HHS Public Access

Author manuscript

Pharmacol Ther. Author manuscript; available in PMC 2020 December 01.

Published in final edited form as:

Pharmacol Ther. 2019 December ; 204: 107400. doi:10.1016/j.pharmthera.2019.107400.

Biochemical and Physiological Importance of the CYP26 Retinoic Acid Hydroxylases

Nina Isoherranen, **Guo Zhong**

Department of Pharmaceutics, School of Pharmacy, University of Washington, Seattle WA

Abstract

The Cytochrome P450 (CYP) family 26 enzymes contribute to retinoic acid (RA) metabolism and homeostasis in humans, mammals and other chordates. The three CYP26 family enzymes, CYP26A1, CYP26B1 and CYP26C1 have all been shown to metabolize all-*trans*-retinoic acid (*at*RA) as well as 9-*cis*RA and 13-*cis*RA isomers and primary metabolites 4-OH-RA and 4-oxo-RA with high efficiency. While no crystal structures of CYP26 enzymes are available, the binding of various ligands has been extensively explored via homology modeling. All three CYP26 enzymes are inducible by treatment with *at*RA in various prenatal and postnatal tissues and cell types. However, current literature shows that in addition to regulation by *at*RA, CYP26 enzyme expression is also regulated by other endogenous processes and inflammatory cytokines. In humans and in animal models the expression patterns of CYP26 enzymes have been shown to be tissue and cell type specific, and the expression of the CYP26 enzymes is believed to regulate the formation of critical *at*RA concentration gradients in various tissue types. Yet, very little data exists on direct disease associations of altered CYP26 expression or activity. Nevertheless, data is emerging describing a variety of human genetic variations in the CYP26 enzymes that are associated with different pathologies. Interestingly, some of these genetic variants result in increased activity of the CYP26 enzymes potentially leading to complex gene-environment interactions due to variability in dietary intake of retinoids. This review highlights the current knowledge of structure-function of CYP26 enzymes and focuses on their role in human retinoid metabolism in different tissues.

Keywords

Retinoic acid; Cytochrome P450; vitamin A; Structure-function

Corresponding author: Nina Isoherranen, Department of Pharmaceutics, School of Pharmacy, University of Washington, Health Science Building, Room H-272M, Box 357610, Seattle, Washington 98195-7610 USA, Phone: 206-543-2517; Fax: 206-543-3204; ni2@uw.edu.

Conflict of Interest Statement.

Nina Isoherranen holds a US Patent number: 9963439 on CYP26 Inhibitors. She has received honorariums from Pfizer and Genentech and is a consultant for Boehringer Ingleheim. Guo Zhong has no conflicts of interest to declare.

1. Introduction: Vitamin A and retinoic acid homeostasis and signaling

Vitamin A (retinol) is an essential micronutrient that is obtained from the diet either in the form of retinyl esters or β-carotene (Blomhoff and Blomhoff, 2006). While retinyl esters are predominantly present in animal-based foods such as meats and milk products, β-carotene is mainly obtained from orange vegetables such as carrots. Mammals have generally evolved to survive prolonged periods with diet deficient in vitamin A (Napoli, 2012). This survival is ensured by the storage of vitamin A in the liver in the form of retinyl esters, mostly retinyl palmitate. The majority, about 70%, of total body retinyl ester stores are in the liver with the remaining stores in the eyes, lungs, skin, testes, spleen and adipose (O'Byrne and Blaner, 2013). In particular, due to the size of the organ, the adipose tissue has been estimated to account for as much as 15-20% of total body retinyl ester stores (O'Byrne and Blaner, 2013). In the liver, retinyl esters are stored in the stellate cells and the storage is regulated by the action of the esterification enzyme lecithin retinol acyltransferase (LRAT) which functions in an Acyl-CoA independent manner (Figure 1) (Ong et al., 1987). LRAT is an enzyme localized in the endoplasmic reticulum in the stellate cells in the liver and it functions to esterify retinol obtained from hepatocytes with palmitic acid (O'Byrne and Blaner, 2013). LRAT expression and role in vitamin A storage has also been shown in lung and retina (Amengual et al., 2012). LRAT activity in the small intestine and the interactions of LRAT with cellular retinol binding protein II (CRBPII) appear to play a particularly important role in the absorption of vitamin A and the esterification of retinol for chylomicron-mediated absorption (O'Byrne and Blaner, 2013). Yet, LRAT is not expressed in all tissues that have retinol esterification activity. In the testis, significant activity similar to LRAT activity has been demonstrated in microsomes prepared from cultured Sertoli cells (Schmitt and Ong, 1993). However, LRAT mRNA was localized to early spermatids in the testis and not to Sertoli cells (Vernet et al., 2006) suggesting Sertoli cell acetyltransferase activity maybe a different enzyme than LRAT. Similarly, in the adipose tissue, it appears that other enzymes exist that contribute to retinol esterification (Wongsiriroj et al., 2014). acyl-CoA:retinol acyltransferase (ARAT) activity is found in the absence of LRAT activity and other enzymes such as DGAT1 have been suggested to be responsible for the ARAT activity in tissues such as the adipose and intestine (Orland et al., 2005). However, the exact identity of the full complement of retinol esterification enzymes in the different tissues in the body remains to be determined.

The release of vitamin A from liver stores is mediated by retinyl ester hydrolases (REH) that cleave retinyl esters to retinol (O'Byrne and Blaner, 2013). Enzymes that have been shown to possess retinyl ester hydrolase activity include PNPLA3 and carboxylesterases (Pirazzi et al., 2014), but the enzymes that quantitatively are most important for the hydrolysis of retinyl esters have not been defined. For example, lipoprotein lipase cleaves retinyl esters and facilitates retinol uptake into adipose tissue (Blaner et al., 1994). After retinyl ester hydrolysis in the liver, retinol is transferred to hepatocytes where it can bind to the retinol binding protein (RBP4) in the endoplasmic reticulum. The retinol-RBP4 complex is then secreted into circulation being the main circulating form of vitamin A and the form that acts as a transport system to supply various tissues with vitamin A as needed. Circulating concentrations of retinol are around 1μM and these concentrations are found entirely as

retinol bound to RBP4. While retinol can cross membranes via passive diffusion, it is also believed that RBP4-retinol directly interacts with the membrane transporter STRA6 which then takes retinol up into target cells (Kawaguchi et al., 2007; Chen et al., 2016).

In general, retinol is believed to be devoid of biological activity although some direct biological actions of retinol in mitochondrial electron transfer have recently been proposed (Hammerling, 2016a; b). For classic retinoid activity and signaling retinol must be biotransformed to the active form all-*trans*-retinoic acid (*at*RA) (Figure 1). The oxidation of retinol to *at*RA is driven by a combination of oxidative and reductive enzymes that regulate retinoic acid signaling (Napoli, 1996, 2012). Retinol can be oxidized to retinaldehyde by the retinol dehydrogenases (RHD) and Dehydrogenase/reductase (Dhrs) enzymes but this reaction is reversible and retinaldehyde is also reduced back to retinol by enzymes in this same family (Kedishvili, 2013). This reversible reaction is critically important as β-carotene absorbed from the diet is cleaved in the intestine to retinaldehyde and subsequently contributes to the vitamin A storage through the reduction of retinaldehyde to retinol. As the retinol to retinaldehyde biotransformation is reversible, the first step in *at*RA synthesis is essentially equilibrative and hence challenging to characterize kinetically. However, as shown with the LRAT enzyme, cellular retinoid binding proteins contribute also to the regulation of the retinaldehyde-retinol equilibrium via interactions with the RDH enzymes (Napoli, 2017).

