished data). This number is four-fold lower than that of AKR1C2 under the same conditions (unpublished data), indicating AKR1D1 retains the slow cofactor release characteristics as the other AKR1 enzymes. In the stopped-flow under multiple turnover conditions, burst-phase kinetics were observed at saturating substrate concentration for the “fast” substrates (aldosterone, cortisone, and testosterone, $k_{cat} > 8\ min^{-1}$, pH 6) but not for the “slow” substrates 7α-hydroxy-4-cholesten-3-one and cholestenone, $k_{cat} < 2\ min^{-1}$, pH 6) (Y. Jin, unpublished data), indicating the rate limiting step of AKR1D1 is also substrate dependent. For the fast substrates, the overall rate of 5β-reduction is limited by the release of either the steroid or the cofactor product, whereas for the slow substrates the chemical event governs the reaction rate.

b) Substrate specificity—AKR1D1 accepts various C_{18}–C_{27} Δ^4-3-ketosteroids, including sex steroids, glucocorticoids, mineralocorticoids, and bile acid precursors as substrates with a pH optimum at 6.0 [89]. The enzyme is promiscuous with respect to the side chain at the C-17 position. In addition, AKR1D1 also reduces $\Delta^{1,4}$-dienes and likely plays a role in the metabolism of synthetic androgens and glucocorticoids. Under steady-state conditions, AKR1D1 exhibits significantly different k_{cat} (2.0–11.7 min^{-1}) and K_m (0.3–15.1 μM) values for different steroid substrates. The catalytic efficiency of the most favored substrate Δ^4-androstene-3,17-dione is over 30-fold higher than the least favored substrate cortisol [89]. Substrate inhibition was observed for C_{18}, C_{19}, and some of the C_{21} steroids but not C_{27} steroids. More extensive substrate screening to include nonsteroidal substrates has not been performed with AKR1D1. But plant progesterone 5β-reductase was shown to use the 5β-reduction machinery to reduce monocyclic enones and acyclic enoate esters [126]. In addition, AKR1C enzymes, the closest structural homologs to AKR1D1, demonstrate that their active site can accommodate small nonsteroidal molecules such as *S*-tetralol and 1-acenaphthenol. Thus, it is reasonable to assume that the double bond reductase activity of AKR1D1 may not be limited to steroid substrates.

c) Inhibition—Several inhibitors developed for AKR1C enzymes and 5β-reductases have been tested with AKR1D1. AKR1D1 is not inhibited by non-steroidal anti-inflammatory drugs (NSAIDs) such as indomethacin, mefenamic acid, and the related 4-benzoylbenzoic acid [89]. Recently NSAID analogs have been pursued as AKR1C3 inhibitors, where AKR1C3 inhibitors are desirable for treatment of castrate resistant prostate cancer [127]. The lack of cross inhibition of AKR1D1 by AKR1C inhibitors is desired. The 5α-reductase inhibitor finasteride acts as a competitive inhibitor for AKR1D1 [128]. Even though the inhibition potency is much lower for AKR1D1 than for the 5α-reductases, it may be involved in some side effects elicited by finasteride. Primary bile acid chenodexoycholic acid and secondary bile acid ursodeoxycholic acid both act as noncompetitive inhibitors for AKR1D1 [89]. Their potency towards AKR1D1 is in the low micromolar range, similar to the potency for AKR1C1 and AKR1C3 but much lower than the potency for AKR1C2, which is a known human bile acid binding protein and binds bile acids with nanomolar affinity. [129]. AKR1D1 inhibition by bile acids may play a role in the feedback inhibition of bile-acid biosynthesis.

d) Structural Biology—Numerous crystal structures of AKR1D1 have been reported (Table 1). The enzyme possesses the same triose-phosphate isomerase (TIM) barrel core structure composed of eight alternating α-helices and β-strands shared by the other aldo-keto reductase family members. The steroid cavity is situated at the C-terminal of the β-strands surrounded by three long flexible loops. In the productive binding mode, the cofactor NADPH and steroid bind perpendicular to each other (Figure 5A) [120]. The steroid 3-ketone group is anchored to the oxyanion site through hydrogen bonding to Tyr58 and Glu120. The β-face of the steroid is presented to the cofactor with the aid of Trp230 and Tyr132. Thus the Δ^4-double bond is positioned within an appropriate distance and orientation from the cofactor to permit 4-pro-*R* hydride transfer from the nicotinamide ring to the 5β-position of the steroid. The end of the steroid cavity is open to solvent, which also explains the promiscuity of AKR1D1 for substrates with varied C-17 side-chains. Smaller steroid substrates like testosterone and progesterone can be tethered by Tyr132, Ser225, and Asn227 to a non-productive binding mode in which the steroid lies parallel with the cofactor (Figure 5B). This non-productive binding mode provides an explanation for substrate inhibition. This is supported by studies with the Val309Phe mutant. Val309 resides in the non-productive cavity and when it is mutated to the bulkier Phe, substrate inhibition is eliminated [130].

