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Novel activities of CYP11A1 and their potential physiological significance

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Abstract

CYP11A1, found only in vertebrates, catalyzes the first step of steroidogenesis where cholesterol is converted to pregnenolone. The purified enzyme, also converts desmosterol and plant sterols including campesterol and β -sitosterol, to pregnenolone. Studies, initially with purified enzyme, reveal that 7-dehydrocholesterol (7DHC), ergosterol, lumisterol 3, and vitamins D3 and D2 also serve as substrates for CYP11A1, with 7DHC being better and vitamins D3 and D2 being poorer substrates than cholesterol. Adrenal glands, placenta, and epidermal keratinocytes can also carry out these conversions and 7-dehydropregnenolone has been detected in the epidermis, adrenal glands, and serum, and 20-hydroxyvitamin D3 was detected in human serum and the epidermis. Thus, this metabolism does appear to occur *in vivo*, although its quantitative importance and physiological role remain to be established. CYP11A1 action on 7DHC *in vivo* is further supported by detection of 7 steroids in Smith-Lemli-Opitz syndrome patients. The activity of CYP11A1 is affected by the structure of the substrate with sterols having steroidal or 7 -steroidal structures undergoing side chain cleavage following hydroxylations at C22 and C20. In contrast, metabolism of vitamin D involves sequential hydroxylations that start at C20 but do not lead to cleavage. Molecular modeling using the crystal structure of CYP11A1 predicts that other intermediates of cholesterol synthesis could also serve as substrates for CYP11A1. Finally, CYP11A1-derived secosteroidal hydroxy-derivatives and 7 steroids are biologically active when

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Conflict of interest

The authors declare no conflict of interest

administered *in vitro* in a manner dependent on the structure of the compound and the lineage of the target cells, suggesting physiological roles for these metabolites. This article is part of a special issue entitled 'SI: Steroid/Sterol signaling'.

Keywords

CYP11A1; Cholesterol; Plant sterols; Vitamin D; 7-Dehydrocholesterol; Ergosterol

1. Overview of the function and phylogeny of CYP11A1

1.1. Biochemical characterization

CYP11A1, also known as cytochrome P450 side chain cleavage (P450_{scc}), is a mitochondrial enzyme best characterized for its catalysis of the cleavage of the side chain of cholesterol to produce pregnenolone, the common precursor of steroid hormones [1,2]. The reaction involves initial hydroxylation at C22 producing 22*R*-hydroxycholesterol, then subsequent repositioning of the side chain in the active site resulting in a second hydroxylation at C20, producing 20*R*,22*R*-dihydroxycholesterol. This is followed by oxidative cleavage of the C20—C22 bond producing pregnenolone [1–3].

Electrons for these reactions are provided by NADPH *via* a short electron transport chain comprising adrenodoxin reductase and adrenodoxin [1,2,4,5]. The final product, pregnenolone, can leave the mitochondria and be converted to steroid hormones by cell- and gland-specific pathways [2]. The products of these pathways are dependent on cell type- or tissue-dependent expression of the different steroidogenic enzymes.

Recently, the crystal structures of bovine [6] and human CYP11A1 [7] have been reported with either cholesterol, 22*R*-hydroxycholesterol (bovine) or 20*R*,22*R*-dihydroxycholesterol in the active site. These structures reveal that the tight binding of the first intermediate, 22*R*-hydroxycholesterol, appears to be mediated, at least in part, by direct co-ordination of the C22-oxygen to the heme-iron. Comparison of the structures with the different intermediates bound reveals that subtle shifts occur in the positioning of the side-chain in the active site following each hydroxylation, enabling the three sequential oxidations required for side chain cleavage. These crystal structures have also proved useful for docking studies with other substrates of the purified enzyme [7–10] (see later).

In classical steroidogenic tissues such as the adrenal cortex, corpus luteum, and testis, and in non-classical tissues with low or very low steroidogenic activity including brain, skin, gastrointestinal tract, and the immune system, steroid synthesis is regulated by the action of the steroidogenic acute regulatory (StAR) protein or its equivalents (STARD3) that control cholesterol transport to the inner mitochondrial membrane site of CYP11A1 action [2,11–15]. In the classical pathway of cholesterol side chain cleavage, CYP11A1 activity is substrate limited and increased rates of cholesterol delivery cause a corresponding increase in the rate of pregnenolone synthesis. The activity of the StAR protein or STARD3 is elevated by both increased protein synthesis and its posttranslational modifications, predominantly mediated by cAMP-dependent pathways activated by ACTH, angiotensin II or LH [2,11,15].

Studies carried out over a number of years reveal that purified CYP11A1 and/or steroidogenic mitochondria that contain CYP11A1, can act on a range of steroids or secosteroids, other than cholesterol. Arthur et al. [16], reported that bovine, rat, and pig adrenal mitochondria could metabolize analogues of cholesterol with shorter 5C and 7C hydrophobic side chains at slightly higher or comparable rates to cholesterol. However, analogues with polar side chains such as 20 α -hydroxycholesterol, 24-hydroxycholesterol, 25-hydroxycholesterol, or 26-hydroxycholesterol were metabolized to pregnenolone at appreciably higher rates than cholesterol. Mason et al. [17] and Craig et al. [18] subsequently attributed these differences to a slower rate of transfer of cholesterol into the mitochondrion by a cycloheximide-sensitive step (later found to be due to inhibition of StAR protein synthesis), compared to the cycloheximide-insensitive transfer of side-chain hydroxy analogues. Morisaki et al. [19] reported that in a CYP11A1 reconstituted system, cholesterol analogues with a 3C or 4C side chains were poorly metabolized to pregnenolone. Activity then increased with side chain length to a maximum with 7C, then decreased with further increases in side chain length. It has also been reported that adrenal mitochondria or a reconstituted CYP11A1 system can act on halogenated side chain analogues of cholesterol, such as 26-bromocholesterol or 25-fluorocholesterol, converting them to pregnenolone [19,20].

In 1986, Lambeth [21] reviewed the substrate specificity of CYP11A1 with respect to both binding and catalysis, with much of the data coming from synthetic analogues of cholesterol. It was concluded that there was high specificity towards a planar structure near C5 (as provided by a C5=C6 or C4=C5 double bond), and that there was not a great deal of specificity with respect to the side chain. Since that review, a number of new substrates for the purified enzyme have been identified that may be of physiological significance, and it is these substrates that we primarily focus on in this review with a critical analysis of their potential to be of physiological relevance. These new substrates include 7-dehydrocholesterol (7DHC) [8,14,22,23], vitamin D3 [22,24–27], vitamin D2 [9,28,29], ergosterol [10,30], and lumisterol [31]. For both cholesterol and its precursor, 7DHC, CYP11A1 catalyzes three oxidative reactions on their side chain, resulting in cleavage occurring between C20 and C22, producing pregnenolone and 7-dehydropregnenolone (7DHP), respectively, as well as isocaproic aldehyde [1,14,22]. Importantly, catalytic activity on non-traditional substrates such as vitamin D and ergosterol does not result in side chain cleavage, but involves sequential hydroxylations of the side chain including at C17 [[10,24–30], and see below].