The oxidation of retinaldehyde is an irreversible step carried out predominantly by aldehyde dehydrogenase enzymes including ALDH1A1, ALDH1A2, ALDH1A3 and ALDH7A1 (Napoli, 1996, 2012). In addition, several studies have pointed to a role of the aldehyde oxidase (AOX) enzymes in *at*RA synthesis from retinaldehyde, especially in the liver and harderian gland and possibly the testis in mice (Terao et al., 2009; Arnold et al., 2015; Beedle et al., 2018a). However, whether AOX can accept retinaldehyde bound to the CRBPs as a substrate and contributes to regulation of endogenous *at*RA homeostasis remains to be determined. The critical role of ALDH1A enzymes in *at*RA synthesis has been clearly demonstrated in studies using the potent ALDH1A inhibitor WIN18,446. Treatment of mice with WIN18,446 results in significant depletion of *at*RA concentrations in target tissues such as the testis and subsequently halt spermatogenesis (Paik et al., 2014; Arnold et al., 2015). Based on mouse knock-out studies, of the four enzymes, ALDH1A2 and ALDH1A3 are required during embryonic development for the retinoid signaling while ALDH1A1 knock out mice are viable and relatively healthy (Kumar et al., 2012). Recent advances in conditional and organ specific knock-out mouse models have allowed studies of the role of ALDH1A2 and ALDH1A3 in adult and post-natal animals. These studies have highlighted the differences in the role of ALDH1A enzymes during embryonic development and adult animals. For example, ALDH1A2 conditional knock out mice appear to be healthy (Beedle et al., 2018b), suggesting that other critical *at*RA synthesizing enzymes may exist in adult animals to modulate retinoid homeostasis. The ALDH1A enzymes are expressed throughout the body with some tissue and cell type specificity although quantitative ALDH1A protein expression levels throughout the tissues in the body, and the interindividual variability in expression levels is not well known. mRNA and immunohistochemistry analysis are consistent in showing that ALDH1A1 is promiscuously expressed in the majority of human tissues (Sládek, 2003). Based on mRNA analysis, ALDH1A2 is predominantly expressed in

reproductive tissues of the male and female and to a lesser extent in muscle and liver (Sládek, 2003). The role of ALDH1A3 is not well known in adults. While mRNA detection and IHC suggest promiscuous constitutive protein expression (Sládek, 2003) detected levels are generally low (Arnold et al., 2015). Recent advances in mass spectrometry-based protein quantification provide a useful tool to address these discrepancies between protein and mRNA quantification (Arnold et al., 2016), and the quantitative proteomic approach has been successfully applied to characterizing ALDH1A enzyme expression in the human testis (Arnold et al., 2015).

Once *at*RA is synthesized in target tissues it can bind to cellular retinoic acid binding proteins (CRABPs) and be channeled to either nuclear retinoic acid receptors (RARs) (Dong et al., 1999) or to the cytochrome P450 (CYP) 26 enzymes in the endoplasmic reticulum (Nelson et al., 2016; Zhong et al., 2018). The RARs heterodimerize with retinoid X receptors to regulate the transcription of at least 500 genes although RA also modulates gene expression via other indirect mechanisms (Balmer and Blomhoff, 2002). The RARs are ligand activated nuclear receptors and as such their transcriptional activity depends not only on the expression level of the RARs but also on the availability of the ligand, *at*RA. In addition to *at*RA, other retinoids such as the metabolites of *at*RA, 4-OH-RA and 4-oxo-RA, and the isomers 13-*cis*RA and 9-*cis*RA bind to RARs and therefore may contribute to overall retinoid signaling depending on their concentrations in different cell types and organisms (Idres et al., 2002; Topletz et al., 2015).

*at*RA clearance is mainly mediated by cytochrome P450 enzymes and majority of the clearance of endogenous *at*RA is believed to be done by CYP26 family enzymes (Thatcher and Isoherranen, 2009; Ross and Zolfaghari, 2011). However, other CYP enzymes such as CYP3A4, CYP3A5, CYP3A7 and CYP2C8 in humans and Cyp2c22 in rodents have been shown to also catalyze *at*RA hydroxylation although with much lower efficiency than CYP26 enzymes (Marill et al., 2000; Thatcher et al., 2010; Lee et al., 2014). As CYP3A enzymes and CYP2C8 are predominantly expressed in the human liver they are unlikely to contribute to *at*RA clearance in the majority of other *at*RA target tissues. In addition, based on enzyme kinetic analysis and the expression levels of CYP3A4, CYP3A5, CYP2C8 and CYP26A1 in the human liver, CYP26A1 is the main contributor to *at*RA clearance in the adult human liver (Thatcher et al., 2010). However, the expression of CYP26A1 is highly variable in human livers and in some livers the expression is nearly undetectable (Thatcher et al., 2010). In such livers the contribution of CYP26A1 is predicted to be minimal. In contrast, recent analysis of human fetal livers suggests that in human fetal liver CYP3A7 is the main enzyme metabolizing *at*RA (Topletz et al., 2019). This finding highlights an interesting dichotomy of the enzyme expression between adult and fetal liver and suggests that findings of tissue specific roles of CYP26 enzymes in fetal and embryonic tissues cannot be used to predict adult tissue expression patterns. In addition to CYP26 enzymes, an often-ignored elimination pathway of *at*RA is the acyl glucuronidation of *at*RA and subsequent biliary secretion. The uridine glucuronosyltransferase (UGT) 2B7 as well as UGT1A3 have been shown to catalyze the glucuronidation of *at*RA, although the catalytic activity of UGT1A3 was considerably lower than that of UGT2B7 towards *at*RA (Samokyszyn et al., 2000). Glucuronidation of *at*RA has also been observed in rat liver microsomes from uninduced and induced animals, but the UGT isoforms in the rat liver

contributing to *at*RA clearance have not been identified (Sass et al., 1994; Genchi et al., 1996). Unfortunately, no systematic studies have tested *at*RA glucuronidation by a panel of human UGT enzymes and as such the number of UGT enzymes that can glucuronidate *at*RA remains unknown. After administration of *at*RA, it and some of its oxidized metabolites are recovered as glucuronides in bile and feces demonstrating the in vivo importance of this elimination pathway (Vane et al., 1990). Since UGT enzymes are expressed in nearly all major organs and tissues in the body it is possible that they contribute significantly to *at*RA clearance. However, the quantitative importance of UGT enzymes in the clearance of endogenous *at*RA has not been evaluated and this metabolic pathway needs further study.

2. Biochemical characterization of CYP26s

2.1 CYP26 enzyme family and sequence similarity

The family of CYP26 enzymes consists of three members, CYP26A1, CYP26B1 and CYP26C1. The cDNA of CYP26A1 (P450RAI-1) was first isolated from zebrafish and the enzyme was assigned as a cytochrome P450 enzyme due to the heme binding domain in the sequence (White et al., 1994, 1996). CYP26A1 was characterized as a RA hydroxylase as it was found to catalyze hydroxylation of RA and the gene expression was inducible by exposure to exogenous RA (White et al., 1996). Later CYP26A1 was also identified in humans (Ray et al., 1997; White et al., 1997) and other species (Fujii et al., 1997; Swindell et al., 1999; Wang et al., 2002; Kruger et al., 2005). The cloning and characterization of CYP26A1 led to the search for other CYP26 enzymes that could metabolize *at*RA, and cloning of CYP26A1 was followed by the identification of CYP26B1 (P450RAI-2) (White et al., 2000; MacLean et al., 2001; Zhao et al., 2005) and CYP26C1 (Tahayato et al., 2003; Taimi et al., 2004). The existence of a fourth member of the family, CYP26D1, has also been proposed in zebrafish but this enzyme has been shown to be the same as CYP26C1 (Gu et al., 2005, 2006).

The CYP26 enzymes are present in all chordates and appear to be evolutionarily highly conserved. It has been proposed that the conservation of the CYP26 enzymes is due to the critical role that RA signaling plays in vertebrate development (Carvalho et al., 2017). For example, CYP26 enzymes and the subsequent regulation of *at*RA gradients in the embryo are critical in the formation of the anterior-posterior body axis. In general, the complement of genes related to *at*RA- signaling has diversified in the animal lineages due to gene duplications and losses, but genetic analysis suggests that CYP26 enzymes were already present in protostomes, and in the last common ancestor of bilaterians (Albalat and Cañestro, 2009). Furthermore, using phylogenetic analyses, evolutionary proximities of animal CYP26 enzymes and cyanobacterial CYP120 enzyme were discovered likely due to a lateral gene transfer (Millard et al., 2014). CYP120 was in fact the first non-animal *at*RA hydroxylase identified, and the CYP120 enzyme from *Synechocystis* has been shown to metabolize *at*RA in the β-ionone ring similar to CYP26A1 (Ke et al., 2005; Kühnel et al., 2008; Alder et al., 2009).

The *CYP26* genes have undergone evolutionary diversification in vertebrates. It has been proposed that phylogenetic separation of *CYP26* genes was a result of two whole gene duplications occurring during the vertebrate evolution, one before cyclostome-gnathostome

split and one after (Carvalho et al., 2017). The early phylogenetic separation caused diversification of *CYP26A1* from *CYP26B1/C1*, resulting in two independent clades within the *CYP26* family, while the additional whole gene duplication led to separation of *CYP26B1* and *CYP26C1* genes from ancestral *CYP26B1/C1* (Fig.3) (Carvalho et al., 2017). However, no specific evolutionary pressures that may have led to the development of the three separate CYP26 enzymes have been proposed as yet, possibly because the specific roles of the individual CYP26 enzymes are still not known. However, the conservation of the CYP26 enzymes through the evolution of mammals points to the critical significance of retinoid signaling and regulation of *at*RA concentrations during embryonic development as well as other processes in adult animals.