The active site of AKR1D1 is fine tuned for 5β-reduction and its function is precisely controlled by a single residue, Glu120. Glu120 is also the most prominent residue that distinguishes 5β-reductase from the other AKR family members in sequence alignment. A Glu120His mutation abolishes 5β-reductase activity and perfectly converts the enzyme into a highly efficient 3β-hydroxysteroid dehydrogenase [131]. Crystal structures of the Glu120His mutant reveal that the residue controls enzyme function by manipulating the relative position between the steroid and cofactor. With the smaller glutamate in position, the steroid can reach “deeper” into the active site to bring the steroid C5 carbon within hydride transfer distance to the cofactor (Figure 6A). Once substituted by histidine, the bulky imidazole side chain pushes the steroid away so that only the steroid C3 carbon can be reduced. In this new position the steroid C3 ketone group in the Glu120His mutant superimposes on the corresponding group in ketosteroids bound in the oxyanion hole in the AKR1C enzymes (Figure 6B). This Glu120His mutation sets an excellent example of how a single amino acid governs enzyme function and emphasizes that the presence of the glutamate residue is essential when assigning a newly identified *AKR* gene as a 5β-reductase.

e) Chemical mechanism—5β-Reduction is an irreversible double bond reduction of Δ^4-3-ketosteroids. During the chemical event, the hydride at the 4-pro-*R* position on the nicotinamide ring is stereospecifically transferred to C5 position of the steroid on the β-face, which is facilitated by a highly conserved Tyr58 that acts as a general acid and Glu120 that acts as a "superacid" to enolize the α,β-unsaturated ketone group of the steroid (Figure 7) [120]. In the mechanism of rat 3α-hydroxysteroid dehydrogenase (AKR1C9), Asp53, Tyr58, Lys87, and His120 (AKR1D1 numbering) are collectively called the catalytic tetrad. The mechanism depicts Tyr58 as the general acid/base, where His120 facilitates proton donation from tyrosine in the reduction direction, and Lys87 and Asp53 facilitates proton removal by the tyrosine in the oxidation direction. In the structure of AKR1D1, Lys87 and Asp53 remain in hydrogen bond network with Tyr58 and Glu120. Faucher et al. proposed that Lys87 and Asp53 play roles in the proton relay by shuttling protons from Tyr58 [132]. However, this mechanism has yet to be supported by pH-rate studies.

4. Future directions

The physiological functions of 5β-reduced steroids other than bile acids still remain to be fully elucidated along with their modes of action. Wild type AKR1D1 can be used as a synthon to catalyze the formation of A/B *cis* ring fusions in steroid substrates, which is difficult to perform chemically. AKR1D1 mutations have been associated with bile acid deficiency. With the onset of whole exome sequencing, SNPs are likely to be revealed in AKR1D1 that can be mapped to the existing crystal structure. Examination of these mutants by site-directed mutagenesis will determine whether they affect enzyme function or folding and could be deleterious to health. Defects in AKR1D1 activity may also compromise glucocorticoid metabolism and as a result the loss of enzyme activity could play a role in obesity. The basis of the tissue specific expression of 5β-reductase needs to be elucidated to determine whether this is related to tissue specific transcription factors or epigenetic imprinting.