1.2. Molecular biology and phylogenesis

The CYP11A1 gene is only found in vertebrates. The first cloning of the CYP11A1 gene was reported in 1984 by John et al. [32] and involved differential hybridization screening of bovine cDNA libraries. This facilitated the cloning and sequencing of the gene from other species including a number of mammals as well as species of birds, amphibians, and fish. Phylogenetic analysis of CYP11A1 reveals segregation into three broad groups comprising teleosts, mammals, and the stingray which split early in vertebrate evolution [33,34]. Stingray is a primitive vertebrate of the elasmobranch subclass and its CYP11A1 is highly divergent from both mammalian and teleost forms of the enzyme, with the southern stingray

(*Dasyatis americana*) form displaying 48% identity with teleost species of CYP11A1 and 39–40% with mammals [33,34]. Identity between these three major subclasses is higher in known functional regions of the enzyme. For example, stingray CYP11A1 shows greater than 60% identity with the other forms in the putative adrenodoxin binding domain and the heme binding domain [34]. Of the 4 basic residues implicated in adrenodoxin binding to bovine CYP11A1 (Lys423, Lys425, Arg465, and Arg466), the two arginine residues are found in similar positions in all CYP11A1 proteins, but the two lysines are not well conserved [33,35–37]. The putative steroid binding domain and heme binding domain of CYP11A1 is well conserved from teleosts to the frog, chick, and mouse forms of CYP11A1 [38]. The N-terminal mitochondrial target sequence is moderately conserved among mammalian species (approximately 50% identity) but poorly conserved when rainbow trout (*Oncorhynchus mykiss*) and stingray proteins are compared [34].

As might be expected, there is high amino acid sequence similarity in CYP11A1 between closely related mammalian species. For example, in primates baboon (*Papio ursinus*) CYP11A1 displays 98% identity with human CYP11A1 [39]. For more distantly related mammalian species, identities are typically above 70%. For example, sheep CYP11A1 shares 73% identity with human CYP11A1 and 72% with the rat enzyme [40]. Human CYP11A1 shares 80% identity with hamster (*Mesocricetus auratus*) CYP11A1 and 72% with the bovine enzyme [41,42].

Two CYP11A genes have been identified in zebrafish (*Danio rerio*), CYP11A1 and CYP11A2 [43,44]. This appears to have arisen from a relatively recent genome duplication, with up to 30% of the zebrafish genome being duplicated, including other cytochromes P450 involved in steroid biosynthesis [44]. Zebrafish CYP11A1 and CYP11A2 share 80% amino acid sequence identity. Zebrafish CYP11A2 has higher amino acid sequence homology than CYP11A1 to other vertebrates, including higher homology to other teleosts (72–73% identity *versus* 64–66% identity for zebrafish CYP11A1). Zebrafish CYP11A2 appears to be the functional equivalent of CYP11A1 in other vertebrates, being expressed in the interrenal gland, gonads, and brain. Zebrafish CYP11A1 is expressed in the early embryo and only in the gonads of the adult [44].

Despite the ever increasing number of CYP11A1 sequences from different vertebrate species being reported, little is known about any differences in function of the enzyme between species, particularly catalytic properties. All are assumed to convert cholesterol to pregnenolone to initiate *de novo* steroidogenesis. Non-mammalian forms of the enzyme are particularly poorly characterized. The conversion of cholesterol to pregnenolone by mitochondria isolated from the testis of brook trout (*Salvelinus fontinalis*) has been reported [45]. Rainbow trout (*O. mykiss*), Japanese eel (*Anguilla japonica*), and chicken CYP11A1 have been expressed in COS cells and shown to convert 25-hydroxycholesterol to pregnenolone [33,46,47]. Knockdown of CYP11A2 expression in zebrafish was shown to reduce both pregnenolone and cortisol concentrations. Only human and bovine CYP11A1 have been purified, from both steroidogenic tissues and following expression in *Escherichia coli*, and their catalytic properties fully compared [1,14,48,49]. These forms share 72% amino acid identity [42] and do show some differences in catalytic properties. They display similar K_m values with cholesterol as substrate. The values of K_{cat} vary depending on the

source of the enzyme and the purification procedure employed making it difficult to accurately compare these parameters [14,48,49]. A clear difference between these species is that human CYP11A1 shows poor catalytic efficiency for the metabolism of 25-hydroxycholesterol with its K_{cat}/K_m value being 6.6-fold lower than that for the bovine enzyme [48].

2. 7DHC as a substrate for CYP11A1

2.1. Overview of cholesterol synthesis

Cholesterol balance is maintained by biosynthesis in the liver and extrahepatic tissues (the endogenous pathway) (Fig. 1) [50,51] and through absorption of dietary and biliary cholesterol (the exogenous pathway). Cholesterol biosynthesis can be divided into pre- and post-squalene pathways.

2.1.1. Pre-squalene pathway of cholesterol synthesis—Synthesis of the 27 carbon cholesterol molecule includes approximately 30 enzymatic reactions with all of the carbon atoms originally derived from the two-carbon acetate group of acetyl-CoA. Synthesis of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) is catalyzed by HMG-CoA synthase with acetyl-CoA and acetoacetyl-CoA serving as substrates. HMG-CoA is converted to mevalonate by membrane-bound HMG-CoA reductase, which is the rate-limiting and irreversible step of cholesterol biosynthesis. Mevalonate is then activated by two successive phosphorylations, yielding 5-pyrophosphomevalonate. Mevalonate is decarboxylated to isopentenyl pyrophosphate, an activated isoprenoid molecule which is in equilibrium with its isomer, dimethylallyl pyrophosphate. One molecule of isopentenyl pyrophosphate condenses with one molecule of dimethylallyl pyrophosphate to form geranyl pyrophosphate. This further condenses with another isopentenyl pyrophosphate molecule to yield farnesyl pyrophosphate. Two molecules of farnesyl pyrophosphate then condense to generate squalene through the action of squalene synthase in the endoplasmic reticulum.

2.1.2. Post-squalene pathway of cholesterol synthesis—Squalene is converted into the first sterol, lanosterol (4,4,14 α -trimethylcholesta-8(9), 24-dien-3 β -ol), by squalene epoxidase and oxidosqualene cyclase. Lanosterol is converted to cholesterol through a multistep process including the oxidative removal of three methyl groups at C4 α , C4 β , and C14, which converts the C30 molecule lanosterol to C27 cholesterol; isomerization of the ⁸⁽⁹⁾ double bond to a ⁷ double bond; desaturation to form a ⁵ double bond; and finally, reduction of ¹⁴, ²⁴, and ⁷ double bonds [52].