For a given CYP26 isoform, protein sequences are highly conserved between species. For example, CYP26A1 sequence similarity is higher than 65% between human, mouse and zebrafish, CYP26B1 sequence similarity is > 70% and CYP26C1 >50% (Thatcher and Isoherranen, 2009). On the other hand, within a species sequence conservation among the three CYP26 family members is much lower. Human CYP26A1 shares only 42 and 43% sequence similarity with human CYP26B1 and CYP26C1, respectively, while protein sequences are 51% similar between human CYP26B1 and CYP26C1 (Fig.4) (White et al., 2000; Taimi et al., 2004). For mouse and zebrafish, the three CYP26 isoforms also share only 40-50% protein sequence similarity within the species (MacLean et al., 2001; Tahayato et al., 2003; Zhao et al., 2005). This lack of sequence similarity is striking as all three enzymes appear to be *at*RA hydroxylases and carry out essentially the same metabolic function. Such functional redundancy is quite unusual with CYP enzymes that conduct endogenous metabolism of a single substrate. For example, CYP19, CYP17 and CYP24 lack multiple family members. The lack of sequence homology is also surprising in the context of CYP biochemistry as typically even small differences in protein sequence, such as those observed between CYP3A4 and CYP3A5 or between CYP2C9, CYP2C19 and CYP2C8, result in distinct differences in substrate specificity and catalytic activity. It can be speculated that multiple CYP26 enzymes are required to metabolize different retinoids, to interact with retinoid binding proteins or to respond differently to environmental and endogenous regulatory signals, but further studies of the enzyme structure-function and expression patterns are needed to provide insight to the evolutionary pressures and functional reasons for the conservation of three members of the CYP26 family.

2.2 Kinetics of retinoid metabolism, substrate specificity and metabolite formation

The CYP26 enzymes are collectively believed to be the main *at*RA hydroxylases, and the endogenous substrate of all three enzymes has been assumed to be *at*RA. Indeed, all three CYP26 enzymes catalyze the oxidation of *at*RA to several primary hydroxylation products. However, the two other RA isomers, 13-*cis*RA and 9-*cis*RA have also been shown to be substrates of CYP26 enzymes together with the metabolites of *at*RA such as 4-OH-RA and 4-oxo-RA. The metabolite profiles of human CYP26 enzymes were first explored in transiently transfected cells with RA isomers as substrates (White et al., 1997, 2000; Taimi et al., 2004). Later, recombinant human CYP26 enzymes were successfully expressed in baculovirus-infected insect cells and enzyme kinetics of various retinoids as substrates were characterized using recombinant human CYP26 enzymes reconstituted with the CYP

reductase in insect cell microsomes (Table 1) (Lutz et al., 2009; Topletz et al., 2012; Zhong et al., 2018). All CYP26 enzymes hydroxylate RA isomers predominantly on the C4 position on the β-ionone ring possibly due to the energetic favorability of this position for oxidation due to the conjugated double bond structure in RA isomers. The 4-OH-RA is also the main metabolite formed from *at*RA by other CYP enzymes such as CYP3A4 and CYP2C8. Aside from 4-OH- metabolites, 18-OH-, 16-OH- and 5,6-epoxy-metabolites have been suggested and identified as other primary metabolites from RA isomers formed by CYP26 enzymes (Fujii et al., 1997; White et al., 1997; Lutz et al., 2009; Topletz et al., 2012; Zhong et al., 2018). Of these metabolites the 18-OH-RA and 16-OH-RA were detected as oxidation products with the recombinant CYP26 enzymes (Thatcher et al., 2011; Topletz et al., 2012). These primary hydroxylated metabolites are sequentially oxidized by CYP26 enzymes to a 4-oxo-RA and variety of di-hydroxy (e.g. 4,16- OH_2- and 4,18-OH_2-RA) and oxo-hydroxy metabolites (e.g. 4-oxo-16-OH- and 4-oxo-18-OH-RA) (Lutz et al., 2009; Thatcher et al., 2011; Shimshoni et al., 2012; Topletz et al., 2012; Zhong et al., 2018). Of note, the formation of 4-oxo-*at*RA is oftentimes observed in CYP26-expressing systems (e.g. transiently transfected cells and liver microsomes). However, the formation of 4-oxo-*at*RA likely requires an alcohol dehydrogenase enzyme in tissue and cell systems for the second oxidization step after initial 4-hydroxylation. Studies in HepG2 cells have shown that 4-oxo-*at*RA formation from 4-OH-*at*RA, is mainly mediated by NAD^+-dependent enzymes and not by CYP26A1 (Topletz et al., 2015), and early studies in hamster liver microsomes showed that 4-oxo-RA formation was mediated by an ADH enzyme (Roberts et al., 1980). However, the identity of the enzyme(s) forming 4-oxo-RA remains to be determined. The formation of the 4-oxo-RA is of particular interest as this metabolite appears to be active in terms of RAR binding and it is found at similar concentrations with *at*RA in human liver but not in mice (Zhong, Kirkwood, et al., 2019), suggesting some important species differences in retinoid metabolism. The fact that 4-oxo-RA formation is likely not mediated by CYP26 enzymes is also relevant for understanding the biological role of CYP26 enzymes. Of the CYP26 family enzymes, CYP26A1 has been shown to be mainly an enzyme that clears active retinoids rather than an enzyme forming active retinoids such as 4-oxo-RA (Topletz et al., 2015). It is likely that the same is true for CYP26B1 and CYP26C1.

All three human CYP26 enzymes have high affinity towards *at*RA as a substrate with K_m values in the low nanomolar range (Table 1). This high affinity is interpreted as an indication of the biological role of the CYP26 enzymes as *at*RA hydroxylases, and together with the fact that CYP26 enzymes are generally inducible by *at*RA, the high substrate affinity is considered as the main evidence that CYP26 enzymes are biologically significant *at*RA hydroxylases. Other *at*RA metabolizing CYP enzymes (e.g. CYP2C8, CYP3A4, CYP3A5 and CYP3A7) show K_m values in the micromolar range for *at*RA (Thatcher et al., 2010), demonstrating that *at*RA has a low affinity towards these CYPs, and consequently the intrinsic clearances (Cl_{int}) by these enzymes are relatively low. Among the three CYP26A1 enzymes, CYP26A1 has the highest catalytic activity towards *at*RA indicated by the highest Cl_{int}, while CYP26B1 appears to be a high affinity low capacity *at*RA hydroxylase and exhibits the lowest Cl_{int} of *at*RA amongst the three CYP26s (Table 1). The metabolite patterns formed by the CYP26 enzymes also differ slightly between the three family members. CYP26A1 hydroxylates *at*RA nearly as efficiently at C-16 and C-18 as in C-4

while CYP26B1 and CYP26C1 predominantly form the 4-OH-RA metabolite (Table 1). Whether these metabolite profiles have biological significance in terms of the sequential metabolism or potential biological activity of the metabolites remains to be defined. The 4-OH-RA can be further oxidized to 4-oxo-RA while the 18-OH-RA and 16-OH-RA metabolites likely undergo glucuronidation or sequential hydroxylation by CYP26 and are efficiently eliminated from the body following the initial hydroxylation by CYP26A1. In addition to different metabolite profiles, the three CYP26 enzymes appear to have different specificity towards the stereochemistry of C-4-hydroxylation. The dominant 4-OH-metabolite formed by human CYP26A1 is (4*S*)-OH-RA while CYP26C1 converted *at*RA preferably to (4*R*)-OH-*at*RA (Shimshoni et al., 2012; Zhong et al., 2018). CYP26B1 forms both (4*S*)-OH-*at*RA and (4*R*)-OH-*at*RA, with a 4R/4S ratio of 1:1-1:2 (Topletz, 2013). The metabolite profile and stereospecificity of the 4-hydroxylation of *at*RA offer insights into the ligand binding to the CYP26 enzymes and the possible differences in the active sites of these enzymes, and has been used in homology modeling of the CYP26 structures (*vide infra*).