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Abbreviations

AKR	aldo-keto reductase
AKR1C1-AKR1C4	human hydroxysteroid dehydrogenase
AKR1D1	human steroid 5β-reductase
AKR1D2	rat steroid 5β-reductase
CAR	constitutive androstane receptor
ERK	extracellular-signal regulated kinase
FXR	farnesoid X receptor
JNK	c-Jun NH_2-terminal kinase
LXR	liver X receptor
PXR	pregnane X receptor
SREBP	sterol regulatory element binding protein
SHP	short heterodimer partner

TIM	triose phosphate isomerase

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Highlight Points

- 5β-reduced steroids are metabolites of all Δ^4-3-ketosteroids
- 5β-reduced androstanes, pregnanes, and cholanes perform biological functions
- All mammalian bile-acids are 5β-cholanes
- Aldo-keto reductase (AKR) 1D1 is the only human 5β-reductase
- Structure-function studies on AKR1D1 provide a basis for bile-acid deficiency

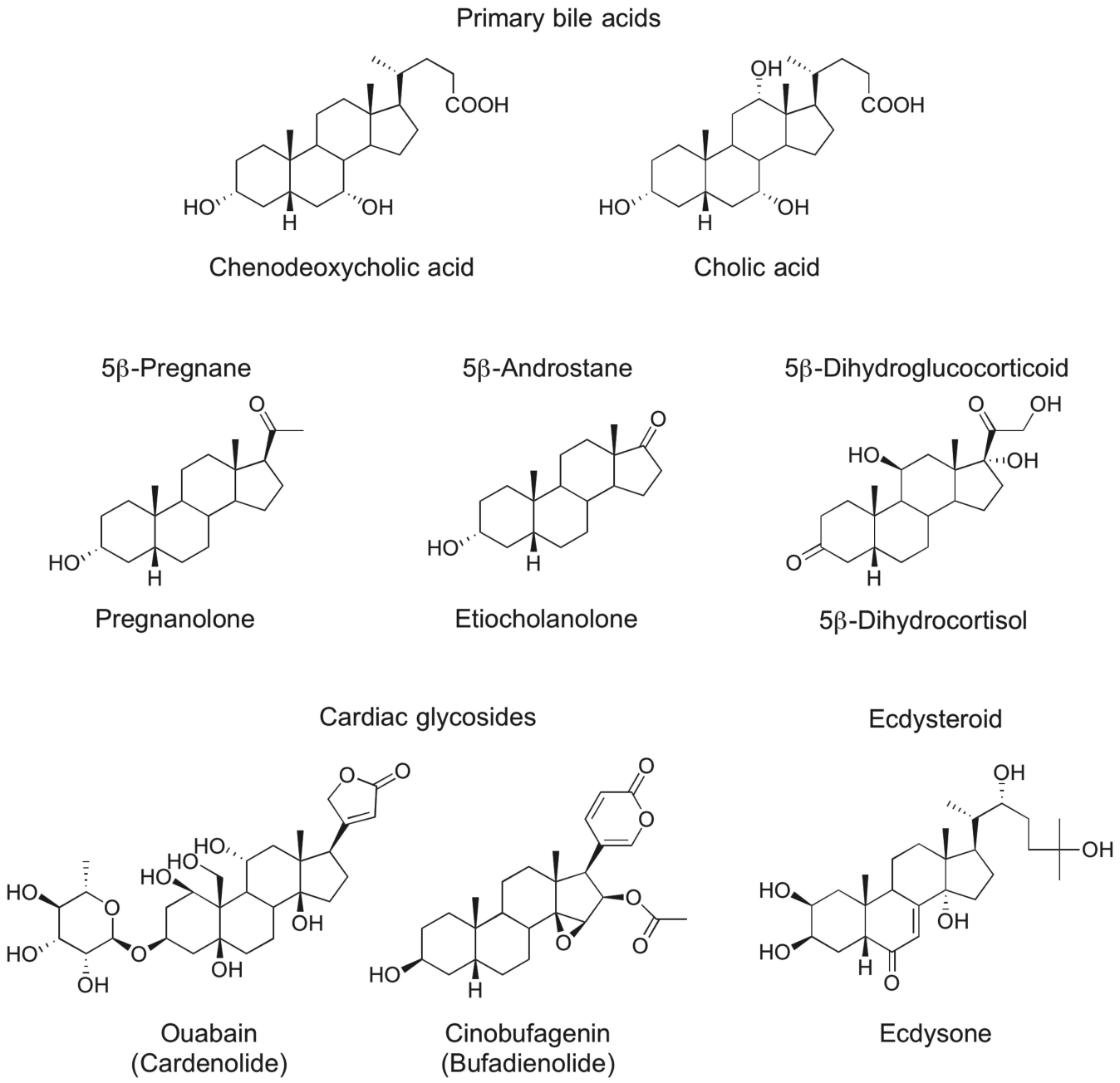

Figure 1.
Structures of biologically active 5β-reduced sterols and steroids.