The saturation of the C-24 double bond of lanosterol can occur at multiple points in the pathway, creating two immediate precursors for cholesterol, desmosterol [cholesta-5(6), 24-dien-3 β -ol] and 7-dehydrocholesterol [cholesta-5,7-dien-3 β -ol], (7DHC) [50,53,54]. The final steps in cholesterol biosynthesis might be tissue specific [55]. In both alternative pathways, lanosterol is demethylated to form zymosterol (5 α -cholesta-8,24-dien-3 β -ol) in a series of enzymatic reactions catalyzed by lanosterol 14- α -demethylase [CYP51A1] (2 in Fig. 1); 3 β -hydroxysterol ¹⁴-reductase (3 in Fig. 1) and C4 demethylation complex (4 in Fig. 1). This complex consists of three enzymes (C4-sterol methyloxidase, C4-sterol decarboxylase (3 β -hydroxysterol dehydrogenase) [NSDHL], and 3-ketoreductase) that

catalyse the demethylation of 4,4-dimethylcholesta-8-en-3 β -ol and 4,4-dimethylcholesta-8,24-dien-3 β -ol (Fig. 1). If reduction of the Δ^24 -bond occurs early, cholesterol is synthesized *via* the Kandutsch–Russel pathway (favored in most tissues) using 7-dehydrocholesterol as the immediate precursor of cholesterol. In this pathway zymosterol is metabolized sequentially by a 3 β -hydroxysterol Δ^24 -reductase (8 in Fig. 1), 3 β -hydroxysterol Δ^8, Δ^7 -isomerase (5 in Fig. 1), and lathosterol 5-desaturase (6 in Fig. 1) to yield 7-dehydrocholesterol, which is reduced by the 3 β -hydroxysterol Δ^7 -reductase (7 in Fig. 1) at the Δ^7 position to form cholesterol (Fig. 1).

In the Bloch pathway reduction of the Δ^24 -bond occurs as the last enzymatic step that forms cholesterol from desmosterol. Zymosterol is converted to 7-dehydrodesmosterol by the 3 β -hydroxysterol Δ^8, Δ^7 -isomerase (5 in Fig. 1) and lathosterol 5-desaturase (6 in Fig. 1). 7-Dehydrodesmosterol is metabolized to desmosterol by the 3 β -hydroxysterol Δ^7 -reductase (7 in Fig. 1) and then by the 3 β -hydroxysterol Δ^24 -reductase (8 in Fig. 1) to form cholesterol (Fig. 1).

2.2. In vitro and in vivo models of 7DHC metabolism by CYP11A1

7DHC was first identified as a substrate for purified bovine CYP11A1 by Guryev et al. [22]. Subsequent studies performed with purified bovine and human recombinant CYP11A1 and isolated mitochondria from the adrenal glands and placentae have demonstrated the following pathway for the metabolism of 7DHC: 7DHC \rightarrow 22-hydroxy-7DHC[22(OH)7DHC] \rightarrow 20,22-dihydroxy-7DHC[20,22(OH) $_2$ 7DHC] \rightarrow 7DHP [8,14,22,23]. In fact, human CYP11A1 has a slightly higher catalytic efficiency with 7DHC as a substrate (higher K_{cat}/K_m) than with cholesterol, indicating that 7DHC can be used preferentially by the enzyme depending on its availability [8,14]. The rate of 7DHC transfer to the inner mitochondrial membrane site of CYP11A1 action by the StAR protein is also slightly higher than that for cholesterol [8]. The definitive chemical structures of the intermediates of 7DHC metabolism by CYP11A1 (22(OH)7DHC and 20,22(OH) $_2$ 7DHC), and the final product (7DHP), were established by NMR spectrometry, UV spectra, mass spectrometry, and chemical synthesis of the standards [8,14,22,56,57].

Further support for the above pattern of 7DHC conversion to 7DHP came from molecular modeling using the crystal structure of human CYP11A1 bound to 20R,22R-dihydroxycholesterol [8]. 7DHC was docked to the crystal structure of CYP11A1 in place of the 20R,22R-dihydroxycholesterol. It showed that the C22 hydrogen was closer to the heme iron than the hydrogen at C20, consistent with initial hydroxylation at C22 being favored. Consistent with the above sequence of reactions, docking scores were highest for the 22-hydroxyderivatives of both 7DHC and cholesterol, with the 20,22-dihydroxyderivatives having intermediate docking scores, however, higher than for cholesterol or 7DHC [8]. This modeling is consistent with the known relative binding strengths for cholesterol, 7DHC and their intermediates, and with the direct coordination of the 22R-hydroxyl group to the heme iron [6,7].

It should be noted that we also detected production of two other hydroxy-7DHC metabolites by human CYP11A1 of which the identity remains to be established [8]. Neither of them corresponded to 20(OH)7DHC as demonstrated by their different retention times. It is

expected that the hydroxyl groups are added to the side chain, most likely at positions C23 and C17 since these represent known sites of vitamin D3 hydroxylation by CYP11A1 [25].

Experiments performed with adrenal glands isolated from both males and females of rats, pigs, rabbits, and from male dogs showed that adrenal fragments incubated *ex-vivo* with 7DHC transform it in a sequential manner to 7DHP, with 22(OH)7DHC and 20,22(OH)₂7DHC serving as consecutive intermediates of the pathway [23]. The involvement of CYP11A1 in this transformation was confirmed by the inhibitory effect of DL-aminoglutethimide. The metabolism of 7DHC was stimulated by forskolin indicating the involvement of cAMP dependent pathways, as seen for cholesterol [23]. Studies with purified mitochondria from rat skin also revealed the transformation of exogenous 7DHC to 7DHP, consistent with the known expression of CYP11A1 in skin [23]. The most recent and extensive studies performed on human placentae and bovine adrenal glands, clearly confirmed the above studies and provided additional evidence that CYP11A1-dependent 7DHC metabolism is possible in the human placenta *in vivo* [8]. Final *in vitro* evidence that 7DHC is metabolized to 7DHP in the skin was obtained using cultured human (HaCaT) epidermal keratinocytes, and primary pig epidermal keratinocytes where products were detected without the addition of exogenous 7DHC [8].

The above findings are consistent with a well described phenomenon of increased accumulation of 7DHC, and production of 7DHP and its downstream steroid metabolites in Smith-Lemli-Opitz syndrome (SLOS), characterized by a deficiency of 7DHC Δ^7 -reductase [58–61]. Similarly, *in vivo* production of 7DHP occurs during synthesis of equilin (Δ^7 -estrone) in horses [62]. The production of 7DHP in SLOS and during the synthesis of equilin is attributed to CYP11A1 action on 7DHC [61]. 7DHP may then undergo sequential transformation to the hydroxy-derivatives of 5,7-steroidal dienes or 7-dehydroprogesterone by classical steroidogenic enzymes, as demonstrated by accumulation of 7DHP, its hydroxy-derivatives (including 21-hydroxy-, 17-hydroxy-, 20-hydroxy-, and 17,20-dihydroxy-7DHP) and 7-dehydroprogesterone in body fluids of SLOS patients [60,63,64]. Further support for 7DHP serving as a substrate for CYP17A1 is the production of 17(OH)7DHP and Δ^7 -dehydroepiandrosterone [65]. CYP21A2 does not appear to use 7DHP as a substrate, similar to its inability to act on pregnenolone [65]. In accordance with this, our studies with pig adrenal glands showed the transformation of 7DHP to 17(OH)7DHP during *ex-vivo* incubations, but 21(OH)7DHP could not be detected [23]. *Ex-utero* incubations of human placentae [8] and *ex-vivo* incubations of pig adrenal glands [23] with 7DHP resulted in its transformation to 7-dehydroprogesterone. This finding was further supported by incubations with partially purified 3 β HSD [23] and placental microsomes where 7-dehydroprogesterone was detected and sufficient of this product made to confirm its structure by NMR [8]. Additional products of the placental metabolism of 7DHP included 20-hydroxypregnenolone [8]. Incubation of 7DHP with skin microsomes generated two new products which likely had a 4,6-diene structure [23]. Details of the metabolism of 7DHP in skin cells represents an exciting area for future research since this organ shows steroidogenic activity and expresses the major classical steroidogenic enzymes [15,66] that can be regulated locally by endogenous hormonal signals [10,67]. Furthermore, Δ^7 -steroids produced in the skin may undergo photochemical transformation after exposure to the UVB, to the corresponding

secosteroids as originally proposed [14], with further *in vitro* experiments and detailed discussion presented in [56,57,68,69].