The other RA isomers, 9-*cis*-RA and 13-*cis*-RA also bind CYP26 enzymes although the binding affinity has been suggested to be lower than that of *at*RA (Helvig et al., 2011). All three CYP26 enzymes also metabolize 13*cis*-RA and 9-*cis*-RA with 4-OH-RA being the dominant metabolite, although the kinetics of 13-*cis*-RA hydroxylation have only been characterized with CYP26C1 (Lutz et al., 2009; Thatcher et al., 2011; Diaz et al., 2016; Zhong et al., 2018). Of the three enzymes, CYP26C1 has the highest affinity towards 9-*cis*RA and CYP26A1 and CYP26B1 showed approximately 13- and 55-fold higher K_m values than CYP26C1 towards 9-*cis*RA, respectively (Table 1). Somewhat surprisingly, the Cl_{int} of 9-*cis*RA by CYP26C1 was nearly as high as that of *at*RA by CYP26A1 while the Cl_{int} towards *at*RA by CYP26C1 was only about 10% of that by CYP26A1. This finding prompted the hypothesis that high clearance of 9-*cis*RA by CYP26C1 may be the reason why this RA isomer cannot usually be detected in biological samples. In terms of the stereochemistry of the 4-OH-metabolites with 13-*cis*RA and 9-*cis*RA as substrates, human CYP26A1 metabolized 13-*cis*RA to both (4*S*)- and (4*R*)-OH-13*cis*RA (ratio 1:1), and 9-*cis*RA to (4*S*)-OH-9*cis*RA (Zhong et al., 2018). CYP26C1 converted 13-*cis*RA to both (4*S*)- and (4*R*)-OH-13-*cis*RA (ratio 1:1), and 9-*cis*RA to (4*S*)-OH-9-*cis*RA (Zhong et al., 2018). Whether this stereoselectivity has any biological significance remains to be determined.

Other than RA isomers, human CYP26 enzymes also effectively metabolize 4-OH-, 18-OH- and 4-oxo-*at*RA (Table 1) and the sequential metabolism of *at*RA is characteristic for these enzymes. The K_m values of 4-OH-*at*RA and 18-OH-*at*RA with CYP26A1 and CYP26B1 were all in the nanomolar range, indicating similar affinity of these substrates as *at*RA (Shimshoni et al., 2012; Topletz et al., 2015). In contrast, CYP26C1 was most efficient in clearing 4-oxo-*at*RA showing 10-fold higher Cl_{int} towards this substrate than other two CYP26 enzymes (Zhong et al., 2018). Both CYP26A1 and CYP26B1 preferred (4*S*)-OH-*at*RA as a substrate over (4*R*)-OH-*at*RA (Table 1) to yield dihydroxylated metabolites.

2.3 Interactions of CYP26 enzymes with retinoid binding proteins

Due to the lipophilicity of retinoids and their high affinity to cellular retinoid binding proteins, it is generally believed that retinoids are bound to binding proteins and get

delivered to the nucleus to activate RA signaling, and to corresponding enzymes for further metabolism by the binding proteins (Napoli, 2017). The biological importance of retinoid metabolizing enzymes largely depends on whether protein-protein interactions and substrate channeling exist between cellular binding proteins and the metabolizing enzymes. It has been shown that *at*RA and 4-oxo-*at*RA both have high affinities to cellular retinoic acid binding proteins (CRABPs), CRABP I and II (K_d < 20 nM; (Fiorella and Napoli, 1991, 1994). In addition, before the CYP26 enzymes were identified, it was shown that the CRABPs interact with the microsomal enzymes that metabolize *at*RA in rat testis microsomes and impact the metabolite formation and *at*RA clearance (Fiorella and Napoli, 1991, 1994; Napoli et al., 1991). Based on current knowledge of the enzymes expressed in the testis and hydroxylating *at*RA, it is likely that these early findings were characterizing the interactions between CRABPs and CYP26 enzymes. Studies with recombinant CYP26B1 and CYP26C1 have subsequently confirmed the observations that were made in rat testis microsomes, demonstrating that the CYP26 enzymes interact with CRABPI and CRABPII (Nelson et al., 2016; Zhong et al., 2018). The kinetic analysis of the interactions between CRABPs and CYP26B1 suggested that not only does CYP26B1 interact with CRABP-*at*RA (holo-CRABP) to accept *at*RA as a substrate via substrate channeling, but also that apo-CRABP inhibits the activity of CYP26B1 (Nelson et al., 2016). As *at*RA binding with the CRABPs is tight and essentially in the presence of CRABPs no free *at*RA is present in the incubations, the kinetic observations strongly support direct substrate channeling between the CRABPs and CYP26 enzymes. The apparent K_m values of CRABP-bound *at*RA were either lower or similar to free *at*RA, an observation that indicates a much lower K_m value for free *at*RA in the presence of CRABPs if free drug hypothesis is assumed. The k_{cat} values were significantly decreased for CYP26 enzymes in the presence of CRABPs also suggesting that the CRABPs act as noncompetitive inhibitors of the CYP26 enzymes (Table 1) (Nelson et al., 2016; Zhong et al., 2018). In addition to *at*RA channeling, the experiments with recombinant CYP26C1 suggest that CRABPs may also deliver *at*RA metabolites for further oxidation. When 4-oxo-*at*RA was bound with CRABPI or CRABPII the K_m and k_{cat} values were lower when compared with free 4-oxo-*at*RA suggesting similar protein-protein interactions as with *at*RA (Table 1) (Zhong et al., 2018). In contrast, CRABPI and CRABPII had no impact of 9*cis*RA metabolism by CYP26C1.

2.4 Homology models, ligand interactions and structural information

At present there are no crystal structures of the CYP26 enzymes and much of the understanding of the CYP26 structures comes from general CYP crystal structures and homology modeling of CYP26s. Due to the potential of CYP26 inhibition as a therapeutic target (Nelson et al., 2013), there is considerable interest in gaining more insight into the structures of the CYP26s and in particular to the potential differences in the active site architecture between the individual CYP26 enzymes. Experimentally, differences in inhibitor binding to the different CYP26 enzymes have been observed. For example talarozole and ketoconazole were much more potent inhibitors of CYP26A1 (IC_{50}<10 nM for talarozole and 0.55 μM for ketoconazole) and CYP26B1 (IC_{50}<10 nM for talarozole and 0.59 μM for ketoconazole) than of CYP26C1 (IC_{50}~3.8 μM for talarozole and 124 μM for ketoconazole) (Thatcher et al., 2011; Diaz et al., 2016; Foti, Diaz, et al., 2016; Zhong et al., 2018), and several relatively selective CYP26A1 inhibitors have been identified (Diaz et al.,

2016). To explore the structural similarities and active site characteristics of the CYP26 enzymes, homology models of CYP26A1, CYP26B1 and CYP26C1 have been constructed. These homology models have then been used to help understand protein structures, substrate binding, impact of genetic polymorphisms on CYP26 activity, and to facilitate design of CYP26 inhibitors or retinoic acid metabolism blocking agents (RAMBAs). A summary of the regions within CYP26 protein sequences together with amino acids proposed to interact with *at*RA based on CYP26 homology models are shown in Figure 4.

The first homology models of CYP26A1 were constructed based on the crystal structures of human CYP3A4 (PDB 1TQN), CYP2C8 (PDB 1PQ2) and CYP2C9 (PDB 1R90) using a single template alignment approach (Gomaa et al., 2006). The models based on the three templates could not be differentiated based on computational model validation methods nor based on *at*RA docking that predicted the oxidation of C-4 in all models. The best homology model was chosen based on ligand docking. The classic CYP26 inhibitor R115866 (talarozole), could only be docked to the CYP3A4 based homology model (Gomaa et al., 2006). The active site of CYP26A1 was then optimized via molecular dynamics simulations with talarozole in the active site resulting in the final model. Unfortunately, it is unclear whether the docking of *at*RA into this final model would predict the stereospecificity of 4-hydroxylation and the experimentally observed oxidation of C-16 and C-18 in addition to C-4 (Gomaa et al., 2006). Nevertheless, the developed model was subsequently successfully used to rationalize binding orientations and design of CYP26A1 inhibitors (Gomaa et al., 2008; Pautus et al., 2009).

Another CYP26A1 homology model in parallel with a CYP26B1 homology model was developed using human CYP3A4 (PDB 1TQN) structure as the major template in addition to CYP2C8 (PDB 1PQ2) and CYP2C9 (PDB 1OG2) structures (Karlsson et al., 2008). These CYP26A1 and CYP26B1 homology models were also evaluated based on *at*RA and inhibitor docking, although minimal experimental data was available at the time regarding the different inhibition potencies of the docked compounds towards CYP26A1 and CYP26B1. Minor differences of the CYP26A1 model in comparison to the previous CYP26A1 model were found. The C-4 of *at*RA was predicted to be closer to the heme iron in the CYP26A1 model than in the CYP26B1 model (Karlsson et al., 2008), a finding likely in agreement with the later observed lower catalytic activity of CYP26B1 in comparison to CYP26A1 (Topletz et al., 2012). Interestingly, the developed homology models suggested some distinct differences in the active site architecture between CYP26A1 and CYP26B1, with the CYP26B1 active site having more hydrogen bonding interactions than CYP26A1, providing potential avenues to developing inhibitors that are selective towards the different CYP26 enzymes (Karlsson et al., 2008). One may speculate that the higher number of hydrogen bonding interactions in the CYP26B1 active site in comparison to CYP26A1 could explain the higher affinity of *at*RA to CYP26B1 in comparison to CYP26A1 that was later observed (Topletz et al., 2012).