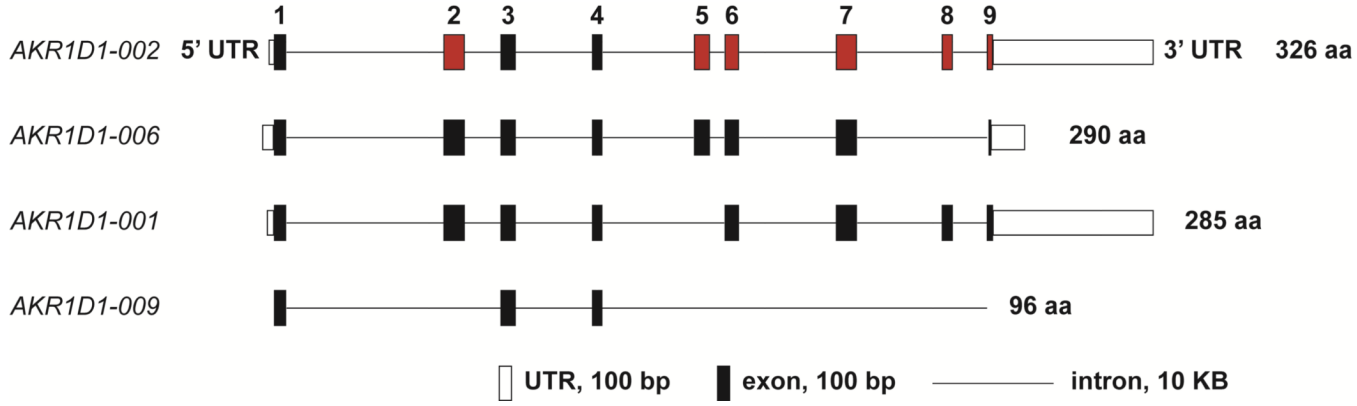

Figure 2.

AKR1D1 gene structures and splice variants. The nine exons are represented as filled boxes and numbered. The constitutive exons are shown in black and the alternatively spliced exons are in red.