3. Diversity in sterol substrates acted on by CYP11A1

3.1. Cholesterol esters

Cholesterol is stored esterified to fatty acids with some cholesterol also undergoing sulfation. Cholesterol sulphate is reported to have some regulatory activities, including promoting keratinocyte differentiation in the skin [70]. Human and bovine CYP11A1 have been shown to cleave the side chain of cholesterol sulfate, with lower catalytic efficiency than cholesterol for the purified enzymes [71], but at higher rates than cholesterol by CYP11A1 in ovarian mitochondria, due to its more rapid movement to the inner mitochondrial membrane [72]. Thus, pregnenolone sulfate which is an active neurosteroid in the brain [73], may be produced by side chain cleavage of cholesterol sulfate by CYP11A1 rather than by sulfation of pregnenolone. Purified CYP11A1 can also act on short chain cholesterol esters converting them to their corresponding pregnenolone esters, but activity is very low when the chain length exceeds 4 carbons [71].

3.2. Lumisterol 3

Recently, it has been reported that purified bovine and human CYP11A1 can metabolize lumisterol 3 with a catalytic efficiency approximately 20% of that for cholesterol [31]. Lumisterol 3 is produced in the skin by photochemical transformation of pre-vitamin D3 during prolonged periods of UV exposure (see Section 4.1). The reaction involves reformation of the intact B ring with C9 and C10 in a 9 β ,10 α -configuration, making it a stereoisomer of 7DHC [74,75]. CYP11A1 initially hydroxylates lumisterol 3 at C22 or C24. 22-Hydroxylumisterol is then hydroxylated at C20 producing 20,22-dihydroxylumisterol 3 and some of this undergoes hydroxylation at other sites while some undergoes cleavage to pregnalumisterol [31]. Fragments of pig adrenal glands incubated with lumisterol also produce these products suggesting that this CYP11A1-catalysed reaction can occur *in vivo*. Pregnalumisterol and some synthetic hydroxylumisterol 3 derivatives exhibit biological activity [76,77] further suggesting that metabolism of lumisterol by CYP11A1 in the skin may produce metabolites with physiological activity.

3.3. Hydroxysterols and plant sterols

Purified bovine and human CYP11A1 and steroidogenic mitochondria can act on a number of other naturally occurring sterols besides cholesterol and its precursor, 7DHC. These include cleaving the side chains of 24-hydroxycholesterol, 25-hydroxycholesterol, and 27-hydroxycholesterol (discussed above) [16,17,19,48]. These sterols are produced from the metabolism of cholesterol by CYP46A1, cholesterol 25-hydroxylase, and CYP27A1, respectively, as intermediates in bile acid synthesis, but some are released into the bloodstream [78]. Desmosterol (5,24-cholestadien-3 β -ol, see Fig. 1), which has recently come to prominence, is also converted to pregnenolone by bovine, rat, and human CYP11A1 [16,17,19], with similar catalytic efficiency to that seen for cholesterol [48]. Regulated accumulation of desmosterol appears to play a homeostatic and anti-inflammatory

role in respect to macrophage activation in atherosclerotic lesions [79] and also plays a role in epidermal barrier function in the skin [80–82], an organ expressing CYP11A1 [14,83].

Human and bovine CYP11A1 can also cleave the side chain of the plant sterols, campesterol (24 α -methylcholesterol), and β -sitosterol (24 β -ethylcholesterol) producing pregnenolone [19], with catalytic efficiencies comparable to cholesterol for campesterol and 5–10 fold less for β -sitosterol [48]. These sterols are available through the diet of mammals although the quantitative importance of their metabolism *in vivo* remains to be established.

3.4. Ergosterol

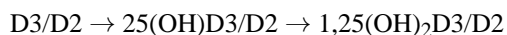
Purified human CYP11A1 acts on the fungal membrane sterol, ergosterol, also available through the diet. No cleavage of the side chain occurs due to the presence of the double bond at C22. Rather, the C22=C23 double bond undergoes epoxidation to produce 20-hydroxy-22,23-epoxy-22,23-dihydroergosterol. The other major product is 22-keto-23-hydroxy-22,23-dihydroergosterol. The catalytic efficiency for the metabolism of ergosterol by human CYP11A1 is the same as that for cholesterol [10]. Bovine CYP11A1 also acts on ergosterol but the product profile varies compared to that seen for human CYP11A1, with major products including 24-hydroxyergosterol and 17 α ,24-dihydroxyergosterol [30]. 17 α ,24-Dihydroxyergosterol is biologically active on cultured human skin cells, inhibiting DNA synthesis, but the other products remain to be tested, as does any *in vivo* function.

4. Vitamins D3 and D2 as the substrates for CYP11A1

4.1. Photobiology of vitamin D

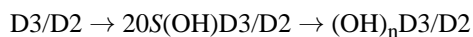
The vast majority of vitamin D3 (D3) in humans is produced in the skin after absorption of UVB energy by the B ring of 7DHC leading to its opening to produce the pre-vitamin D3 intermediate which then undergoes a temperature-dependent isomerization to vitamin D3. Further, UV irradiation of pre-vitamin D3 results in the formation lumisterol 3, tachysterol 3, and isotachysterol 3 [84–86] (Fig. 2). Similarly, vitamin D2 (D2), lumisterol 2, tachysterol 2, and isotachysterol 2 are produced by the action of UVB on ergosterol derived from fungi and phytoplankton [85,87] (Fig. 2). The relative photochemical production of the above steroids and secosteroids depends on UVB energy, temperature, and physicochemical environment.

Until recently, it was widely believed that D3 and D2 were solely activated as follows:



through the sequential hydroxylations by CYP27A1 or CYP2R1 on C25, and by CYP27B1 on C1 [85,88–97]. At the systemic level, 25 and 1 α -hydroxylations take place in liver and kidney, respectively. However, both reactions occur in the epidermis to produce fully active 1,25-dihydroxyvitamin D (1,25 (OH)₂D) [87,98,99]. 1,25(OH)₂D is inactivated by CYP24A1 by hydroxylation at C24 with further oxidation to calcitriolic acid [100–102].

Recently, we discovered that the CYP11A1 hydroxylates the side chain of D3 and D2 in a sequential fashion:



The products can be further hydroxylated by CYP27B1, CYP27A1, and CYP24A1 (reviewed in [103], and see below), which is an alternative to the classical pathway (see above), although its physiological importance remains to be established.