The CYP26A1 and CYP26B1 active sites have subsequently been compared (Foti, Isoherranen, et al., 2016) using homology models of CYP26A1 and CYP26B1 developed based on the crystal structure of CYP120 (PDB 2VE3) (Kühnel et al., 2008). The CYP120 structure provides an advantage over the CYP3A4 template as CYP120 has a 33% sequence

similarity with CYP26A1 and 35% sequence similarity with CYP26B1 (Foti, Isoherranen, et al., 2016) in comparison to 24-26% similarity between CYP26s and CYP3A4 (Gomaa et al., 2006; Karlsson et al., 2008). It should be noted that a corrected sequence of CYP26B1 with two amino acid changes from the originally cloned CYP26B1 sequence was used for this later CYP26B1 model (Foti, Isoherranen, et al., 2016). The study predicted similar active site volumes of the CYP26A1 and CYP26B1 and similar hydrophobic binding sites, but some differences in the residues that interacted with the carboxylic acid moiety of *at*RA (Foti, Isoherranen, et al., 2016). Both the CYP26A1 and CYP26B1 models predicted the stereochemistry of 4-hydroxylation of *at*RA correctly, and the C-16 and C-18 were also predicted as other *at*RA hydroxylation sites by CYP26A1 and CYP26B1 (Foti, Isoherranen, et al., 2016). A unique feature of these models is that a xenobiotic substrate of CYP26 enzymes, tazarotenic acid, was identified and docked into the substrate binding sites of CYP26A1 and CYP26B1. The docking simulations successfully predicted the oxidation sites of tazarotenic acid by CYP26A1 and CYP26B1 providing additional confidence towards the active site architecture of the homology models. In subsequent studies these CYP26A1 and CYP26B1 homology models were used to explore the overlap of xenobiotic inhibitors of CYP2C8 with CYP26A1 and CYP26B1 (Foti, Diaz, et al., 2016), and these docking studies could predict the binding orientations of some of the overlapping inhibitors within CYP26A1 and CYP26B1.

Several other homology models of CYP26A1 have been constructed (Ren et al., 2008; Shimshoni et al., 2012; Sun et al., 2015; Awadalla et al., 2016). Of these models one was made using human CYP3A4 (PDB 1TQN) and mycobacterial CYP51 (PDB 1EA1) as templates (Ren et al., 2008). Notably, in this model the predicted membrane anchor of CYP26A1 (Met1 to Val33), which is located in the N-terminus of CYP26A1, was discarded as this region is not present in the crystal structures of CYP3A4 and CYP51. This CYP26A1 model was further verified by docking talarozole to the active site but no *at*RA docking or prediction of metabolic sites was presented. It was noted that the template CYP51 structure was co-crystallized with fluconazole, a presumed early RAMBA, potentially providing more active site similarity between the CYP26A1 homology model and CYP51 structure (Ren et al., 2008). It is likely that fluconazole binds to CYP26A1 as it has been shown to inhibit tazarotenic acid metabolism by CYP26A1 (Foti, Diaz, et al., 2016), although the inhibition of CYP26A1 mediated 9-*cis*-RA metabolism by fluconazole is equivocal (Thatcher et al., 2011). Another CYP26A1 homology model was developed using a different CYP51 crystal structure (PDB 3JUS) as a template together with the CYP120 co-crystallized with *at*RA (PDB 2VE3) (Ren et al., 2008). With this homology model *at*RA was docked into the active site in a similar orientation as found with the CYP120 crystal structure without further exploration of the binding orientation of *at*RA within the active site. This is noteworthy as it positions the C-2 and C-16,17 of *at*RA closest to the heme iron rather than C-4, and does not predict the metabolic pattern observed with CYP26A1. However, this homology model was used for extensive exploration of inhibitor binding orientations and binding energy with traditional RAMBAs and a series of more recently synthesized CYP26A1 inhibitors (Ren et al., 2008).

In a similar strategy of homology model development, the structures of CYP120 cocrystallized with *at*RA (PDB 2VE3) and CYP2C8 co-crystallized with two 9*cis* RA

molecules in the active site (PDB 2NNH) were used to develop a CYP26A1 homology model (Shimshoni el al., 2012). In this model, the active site architecture of CYP26A1 was optimized using molecular dynamics simulations and energy minimization with *at*RA docked in the active site. The developed homology model was then used to explain the stereospecificity of 4-hydroxylation specifically on the pro-S position in *at*RA C-4 by CYP26A1 and the simultaneous lack of regiospecificity in the site of oxidation of *at*RA in the β-ionone ring. The orientation of *at*RA within the CYP26A1 active site was predicted to be such that the β-ionone ring was on a plane parallel to the heme allowing similar distances of C-16, C-18 and C-4 from the heme iron. This binding orientation, consistent with the observed data of metabolite formation by CYP26A1 was further explored in a homology model developed using solely CYP120 crystal structure as a template (Awadalla et al., 2016). This model was considered improved over the previous CYP26A1 models based on the analysis of Ramachandran plots and other in silico model quality analyses. The stereospecificity of (4S)-OH-RA formation by CYP26A1 was correctly identified by this homology model but the authors did not discuss whether formation of 16-OH- and 18-OH-*at*RA would also have been predicted using this homology model. The authors further explored the validity of the homology model via docking a series of known CYP26A1 inhibitors and comparing the predicted binding energies with observed binding affinities. They concluded that the model could distinguish between weak and potent inhibitors (Awadalla et al., 2016).

Overall the CYP26A1 homology models have identified several key residues within the CYP26A1 active site that consistently in all models appear to interact with *at*RA and other docked ligands. These include Trp112, Phe222, Phe299, Pro371 and Phe374 (Gomaa et al., 2006; Karlsson et al., 2008; Ren et al., 2008; Shimshoni et al., 2012; Awadalla et al., 2016; Foti, Isoherranen, et al., 2016). However, the different homology models predicted divergent residues that would interact with the carboxylic acid moiety of *at*RA. Three of the models have predicted Arg90 to interact with *at*RA carboxylic acid moiety (Karlsson et al., 2008; Awadalla et al., 2016; Foti, Isoherranen, et al., 2016). One model predicted Arg86 to interact with *at*RA carboxylate (Gomaa et al., 2006) and another that Arg64 interacted with the carboxylate moiety (Shimshoni et al., 2012). Further biochemical studies would be needed to explore the importance of these Arg residues in the active site of CYP26A1 to verify the quality of the developed homology models and the identification of active site residues.

Homology models of CYP26B1 and an identified splice variant of CYP26B1 (Elmabsout et al., 2012) were built to explore how the splice-variant of CYP26B1 impacts CYP26B1 structure-function (Saenz-Méndez 2012). The models were constructed based on the CYP120 crystal structure although other templates were also assessed for model building (Saenz-Méndez 2012). The homology model suggested that despite the loss of 75 amino acids in the splice variant, the active site architecture was fairly unchanged in the splice variant CYP26B1 when compared to the full-length protein. The quality of both CYP26B1 models (full length and splice variant) was evaluated based on ligand docking and the authors concluded that the model differentiated between weak and strong binders to the CYP26B1. Similarly, in the splice variant, the modelled distance of *at*RA from the heme iron and the binding energy predicted a reduced catabolic activity of the splice variant

towards *at*RA (Saenz-Méndez 2012). This prediction is consistent with the experimental data (Elmabsout el al., 2012).

There is only one homology model reported for CYP26C1 (Zhong et al., 2018). This homology model was built using the CYP120 (PDB 2VE3), three CYP51 (PDB 4LXJ, 5EQB, 4WMZ) and CYP46A1 (PDB 3MDM) crystal structures as templates and the model was optimized via molecular dynamics simulations. This model was then used to explore potential reasons for the functional differences between CYP26C1 and the other two CYP26 family members. The homology model successfully predicted the sites of metabolism and the stereoselective metabolism of *at*RA, 13-*cis*RA, 9-*cis*RA and 4-oxo-RA observed with recombinant CYP26C1. The CYP26C1 model also predicted the existence of a hairpin loop structure (Lys484-Lys504) unique to CYP26C1, which forced *at*RA to orient differently in the CYP26C1 active site in comparison to *at*RA bound to CYP120A1.