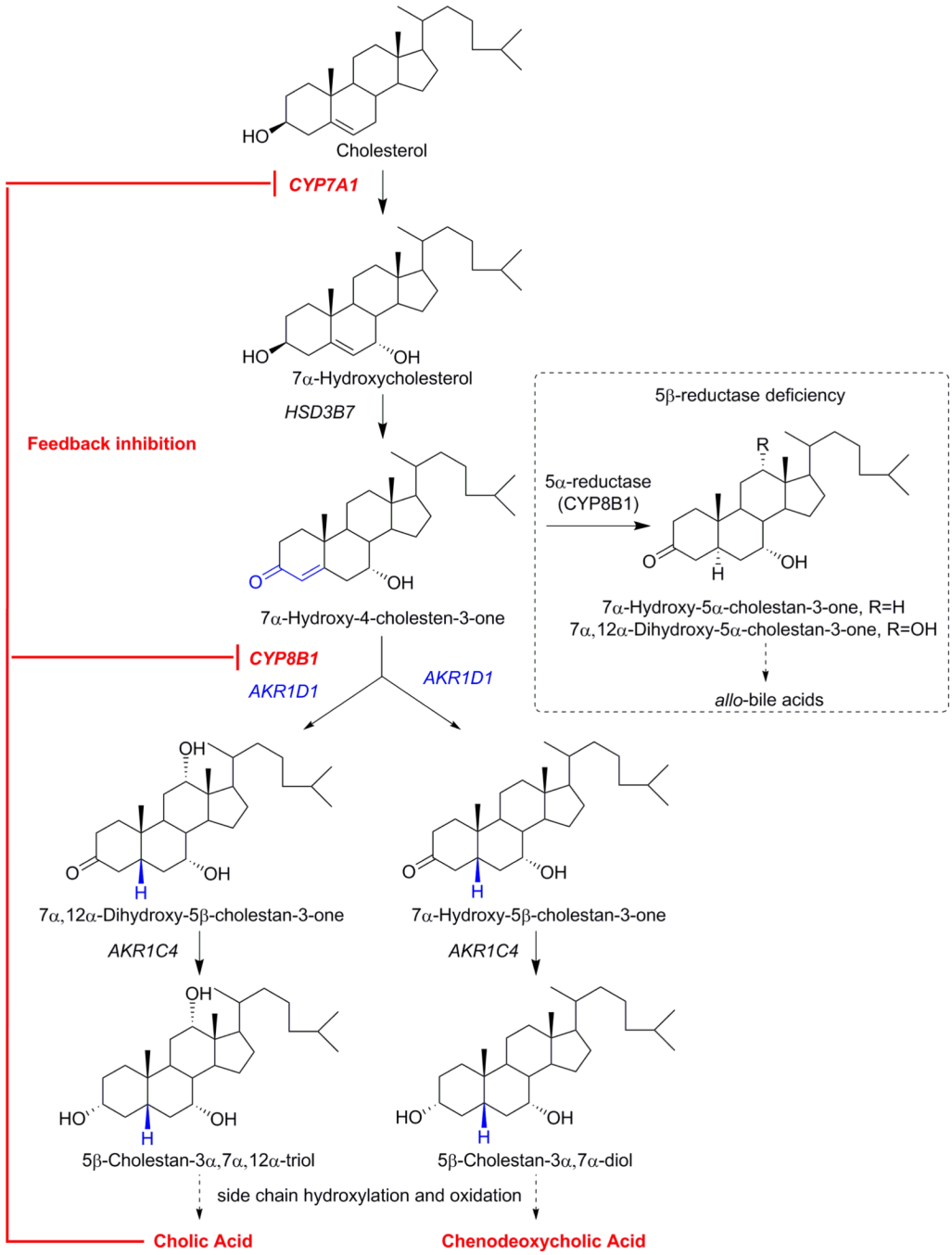

Figure 3.

Bile acid biosynthesis. AKR1D1 associated bile acid deficiency causes accumulation of Δ^4- and *allo*-bile acids and prevents feedback inhibition dependent upon the primary bile acids, cholic acid and chenodeoxycholic acid. *CYP7A1*, cholesterol 7α-hydroxylase; *CYP8B1* sterol 12α-hydroxylase; *HSD3B7*, 3β-hydroxysteroid dehydrogenase. Enzymes are italicized as their gene names.

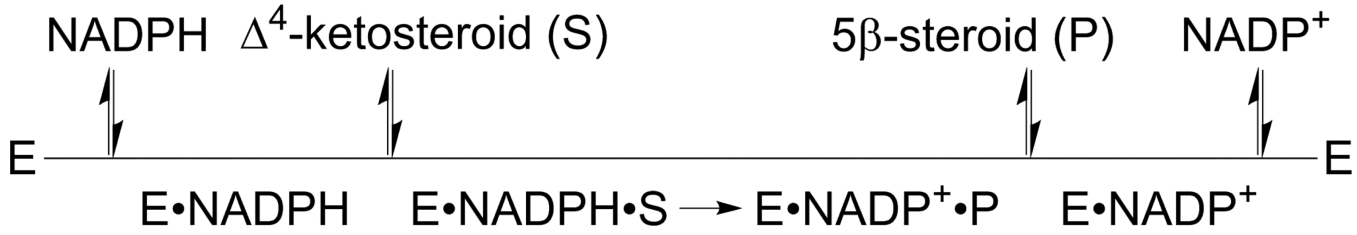

Figure 4.
Ordered bi bi kinetic mechanism for AKR1D1. (S) = substrate, and (P) = product.