4.2. Hydroxylation of vitamin D3 by CYP11A1

4.2.1. *In vitro* models—Initial analyses using purified bovine CYP11A1 demonstrated that it could hydroxylate the side chain of D3 producing 20(OH) D3 and 20,22(OH)₂D3 without cleavage of the side chain occurring [22,24]. Adrenal mitochondria were also shown to produce 20(OH) D3 as the major product and several additional hydroxyderivatives of which 3 of the major ones apparently resulted from the action of CYP11A1 [24]. Subsequent enzymatic analysis revealed that CYP11A1 favors initial hydroxylation of D3 at C20 over C22 (or C23), with 20(OH)D3 representing the first and major metabolite. The absolute configuration of this compound was defined as 20S(OH)D3 by chemical synthesis and NMR analysis [10,22,24,25,103,104]. Support for the initial hydroxylation occurring at C20 was provided by docking of vitamin D3 into the catalytic site of the crystal structure of human CYP11A1 which predicted that C20 is located closer to the heme iron than C22 [7]. However, the conversion of vitamin D3 to 20(OH)D3 by CYP11A1 is less efficient (lower K_{cat}/K_m) than hydroxylation of cholesterol by this enzyme [25], supported by molecular modeling [7], with bovine CYP11A1 metabolizing vitamin D3 more efficiently than the human enzyme [7,25,27]. The structural differences that mediate these differences in catalytic efficiency remain to be established. Nevertheless, CYP11A1 can hydroxylate D3 at C22 to produce 22S(OH)D3 as a minor product, with the structure of this metabolite being confirmed by NMR [26]. The preferred second site of hydroxylation of 20(OH)D3 is at C23 producing 20,23(OH)₂D3 as the second major product of the reaction [25]. 20(OH)D3 is also hydroxylated at C22 and C17 to produce 20,22(OH)₂D3, 17,20(OH)₂D3, and 17,20,23(OH)₃D3 as metabolites [26]. These studies defined the conversions:



as the major pathway of vitamin D3 metabolism by CYP11A1 with



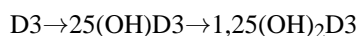
as a minor pathway [103]. There is some flexibility in the order of the observed hydroxylations at C17, C20, C22, and C23 catalyzed by CYP11A1 creating other minor pathways [24,25,103,105].

Importantly, CYP11A1 does not metabolize 25-hydroxyvitamin D3 indicating that hydroxylation at C25 protects the secosteroid against hydroxylation of the side chain by CYP11A1 [24] and therefore, that this enzyme does not compete with CYP27B1 for activation of 25(OH)D3 [103]. Interestingly, CYP11A1 can hydroxylate the biologically inert prodrug, 1(OH)D3, to the biologically active 1,20(OH)₂D3, which could serve as an alternative route to 25-hydroxylation for activation of 1(OH)D3 [106]. The same reaction is mediated by placental mitochondria [27].

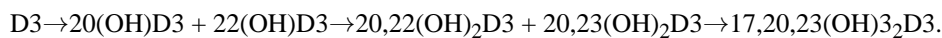
Interestingly, 20(OH)D3 can be metabolized by recombinant human CYP27A1 and rat CYP24A1 [107,108], with production of 20,25(OH)₂D3 and 20,26(OH)₂D3 by CYP27A1 and production of 20,24(OH)₂D3 (major product) and 20,25(OH)₂D3 (minor product) by

CYP24A1. Structures of these compounds were determined by NMR and MS. Other minor dihydroxy-derivatives of which structures remain to be determined were also produced. 20,24(OH)₂D₃, 20,25(OH)₂D₃, and 20,26(OH)₂D₃ can also be 1 α -hydroxylated by CYP27B1 with a catalytic efficiency much higher than for the parent 20(OH)D₃ [109]. In addition, CYP27B1 can 1 α -hydroxylate 20,23(OH)₂D₃ to produce 1,20,23(OH)₂D₃ and 20(OH)D₃ to produce 1,20(OH)₂D₃ [109,110].

4.2.2. *In vivo* models—Most importantly, the *in vivo* studies represented by *ex-vivo* and *ex-utero* incubations of vitamin D₃ with fragments of adrenal glands and human placentae, respectively, clearly demonstrate the CYP11A1-catalyzed metabolism of vitamin D₃ with 20(OH) D₃ being the major metabolite. Less abundant products included 22(OH)D₃, 20,23(OH)₂D₃, 20,22(OH)₂D₃, and 17,20,23(OH)₃D₃ [27]. Both tissues were also shown to express CYP27A1, CYP2R1, and CYP27B1, and to convert vitamin D₃ to 25(OH)D₃, and 25(OH) D₃ to 1,25(OH)₂D₃ [27]. This allowed us to conclude that the adrenal gland and placenta (tissues demonstrating high activity of CYP11A1) metabolize D₃ by both the classical pathway:



and the CYP11A1-dependent pathway:



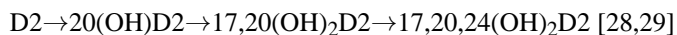
Interestingly, the adrenal gland and placenta supplied with exogenous vitamin D₃ showed higher production of 20(OH) D₃ than 25(OH)D₃. Detection of a monohydroxyvitamin D₃ compound with a retention time corresponding to 20(OH) D₃ in human serum, albeit at a level 20 times lower than that of 25(OH)D₃, indicates that 20(OH)D₃ produced in these organs can enter the systemic circulation [27]. It must be noted that CYP27B1-dependent 1 α -hydroxylation of 20(OH)D₃ and 20,23(OH)₂D₃ was observed in the placenta and adrenals producing 1,20(OH)₂D₃ and 1,20,23(OH)₃D₃, respectively [27]. However, we did not detect 1,17,20(OH)₃D₃ or 1,17,20,23(OH)₄D₃ [27], consistent with enzymatic analyses showing that products with a 17 α -hydroxyl group such as 17,20(OH)₂D₃ and 17,20,23(OH)₃D₃ are not substrates for CYP27B1 [109].

We also detected CYP11A1-dependent metabolism of D₃ in cells with a relatively low expression of this enzyme such as human and pig epidermal keratinocytes [27], and colonic Caco-2 cells [103]. Thus, keratinocytes transformed D₃ to 20(OH)D₃, 22(OH) D₃, 20,23(OH)₂D₃, 20,22(OH)₂D₃, and 17,20,23(OH)₃D₃, with CYP27B1-dependent production of 1,20(OH)₂D₃ and 1,20,23(OH)₃D₃ also being observed [27]. Interestingly, skin cells showed slightly higher production of 22(OH)D₃ and similar or higher production of 1,25(OH)₂D₃ in comparison to 20,22(OH)₂D₃ and 20,23(OH)₂D₃. Also, endogenous production of 20(OH)D₃, 25(OH) D₃, 22(OH)D₃, 20,23(OH)₂D₃, 20,22(OH)₂D₃, 1,25(OH)₂D₃, and 17,20,23(OH)₃D₃ was detected in HaCaT keratinocytes cultured in the presence of 5% serum without additional supplements of D₃ [27]. Caco-2 cells also metabolized exogenous vitamin D₃ to 20(OH)D₃ as the predominant metabolite, with lesser production of 22(OH)D₃ and 25(OH)D₃. These were further metabolized to 20,23(OH)₂D₃, 1,20(OH)₂D₃, and 1,25(OH)₂D₃ [103]. These data indicate that organs expressing lower levels of CYP11A1 such as skin [15,66] and gastrointestinal (GI) tract

[111] can metabolize D3 through the novel CYP11A1-initiated pathway, as an additional and alternative pathway to the classical one.