The homology models of the CYP26 enzymes have provided important information and hypotheses of the structure-function of CYP26 enzymes. However, systemic comparison of the structural differences among the three CYP26 isoforms and the overall homology model predicted differences in the active sites is still needed. In addition, a systematic validation of the homology models with known inhibitors and substrates that are selective for the individual CYP26 enzymes is needed to further explore the potential for developing selective inhibitors of the CYP26 enzymes. With the advances in protein crystallography and other protein structure determination techniques it is likely that more detailed information of the CYP26 structures will become available.

3. Expression patterns of CYP26s in humans and animal models

3.1 Expression patterns in animal models

*at*RA is a highly evolutionarily conserved signaling molecule that plays a critical role in embryonic and fetal development, in body patterning and organogenesis and in mammalian reproduction (McCaffery et al., 2003; Maden, 2007; Duester, 2008; Rhinn and Dolle, 2012). It is then perhaps not surprising that majority of the knowledge of the expression patterns and function of CYP26 enzymes is derived from developmental biology studies and animal models. The expression patterns and role of CYP26 enzymes in regulating *at*RA signaling during embryogenesis have been previously reviewed (Pennimpede et al., 2010; Ross and Zolfaghari, 2011). As detailed in the reviews, the CYP26 knockout mouse models and the observed phenotypes have been instrumental in establishing the importance of the CYP26 enzymes in regulating *at*RA homeostasis. Both the *Cyp26a1*$^{-/-}$ and *Cyp26b1*$^{-/-}$ mice died during gestation or shortly after birth. However, the phenotype of the two mouse models was quite different. The *Cyp26a1*$^{-/-}$ mice exhibited defects of hind-brain patterning, spina bifida and caudal regression as well as sirenomelia and vertebral transformation (Abu-Abed et al., 2001; Sakai et al., 2001). In comparison, *Cyp26b1*$^{-/-}$ mice had skeletal abnormalities and defects that were most pronounced in limb development (phocomelia, oligodactyly, meromelia) and in craniofacial abnormalities (micrognathia, reduced ossification of calvaria, abnormal tooth buds) (Yashiro et al., 2004; Maclean et al., 2009; Pennimpede et al., 2010; Dranse et al., 2011). The *Cyp26c1*$^{-/-}$ mice did not have an apparent phenotype but the

severity of the *Cyp26a1*$^{-/-}$ phenotype was increased in the *Cyp26a1*$^{-/-}$*Cyp26c1*$^{-/-}$ double knockout mice (Uehara et al., 2007).

The role and function of Cyp26 enzymes during embryonic development has also been studied in zebrafish (Emoto et al., 2005; Laue et al., 2008; Spoorendonk et al., 2008). In particular the studies in zebrafish have focused on the link between *at*RA concentration as a posteriorizing signal during body axis establishment and *cyp26* expression (Kudoh et al., 2002). The gene expression pattern of *cyp26a1* was analyzed in developing zebrafish (Kudoh et al., 2002) and *cyp26a1* was found to be expressed in the anterior neural ectoderm in a complementary pattern with *hoxb1b*. The studies showed that Fgf and Wnt signals suppressed the expression of the anterior gene *cyp26a1* in an *at*RA independent manner. In addition, the authors suggested that cyp26a1 was functionally an enzyme that suppressed the expression of posterior genes in the zebrafish embryo via its role as *at*RA depleting enzyme (Kudoh et al., 2002). Consistent with the role of *cyp26a1* in body axis patterning, the phenotype of a zebrafish mutant *giraffe* was shown to be due to mutations in *cyp26a1* resulting in variety of body patterning defects in the fins, tail, spinal cord and hindbrain (Emoto et al., 2005).

Cyp26b1 function during zebrafish development has been assessed in two mutants, the *dolphin* mutant (Laue et al., 2008) and the *stocksteif* mutant (Spoorendonk et al., 2008). The *stocksteif* mutant, associated with *cyp26b1*, showed severe hyperossification in the developing zebrafish vertebral column. This finding led to the discovery that cyp26b1 is expressed in the osteoblasts and that cyp26b1 plays a role in ossification (Spoorendonk et al., 2008). Importantly, the phenotype of the *stocksteif* (cyp26b1) mutant was replicated by treating the zebrafish embryos with the cyp26 inhibitor talarozole (R115866) or with *at*RA. In addition, the expression of Cyp26b1 was also shown in mouse osteoblasts similar to the zebrafish. The role of cyp26b1 in ossification and osteoblasts was independently shown at the same time in the *dolphin* mutant (Laue et al., 2008). The phenotype of the *dolphin* mutant was shown to be caused by the loss of function of cyp26b1 in this mutant, and this mutation was shown to lead to over-ossification of craniofacial bones and axial skeleton. Overall the effects of *cyp26b1* mutation suggested that the depletion of *at*RA by cyp26b1 was necessary to attenuate ossification in vivo likely through osteoblast activity (Laue et al., 2008). Importantly, these findings in zebrafish (fusion of vertebrae) were also replicated in mice via treatment with the Cyp26 inhibitor talarozole (Laue et al., 2008).

The Cyp26 enzymes are generally expressed in embryonic tissues that undergo morphogenesis and the spatial and temporal expression patterns of the three Cyp26 enzymes have been delineated in mouse models. However, understanding of the protein expression patterns of CYP26 enzymes has been limited due to lack of selective antibodies to these enzymes and the variable quality of commercial antibodies (Topletz et al., 2012). Generally, the majority of the characterization of CYP26 enzyme expression in various tissues and species has been done based on mRNA expression and further confirmation of correlation between mRNA and protein expression is needed. The three Cyp26 enzymes appeared to be expressed in distinct sites in gestational stage specific manner (MacLean et al., 2001), and the Cyp26 enzymes were usually not coexpressed in specific tissues at a specific developmental stage. For example, *Cyp26b1* was expressed in the developing murine limb

and limb buds while *Cyp26a1* and *Cyp26c1* were absent from the limbs (MacLean et al., 2001; Pennimpede et al., 2010). Similarly, *Cyp26b1* was expressed in several rhombomeres while *Cyp26a1* was only present in rhombomere 2 in E8.5 (MacLean et al., 2001). In mouse embryos, *Cyp26a1* was expressed in mesenchyme that is neural crest derived while *Cyp26b1* was found in the ectodermal and endodermal areas (MacLean et al., 2001). In mouse embryos, *Cyp26c1* was expressed in the hindbrain, inner ear, first branchial arch and tooth buds (Tahayato et al., 2003). Similarly, in the developing chick embryo *CYP26C1* was shown to have distinct sites of expression in comparison to *CYP26A1* and *CYP26B1* (Reijntjes et al., 2004). However, over the course of development the expression and localization of the Cyp26 enzymes shifts and in general the expression patterns are specific to tissues and gestational age. In addition, the expression patterns across development clearly show Cyp26 enzyme specific patterns and suggests non-redundant roles of these enzymes in specific cell types and tissues.

One of the intriguing characteristics of Cyp26b1 is its expression in fetal gonads. It has been suggested that Cyp26b1 plays a critical role in sex-specific timing of meiotic initiation via modulating the concentrations and actions of *at*RA synthesized by the mesonephros. Strikingly, *Cyp26b1* expression was male-specific by E12.5 in the mice (Bowles et al., 2006) suggesting that Cyp26b1 degrades *at*RA in the developing testis hence preventing the initiation of meiosis. In contrast, in the ovaries meiosis can occur during fetal development due to the lack of Cyp26b1 expression (Bowles et al., 2006). In the same study it was also shown that meiosis occurred earlier than normal during fetal development in the ovaries of the *Cyp26b1*$^{-/-}$ mice (Bowles et al., 2006). In male mouse embryos, *Cyp26b1* was found to be expressed in the Sertoli cells (Bowles et al., 2006), and when *Cyp26b1* was selectively deleted in the Sertoli cells, male germ cells entered the mitotic cycle at E15.5-E16.5 (Li et al., 2009). Importantly, it was found that Cyp26b1 in Sertoli cells not only prevents meiosis but also maintains the mitotic quiescence of the germ cells during embryonic development (Li et al., 2009). These findings of Sertoli-cell specific knockouts are consistent with the characterization of the testes of the global *Cyp26b1*$^{-/-}$ mice, in which relative *at*RA levels were increased in E12.5 and germ cells were virtually absent in the neonates, likely due to apoptotic extinction of the germ cells during development (MacLean et al., 2007).