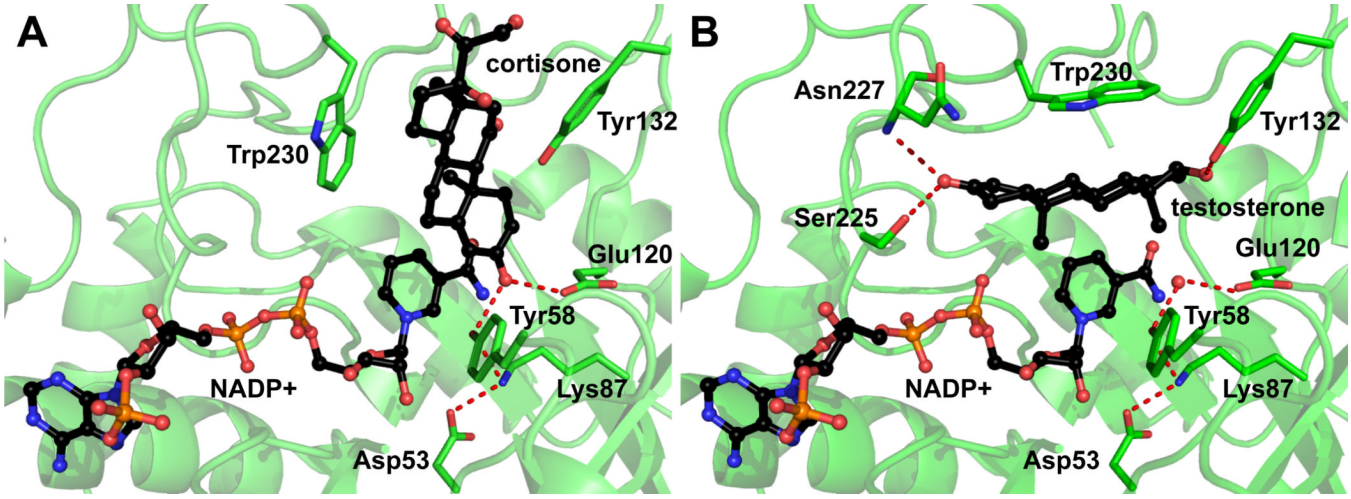

Figure 5.

Crystal Structure of AKR1D1 showing normal (A, PDB: 3CMF) and non-productive (B, PDB: 3BUR) binding modes. The steroids and NADP+ are colored in black. All the other atoms are color-coded as follows: carbon, green; oxygen, red; and nitrogen, blue; phosphor, orange. Hydrogen bonds are indicated by red dashes. All structural figures are prepared using The PyMOL Molecular Graphics System, Version 1.20 Schrödinger, LLC.

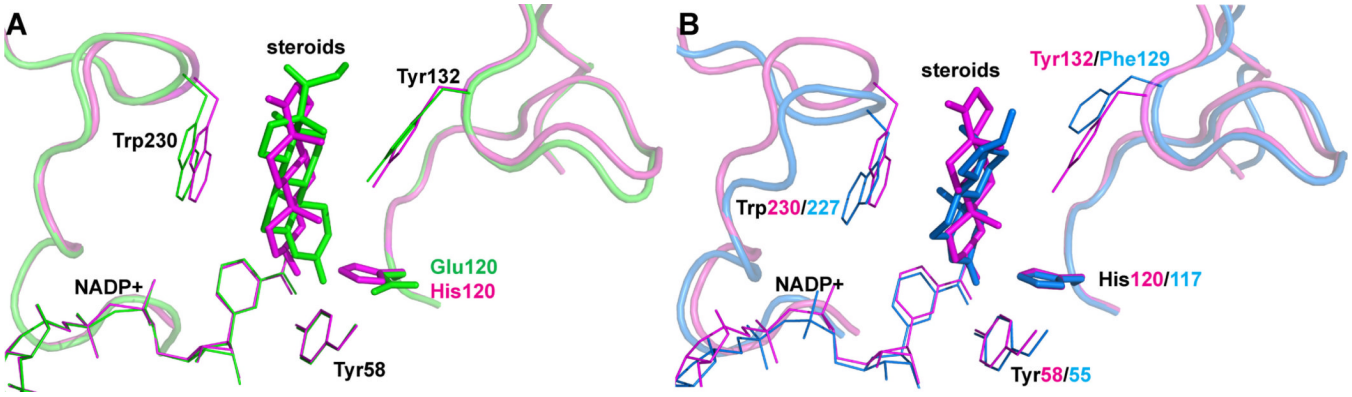

Figure 6.

Crystal Structure of the AKR1D1-Glu120His mutant (magenta) in superposition with WT AKR1D1 (A, green, PDB: 3CMF) and 3α-hydroxysteroid dehydrogenase AKR1C9 (B, blue, PDB: 1AFS). In the AKR1D1 Glu120His mutant structure, the face of the steroid that presents to the cofactor is flipped and the C3 ketone of the steroid does not penetrate as deeply into the active site permitting 3β-hydroxysteroid dehydrogenase activity.