4.3. Metabolism of vitamin D2 by CYP11A1

4.3.1. In vitro models of D2 metabolism—Purified bovine CYP11A1 and adrenal mitochondria hydroxylate the side chain of vitamin D2 without its cleavage, which starts with hydroxylation at C20:



A similar sequence of reactions was seen for recombinant human CYP11A1 [9]. The major product was 20(OH)D2 with lower production of 17,20(OH)₂D2 and 17,20,24(OH)₃D2 and another unidentified D2 metabolite [28,29]. The structure of the above products was defined by NMR. Consistent with the above data were molecular modeling studies using the crystal structure of human CYP11A1 bound to cholesterol [7], which predicted that D2, 20(OH)D2, and 17,20(OH)₂D2 were able to bind to the enzyme with similar docking scores (binding affinity) to cholesterol [9]. In addition, the distances from C22 and C20 to the heme iron for D2 and 20(OH)D2 were similar, and this plus the presence of the C22=C23 double bond predicted that the first hydroxylation would occur at C20 [9].

Although the side chains of D2 and ergosterol are identical, no epoxidation of the double bond between C22 and C23 occurs for D2, indicating its different positioning in the active site. CYP27B1 also 1 α -hydroxylates 20(OH)D2 producing 1,20(OH)₂D2, with an efficiency much lower than for the conversion of 25(OH)D2 to 1,25(OH)₂D2 [112]. In addition, the prodrug, 1 α (OH)D2, could be hydroxylated by recombinant human CYP11A1 to 1,20(OH)₂D2 [9].

4.3.2. In vivo models of D2 metabolism—Importantly, using human placentas and bovine adrenals which express high levels of CYP11A1 [1,2], and human epithelial cells (epidermal keratinocytes and colonic Caco-2 cells) which express relatively low levels of this enzyme [14,15,111,113] we were able to demonstrate the *in vivo* (*ex-vivo*, *ex-utero*, and in cultured cells) transformation of D2 to 20(OH)D2, 17,20(OH)₂D2, and 1,20(OH)₂D2 [9]. The involvement of CYP11A1 in the conversion of D2 to 20(OH)D2, 20(OH)D2 to 17,20(OH)₂D2, and the prodrug 1 α (OH)D2 to 1,20(OH)₂D2 was demonstrated by its almost complete inhibition by 22R-hydroxycholesterol when placental and adrenal mitochondria were used to run the reaction [9]. Placentae *ex-utero*, as well as metabolizing vitamin D2 *via* CYP11A1, also converted D2 to 25(OH)D2 [9]. Placentae *ex-utero* and mitochondria isolated from adrenal glands not only 1 α -hydroxylated 25(OH)D2 to 1,25(OH)₂D2, but also 20(OH)D2 to 1,20(OH)₂D2 [9].

Notably, the amount of 20(OH)D2 produced from D2 was higher than that of the initial product of the classical activation pathway, 25(OH)D2, in placenta and Caco-2 cells, but similar or lower than for 25(OH)D3 in human epidermal keratinocytes. The present data clearly show that after reaching these cells or tissues, D2 can be transformed in a sequential manner to 20(OH)D2 and 17,20(OH)₂D2 through the action of CYP11A1. Similarly the prodrug, 1 α (OH)D2, is transformed to 1,20(OH)₂D2. The latter is consistent with the ability of purified recombinant human CYP27B1 to 1 α -hydroxylate 20(OH)D2 [112].

Epithelial cells expressing low CYP11A1 activity such as epidermal keratinocytes [14,83] and Caco-2 cells [111,113] metabolized D2 through the classical (25-hydroxylase-dependent) and CYP11A1-dependent pathways in a cell type dependent manner [9]. There was significantly higher production of 20(OH)D2 than 25(OH)D2 in Caco-2 cells that was opposite to what was observed in epidermal keratinocytes [9]. Also, production of 20(OH)D2 and 17,20(OH)₂D2 was higher in Caco-2 cells than in HaCaT keratinocytes [9], but higher production of 1,20(OH)₂D2 was seen in HaCaT keratinocytes [9]. In summary, *ex-vivo*, *ex-utero*, and cell culture studies indicate that D2 can be metabolized *in vivo* in tissues expressing CYP11A1, with further modification of products by CYP27B1, with metabolite profiles that are tissues- and cell-type specific [9]. The physiological role of these new pathways remain to be established, but as for the corresponding D3 metabolites, products display biological activity when applied under *in vitro* or *in vivo* conditions (discussed in Section 5).

4.4. Other potential substrates for CYP11A1

Analysis of the cholesterol biosynthetic pathway suggests that many other of its later intermediates besides 7DHC and desmosterol that have a steroidal ring, could serve as substrates for CYP11A1 (Fig. 1). Also, other phototransformation products of 7-DHC (Fig. 2) such as tachysterol could serve as the substrates for CYP11A1. Therefore, we carried out molecular modeling studies using the crystal structures of bovine and human CYP11A1 [6,7]. We observed excellent docking scores for several compounds listed in Table 1 that were comparable to cholesterol or hydroxycholesterol substrates. The docking scores for ergosterol, 7DHC, desmosterol, lumisterol, and vitamins D3 and D2 are consistent with experimental data presented earlier showing that they are substrates for CYP11A1. An excellent docking score for 8-dehydrocholesterol in conjunction with the presence of ⁸-steroids in body fluids of SLOS patients [60,61,63,64], predicts that it is also a substrate for CYP11A1 that undergoes cleavage of its side chain similar to 7-DHC and cholesterol. Interestingly, 7-dehydrodesmosterol and lathosterol intermediates of the cholesterol biosynthetic pathway, as well as tachysterol which is a product of UVB transformation of pre-D, have excellent docking scores suggesting that they could also serve as substrates for CYP11A1 in tissues expressing this enzyme and producing sufficient concentrations of these compounds. The challenge ahead is to test experimentally whether compounds listed in Table 1 or additional intermediates of the cholesterol biosynthetic pathway or other photoproducts of UV induced transformation of 7DHC (Fig. 2) are indeed metabolized by CYP11A1.

5. Concluding remarks

The data reviewed above clearly show that purified CYP11A1, or CYP11A1 in isolated mitochondria or tissue fragments supplied with exogenous substrate, can act on a range of steroids other than cholesterol. Alternative substrates include cholesterol precursors (7DHC and desmosterol), hydroxycholesterols, plant sterols, ergosterol, lumisterol, and vitamins D3 and D2. Thus, the active site of CYP11A1 can accommodate a range of steroids but it should be emphasized that they are all closely related to cholesterol in structure, with the biggest structural variation being for the secosteroids which contain a broken B-ring. The

structure of the substrate affects both the regio-selectivity of hydroxylation and the catalytic efficiency. Cleavage of the side chain usually occurs when only the side chain is modified from that found in cholesterol and the ring system is not, as seen for desmosterol, campesterol (24 α -methylcholesterol), β -sitosterol and various side chain hydroxylated cholesterol derivatives. However, 7DHC with an unmodified side chain and a Δ^7 double bond in the B-ring is a clear exception to this as it undergoes cleavage with high catalytic efficiency. Despite the presence of a Δ^7 double bond in ergosterol it does not undergo cleavage due to the C22=C23 double bond in the side chain making it unfavorable to undergo hydroxylation at C22. Photochemical opening of the B-ring of 7DHC, as seen in D3 (Fig. 3), prevents side chain cleavage from occurring, even when the pattern of hydroxylation would seem to make it possible as no cleavage of 20,22(OH)₂D3 is observed.