The significance of Cyp26 enzymes and retinoid signaling appears to extend to the postnatal gonads. In adult reproductive tract Cyp26 enzyme expression has been fairly well characterized in the mouse testes. Using cell-type specific knockout mouse models it was shown that Cyp26 enzymes and Cyp26b1 in particular play an important role in regulating spermatogenesis (Hogarth et al., 2015). When *Cyp26b1* was knocked out within the Sertoli cells, mild defects in spermatogenesis were observed while knockout in germ cells resulted in no defects in spermatogenesis. When *Cyp26b1* was knocked out in both cell types, histological analysis showed severe defects in spermatogenesis and loss of male fertility (Hogarth et al., 2015). In contrast, when *Cyp26a1* was knocked out either in the Sertoli or germ cells or in both, no significant effects on spermatogenesis were observed. This finding was reproduced in the global tamoxifen induced *Cyp26a1* knock-out mice, in which no change in testis *at*RA concentrations or in spermatogenesis was observed (Zhong, Hogarth, et al., 2019). However, knockout of *Cyp26a1* in addition to *Cyp26b1* in the germ cells appeared to precipitate a testis phenotype. In the animals with a dual *Cyp26a1/Cyp26b1*

germ cell specific knockout, vacuoles were observed in the seminiferous epithelium while in the *Cyp26b1* knockouts no vacuoles were present (Hogarth et al., 2015). In dogs *Cyp26b1* transcripts were detected in testes from young (4 months of age), peripubertal and adult dogs with the highest *CYP26B1* mRNA expression detected in the young dogs (Kasimanickam and Kasimanickam, 2012). In the female reproductive tract, *Cyp26b1* transcripts and protein have been detected in the postnatal ovary and the expression of Cyp26b1 was found to be downregulated by activin and TGF-β (Kipp et al., 2011). Interestingly, *Cyp26b1* expression was lost in the mature follicles and Cyp26b1 enzyme was considered to be capable of decreasing the granulosa cell proliferation by degrading *at*RA, a factor inducing proliferation of this cell type.

*at*RA has a critical role in regulating neuronal patterning, maintaining adult neurons, in neurogeneration and in neuronal differentiation (Maden, 2007), and therefore it is expected that the enzymes responsible for regulating *at*RA concentration gradients such as the ALDH1A and CYP26 enzymes will be expressed in the adult brain in addition to the developing brain. Indeed, both *Cyp26a1* and *Cyp26b1* mRNA appear to be expressed in the adult brain but the expression level of *Cyp26a1* seems variable as it was shown to be low in the mouse and rat brain in some studies (Abu-Abed et al., 2002; Stoney et al., 2016) but robust in the mouse brain in other studies (Ray et al., 1997). It is likely that the detection of *Cyp26a1* in the brain requires or is dependent on analysis of specific brain regions that express *Cyp26a1*. For example, in the rat brain *Cyp26a1* mRNA was detected at low levels in the striatum, meninges, thalamus and cortex but *Cyp26a1* mRNA was absent in the hypothalamus, olfactory bulb, hippocampus and cerebellum (Stoney et al., 2016).

It has been proposed that Cyp26b1 contributes to maintaining necessary retinoid gradients also within the adult rodent brain. In contrast to Cyp26a1, in the rat brain *Cyp26b1* mRNA expression was fairly robust with mRNA detected in all the structures analyzed including the striatum, meninges, thalamus, hypothalamus, olfactory bulb, hippocampus, cerebellum and cortex (Stoney et al., 2016). Interestingly, the *Cyp26b1* expression in the rat hippocampus was shown to be restricted to the hilus of the dentate gyrus between the suprapyramidal and infrapyramidal blades, and chemical inhibition of Cyp26b1 was shown to increase the relative *at*RA levels in the hippocampus. This increase in *at*RA concentrations also decreased cell proliferation, an observation supporting the authors' hypothesis that Cyp26b1 in the hippocampus acts in a paracrine fashion to restrict the distribution and steepness of RA concentration gradients (Stoney et al., 2016). Taken together these findings suggest that Cyp26 enzymes play important roles in maintaining retinoid gradients in the brain but further studies are needed to capture the individual role of Cyp26a1 and Cyp26b1 in maintaining brain tissue health, in neuronal differentiation and in the pathogenesis of neurodegenerative diseases.

The expression and importance of Cyp26 enzymes in adult animals has not been as well studied as fetal and embryonic tissue expression. Surprisingly, in a recent study global conditional knock-out of *Cyp26a1* in adult and juvenile mice was shown to neither have major impact on vitamin A homeostasis and tissue retinoid concentrations nor result in major adverse retinoid toxicities (Zhong, Hogarth, et al., 2019), suggesting that Cyp26b1 is the main Cyp26 enzyme responsible for endogenous *at*RA homeostasis in postnatal animals.

Overall, in the tamoxifen inducible *Cyp26a1* knock out animals the main phenotypic changes were a mild skewing of hematopoiesis and significantly decreased clearance of exogenous *at*RA (Zhong, Hogarth, et al., 2019). These findings are consistent with the assessment of changes in tissue specific *at*RA concentrations in mice after talarozole treatment (Stevison et al., 2017). When Cyp26a1 and Cyp26b1 were inhibited with talarozole in adult mice, following a single dose administration of talarozole, *at*RA concentrations increased transiently in the liver, testis and serum but the magnitude of increase in *at*RA concentrations in serum could only be explained by inhibition of extrahepatic Cyp26b1 (Stevison et al., 2017). Following multiple dose treatment of mice with talarozole, Cyp26a1 expression was significantly induced in the liver and testis leading to lack of changes in *at*RA concentrations in these tissues. Yet, *at*RA concentrations in serum were still significantly elevated, a finding that could only be explained by the inhibition of Cyp26b1 in extrahepatic tissues (Stevison et al., 2017).

Cyp26b1 has been shown to be important in variety of extrahepatic tissues (Chenery et al., 2013; Kurashima et al., 2014). For example, Cyp26b1 was shown to be expressed in skin fibroblasts at higher levels than in colon stromal cells (Kurashima et al., 2014). In addition, *Cyp26b1* mRNA expression was more pronounced than *Cyp26a1* in both tissues. Cyp26b1 was concluded to play a role in Mast cell activation and in skin inflammatory responses by regulating *at*RA concentrations and *at*RA clearance in the skin (Kurashima et al., 2014). As Cyp26b1 was expressed in the skin fibroblasts it was shown to regulate the *at*RA signal that subsequently results in Mast cell maturation locally in the skin. Inhibition of the Cyp26b1 by liarozole resulted in upregulation of the P2X7, the receptor of extracellular ATP in skin Mast cells, resulting in retinoid dermatitis similar to what was observed in mice treated with RA (Kurashima et al., 2014). These studies suggest that Cyp26b1 expression in skin fibroblasts degrades *at*RA and hence reduces the paracrine *at*RA signal that acts on Mast cells to activate inflammatory pathways.

Cyp26b1 has been shown to be the only Cyp26 enzyme expressed in $CD4^+$ and $CD8^+$ T-cells from mouse lymphoid tissues, and Cyp26b1 expression was specific for the $CD44^+$ effector/memory T-cells (Takeuchi et al., 2011). $CD4^+$ T-cells from peripheral lymph nodes or $CD44^-$ naïve T-cells from mesenteric lymph nodes did not show any *Cyp26b1* expression (Takeuchi et al., 2011). Subsequently, Cyp26b1 was shown to play a role in T-cell differentiation (Chenery et al., 2013). In ex vivo experiments with mouse T cells, *Cyp26b1* mRNA was found to be expressed in T-cells and the expression was dependent on the type of T-cells. Naïve CD4+ T-cells did not have any detectable *Cyp26b1* expression but ${}_iT_{reg}$ and T_H17 cells both had Cyp26b1 with T_H17 cells having significantly higher *Cyp26b1* mRNA expression than ${}_iT_{reg}$ cells. This difference in *Cyp26b1* expression was linked to the role of *at*RA signaling in differentiation of Naive CD4+ T-cells. In addition, deletion of *Cyp26b1* in the T-cells led to a greater frequency of IL17 producing T_H17-cells and in $CD4^+CD25^+Foxp3^+iT_{reg}$ cells (Chenery et al., 2013). These findings suggest that increased *at*RA concentrations stimulate IL17 production by T_H17 cells and iTreg responses, and Cyp26b1 acts to degrade this *at*RA and hence limits T_H17 and ${}_iT_{reg}$ cell differentiation. These in vitro findings were shown to be relevant also in vivo in mice as the T-cells isolated from the mice deficient in Cyp26b1 in T-cells failed to induce intestinal inflammation in a mouse model of colitis (Chenery et al., 2013). Hence the authors concluded that Cyp26b1

enzyme expression is critical in intestinal inflammation and T-cell responses despite lack of effect of *Cyp26b1* deletion on homing of the T-cells to the gut. It is likely that this function is due to the regulation of *at*RA concentrations and signaling by Cyp26b1.