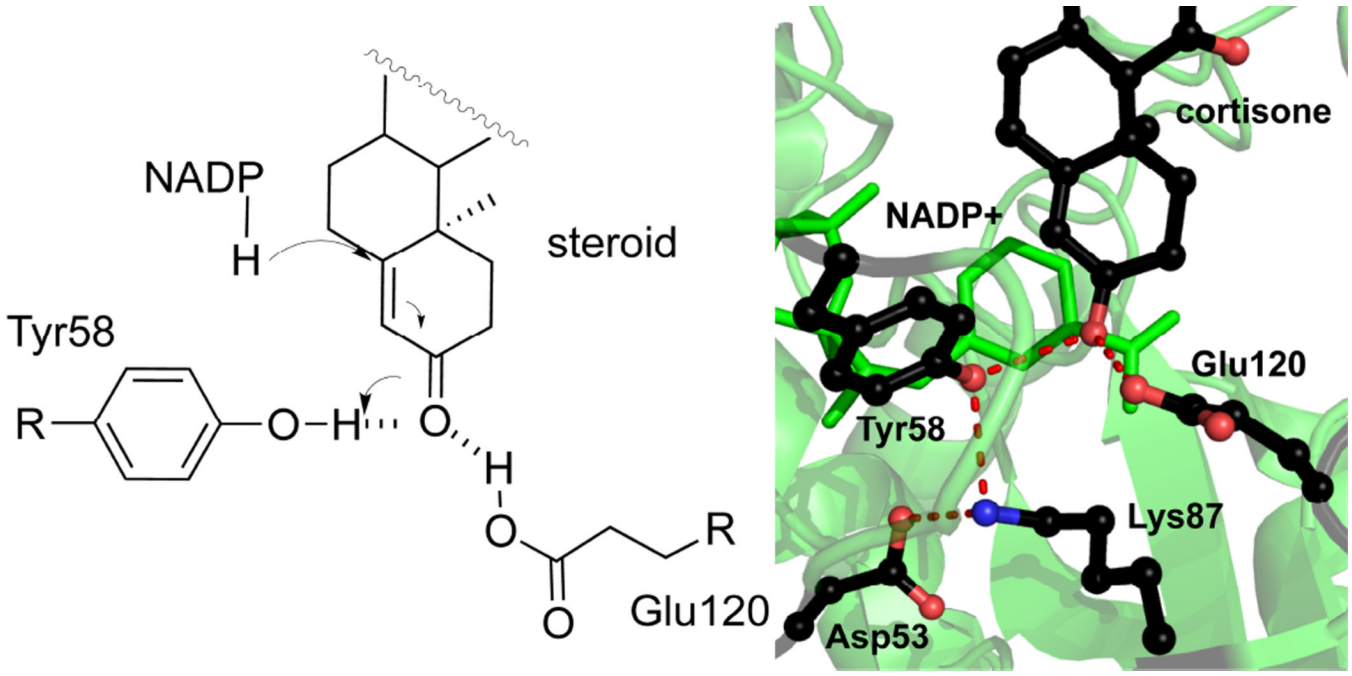

Figure 7.
The chemical mechanism of AKR1D1. Note the steroid is drawn with the α-face towards the viewer and the hydride is transferred to the β-face. The tetrad residues and steroid are colored in black. All the other atoms are color-coded as in Figure 5. Hydrogen bonds are indicated by red dashes.

Table 1

Crystal structures of AKR1D1.

PDB ID	AKR1D1	Cofactor/steroid	Reference
3BV7	WT	$NADP^+$	[120]
3BUV	WT	$NADP^+$	[120]
3BUR	WT	$NADP^+$, testosterone	[120]
3CMF	WT	$NADP^+$, cortisone	[120]
3COT	WT	$NADP^+$, progesterone	[120]
3CAQ	WT	NADPH	[132]
3CAV	WT	$NADP^+$, 5β-pregnane-3,20-dione	[130]
3CAS	WT	$NADP^+$, Δ^4-androstenedione	[130]
3DOP	WT	$NADP^+$, 5β-dihydrotestosterone	[133]
3G1R	WT	$NADP^+$, finasteride	[128]
3UZW	Glu120His	$NADP^+$	[131]
3UZX	Glu120His	$NADP^+$, epiandrosterone, 3α-androstanediol	[131]
3UZY	Glu120His	$NADP^+$, 5β-dihydrotestosterone	[131]
3UZZ	Glu120His	$NADP^+$, Δ^4-androstenedione, testosterone	[131]