Importantly, *in vitro* studies with exogenously added compounds reveal that novel CYP11A1-derived secosteroids and Δ^7 steroids exerts anti-proliferative, pro-differentiation, and anti-inflammatory effects on cultured skin cells, which are dependent on the structure of the compound and the cell lineage [57,104,112,114–119]. Pharmacologically administered 20(OH) D3 shows antifibrotic activities both *in vitro* [57,117,118] and *in vivo* in bleomycin induced scleroderma [118]. It suppresses *in vivo* collagen-induced arthritis (CIA) in a mouse model [103]. It also shows anti-cancer and anti-tumor activities that are dependent on the cell-type lineage [57,77,112,119–121]. Importantly, the novel secosteroids in addition to acting as biased agonists on the vitamin D receptor (showing selectivity for certain receptor mediated effects) [103,112,122], can also interact with ROR α and γ , presumably acting as inverse agonists [123].

A future challenge in this field is a quantitative assessment of the degree of *in vivo* metabolism of endogenous sources of these alternative substrates for CYP11A1, and to determine whether their metabolites are produced at sufficient levels to exert physiological effects. Studies of SLOS patients clearly show that under pathological conditions 7DHC can serve as a substrate for CYP11A1, with downstream Δ^7 steroidal products possibly being responsible for some of the disease symptoms [58–61,63,64]. Tissue specific knockouts of CYP11A1, particularly in skin, are required to define whether metabolism of 7DHC and vitamin D are of physiological importance.

In summary, CYP11A1 shows considerable flexibility with respect to substrate specificity acting on a range of naturally occurring steroid molecules other than cholesterol. It can generate several intermediates and products that are biologically active in a context dependent manner when administered exogenously, but for which physiological roles remain to be defined.

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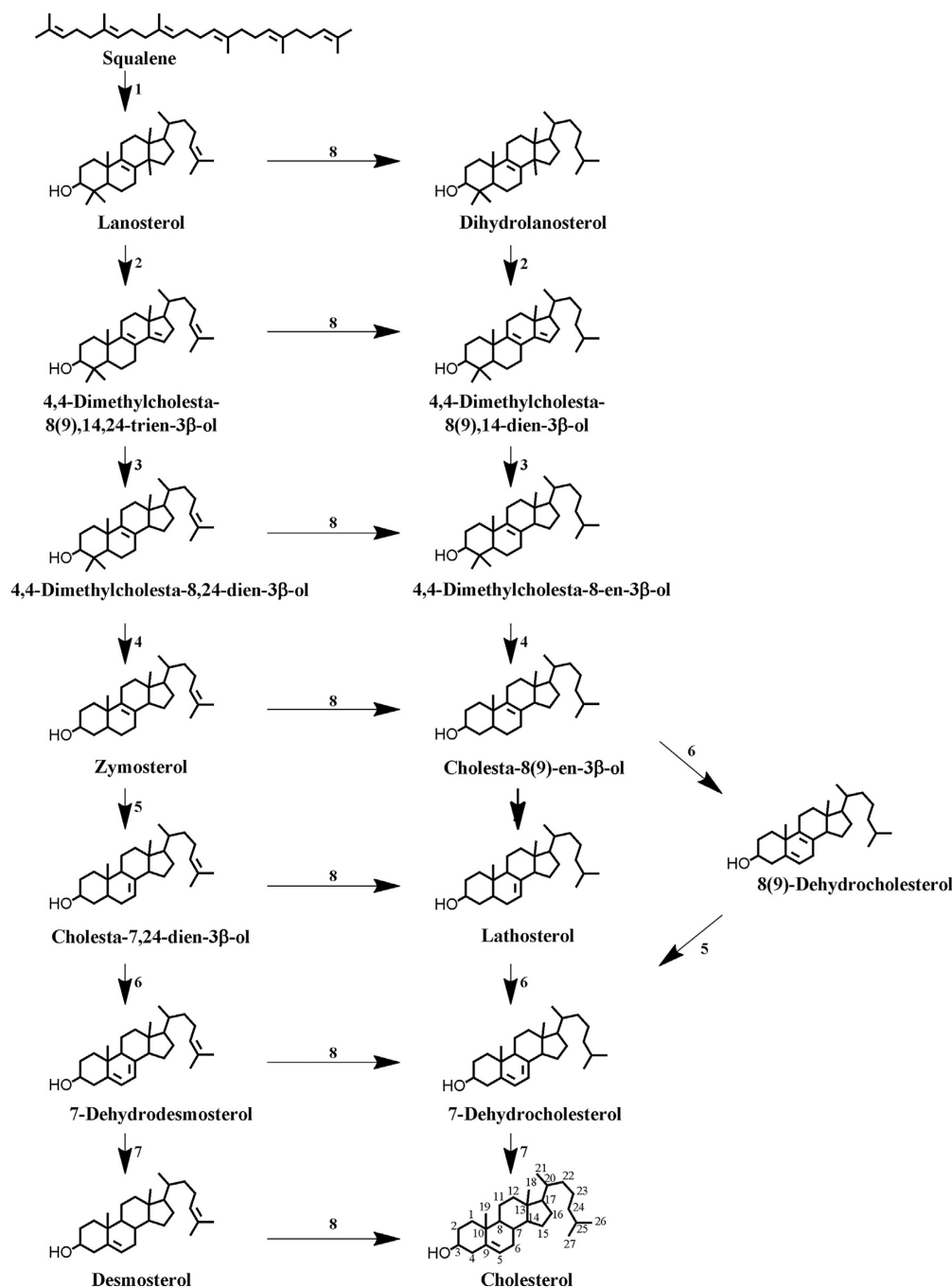


Fig. 1. Cholesterol synthesis: the later steps in the pathway

Enzymes: 1, squalene monooxygenase, and squalene cyclase; 2, lanosterol 14- α -demethylase; 3, 3 β -hydroxysterol ¹⁴-reductase; 4, C4 demethylation complex (C4-sterol methyloxidase, C4-sterol decarboxylase [NSDHL], and 3-ketoreductase); 5, 3 β -hydroxysterol ⁸, ⁷-isomerase; 6, lathosterol 5-desaturase; 7, 3 β -hydroxysterol ⁷-reductase; 8, 3 β -hydroxysterol ²⁴-reductase. See [50,51] for additional detail.

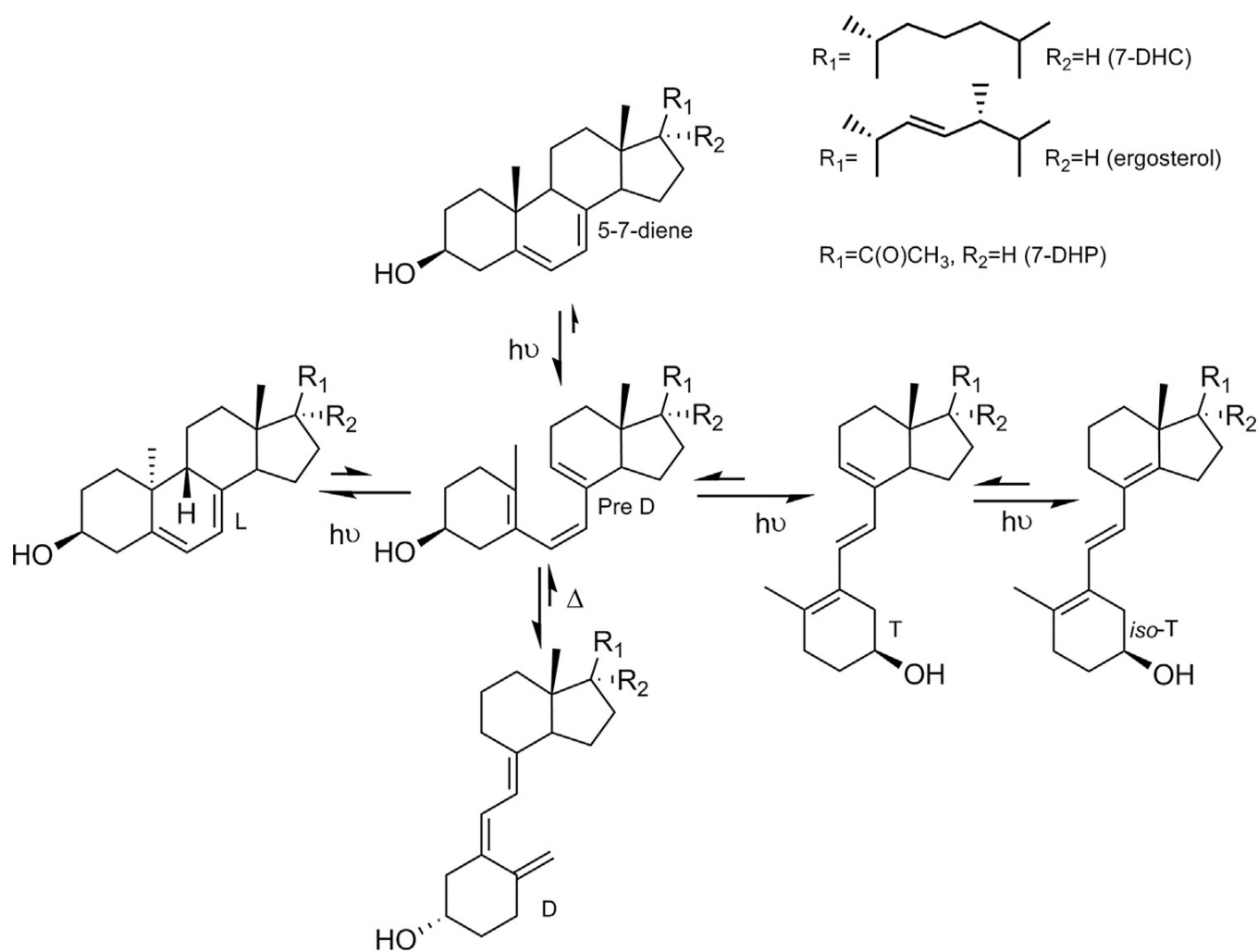
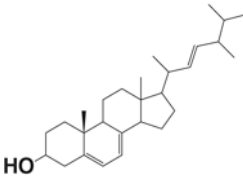
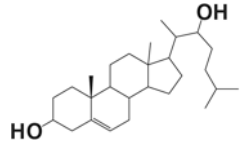
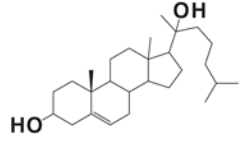
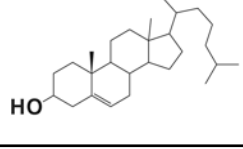
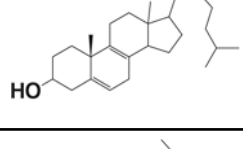
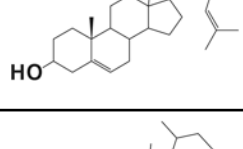
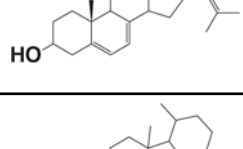
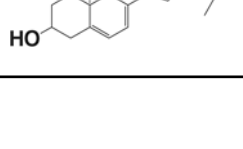


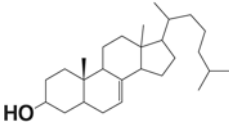
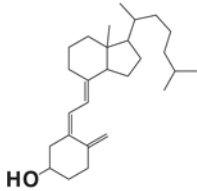
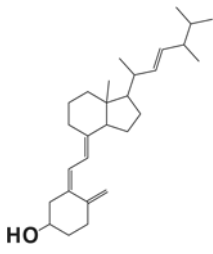
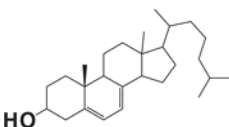
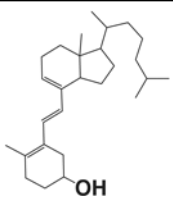
Fig. 2. UVB-induced photolysis of 7-DHC, ergosterol and 7-DHP

D, T, and L represent vitamin D, tachysterol or lumisterol compounds. Note that UVB transformation of 7DHC is similar to that described for 7DHP [56,124].

Table 1

Docking scores of compounds binding to the catalyst site of human or bovine CYP11A1 crystal structures.

Compound name	Chemical structure	Docking score	
		Human CYP11A1 (PDB entry: 3N9Y)	Bovine CYP11A1 (PDB entry: 3MZS)
Ergosterol		-11.97	-11.61
22-Hydroxy cholesterol		-11.94	-10.95
20-Hydroxy cholesterol		-11.68	-10.00
Cholesterol		-11.67	-11.12
8(9)-Dehydrocholesterol		-11.59	-11.02
Desmosterol		-11.54	-10.95
7-Dehydrodesmosterol		-11.41	-11.13
Lumisterol 3		-11.20	-10.85

Compound name	Chemical structure	Docking score	
		Human CYP11A1 (PDB entry: 3N9Y)	Bovine CYP11A1 (PDB entry: 3MZS)
Lathosterol		-11.18	-11.04
Vitamin D3		-11.06	-10.59
Vitamin D2		-10.94	-11.00
7-Dehydrocholesterol		-10.67	-10.18
Tachysterol 3		-10.39	-10.67

Docking methodology description.

The molecular modeling studies were performed with Schrodinger Molecular Modeling Suite 2014 (Schrodinger LLC, New York, NY) as previously described [8,9]. All the compounds were built and prepared by the Ligprep module to generate at most 20 conformations of each ligand. The crystal structures of human CYP11A1 (PDB entry: 3N9Y) or bovine CYP11A1 (PDB entry: 3MZS) were imported into the Protein Preparation Wizard to correct problems including the missed hydrogen atoms and incomplete side chain and loops in the original PDB files. Next, the grids of catalytic sites (where the native ligand binds with enzyme) in the prepared CYP11A1 structures were generated. Then the compounds were docked into the CYP11A1 catalytic site by the Glide module. The best docking complexes were subject to restricted molecular dynamics to release any strains under OPLS-2005 force field.