3.2 Expression patterns in human tissues

Due to the importance of the CYP26 enzymes in regulating *at*RA gradients during fetal development there has been an interest in understanding the expression patterns of CYP26 enzymes in human fetal tissues. In a study of fetal and adult human tissues from individual donors, *CYP26A1* mRNA was detected mainly in the fetal brain and at low levels in the thymus (Xi and Yang, 2008). *CYP26A1* mRNA was detected in the fetal brain in a separate study as well but in that study *CYP26A1* was also clearly detected in fetal heart, kidney, liver and lung (Trofimova-Griffin and Juchau, 1998). The discrepancy between the tissue expression patterns between the two studies is likely due to differences in gestational ages of the donors as the gestational ages of the fetuses were not specified and the CYP26 expression in specific tissues likely changes across fetal development as shown in animal models.

CYP26A1 mRNA expression has also been characterized in human prenatal liver and cephalic tissues of multiple donors of specific gestational ages (Trofimova-griffin et al., 2000). *CYP26A1* mRNA was found both in prenatal cephalic tissues and in the adult brain (Trofimova-griffin et al., 2000). In contrast, but in agreement with the findings of the single donor study (Xi and Yang, 2008), the expression of *CYP26A1* mRNA was detected but not quantifiable in fetal liver and was clearly detectable in the adult liver (Trofimova-griffin et al., 2000). Interestingly, in the prenatal cephalic tissues the gene expression of *CYP26A1* was considerably higher than in the prenatal liver while in adult liver *CYP26A1* expression was higher than in the adult brain (Trofimova-griffin et al., 2000). The expression of *CYP26A1* mRNA was lower in the adult brain when compared to the prenatal brain. Despite the fact that the adult tissues were from a single donor, these findings are consistent with the later study of human tissues (Xi and Yang, 2008), suggesting that *CYP26A1* is predominantly expressed in the prenatal brain and in the adult liver.

In a recent study in a panel of human fetal livers mRNA of both *CYP26A1* and *CYP26B1* were detected although at low levels (Topletz et al., 2019). *CYP26C1* was absent from all fetal livers analyzed (Topletz et al., 2019), a finding in agreement with a previous study in which *CYP26C1* mRNA was only detected in the fetal lung (Xi and Yang, 2008). It is noteworthy, that despite the detection of *CYP26A1* mRNA in the fetal livers, characterization of *at*RA hydroxylation in the fetal liver suggested that CYP3A7 and not CYP26 is mainly responsible for *at*RA clearance in the human fetal livers analyzed (Topletz et al., 2019). These fetal livers were, however, collected from fetuses after approximate day 80 of gestation, a period that is after critical organogenesis stage when CYP26 enzymes are believed to have a major role. As such, it is likely that this analysis does not represent the importance of CYP26 enzymes in human embryonic development and organogenesis.

A gestational age effect in *CYP26* expression in human prenatal tissues was clearly illustrated in a study that assessed the expression of *CYP26B1* in human cephalic tissues using absolute mRNA quantification (Trofimova-Griffin and Juchau, 2002). While robust

CYP26B1 mRNA expression was detected in the cephalic tissues between gestational ages 57 and 110 days, the absolute expression level normalized to GAPDH expression was 5-10 times higher in these early gestational days than in fetal brains of gestational age 112-224 days or adult brain (Trofimova-Griffin and Juchau, 2002). Somewhat surprisingly, *CYP26B1* mRNA was not detected in the fetal brain in a separate study in which *CYP26B1* was detected at highest level in the fetal kidney and muscle and at lower levels in the fetal heart, spleen, thymus, lung and liver (Xi and Yang, 2008). Taken together these studies suggest that the spatiotemporal expression patterns observed in animal models during development also occur in humans.

Several studies have suggested that the expression patterns of CYP26 enzymes in human adult tissues are distinct. CYP26A1 and CYP26B1 are usually not expressed in the same cells and tissues (Xi and Yang, 2008; Tay et al., 2010; Topletz et al., 2012). In adult tissues from individual donors *CYP26A1* mRNA was found only in the liver, placenta and testis while *CYP26B1* mRNA was detected in the brain, kidney, spleen, thymus, placenta, pancreas, prostate, testis, ovary and intestine (Xi and Yang, 2008). This finding of extensive expression of *CYP26B1* mRNA in extrahepatic tissues and predominant expression of *CYP26A1* in the human adult liver was reproduced in a second study in which absolute quantification of *CYP26A1* and *CYP26B1* mRNA was employed in human tissues (Topletz et al., 2012). Generally, of the tissues studied, *CYP26B1* mRNA expression was 10-100 times higher than *CYP26A1* in the human skin, adipose, cerebellum and vein demonstrating particularly notable expression of *CYP26B1* in comparison to *CYP26A1*. These expression patterns in human adult tissues are quite consistent with the original work of *CYP26A1* mRNA detection in individual human tissues where *CYP26A1* mRNA was detected in the temporal cortex, hippocampus, olfactory bulb, placenta and liver (Ray et al., 1997). In addition, *CYP26B1* expression has been detected in kidney arteries, in cultured endothelial and smooth muscle cells and in atherosclerotic lesions (Elmabsout et al., 2012). It is interesting to note that a RA inducible *CYP26B1* splice variant missing exon 2, was also detected in human kidney arteries and cultured endothelial and smooth muscle cells and this splice variant was found to be active although with diminished activity when compared to the full length protein (Elmabsout et al., 2012). It was proposed that both CYP26B1 enzymes play a role in atherosclerotic lesions as the expression of the full length and splice variant *CYP26B1* was 2.5-4.5-fold higher in atherosclerotic lesions than in normal arteries.

A comparison of the data collected of *CYP26A1* and *CYP26B1* expression patterns in fetal and adult tissues suggest that the tissue distribution of CYP26 enzymes in fetal and adult organs is different, likely due to different roles of retinoid signaling during development and adult life. For example, in the human adult liver CYP26A1 is the main, predominant CYP26 enzyme and main *at*RA hydroxylase (Thatcher et al., 2010) while CYP26B1 protein is not detected in the human adult liver (Tay et al., 2010; Topletz et al., 2012). However, some tissues do appear to express both enzymes although it can be speculated that the enzymes are expressed in different cell types within the tissue. For example, when CYP26A1 and CYP26B1 protein expression was evaluated in human tissues by western blot, significant expression was detected in the lung, pancreas, skin and uterus (Topletz et al., 2012). Similarly, *CYP26A1* and *CYP26B1* mRNAs were robustly detected in the bone marrow stroma while it appeared that only *CYP26A1,* although at low levels, was present in the

$CD34^{+}CD38^{-}$ human hematopoietic cells (Ghiaur et al., 2013). This expression pattern of CYP26 enzymes in the bone marrow niche is important as the authors showed that clearance of *at*RA by the CYP26 enzymes in the stroma is important in decreasing retinoid signaling and maintaining the primitive hematopoietic cells. Based on these findings it has also been suggested that CYP26 activity and induction in the bone marrow may be responsible for the drug resistance in hematological conditions responsive to *at*RA such as acute promyelocytic leukemia (APL) and multiple myeloma (Alonso et al., 2017).

CYP26A1 and CYP26B1 have both been detected in adult human brain but at expression levels lower than the human liver (Stoney et al., 2016). Unlike the mouse and rat brain, in the human brain *CYP26A1* transcripts and protein were detected in neurons in the dentate gyrus but not in the microglia or astrocytes. Interestingly in the adult human brain CYP26A1 colocalized with ALDH1A2 in hippocampal neurons and the authors interpreted this in support of an autocrine role of *at*RA in human neurons (Stoney et al., 2016). These findings are somewhat conflicting to the findings in rats and mice where the *Cyp26b1* expression patterns suggested a paracrine role of *at*RA and further studies are needed to define the role of CYP26A1 in the regulation of *at* RA gradients in the brain. The expression of *CYP26B1* was about 2-fold higher in adult brain cerebellum than in the whole brain (Trofimova-Griffin and Juchau, 2002). This expression pattern of *CYP26B1* in the adult whole brain and cerebellum is noteworthy as *CYP26B1* was originally cloned from the retina and upon its discovery was found to be highly expressed in the cerebellum and pons (White et al., 2000). The high mRNA expression of *CYP26B1* in the cerebellum, whole brain, cerebral cortex, hippocampus and temporal lobe has also been confirmed in a separate study which also showed, based on absolute mRNA quantification of matched samples that *CYP26B1* mRNA expression was consistently higher in the brain structures than that of *CYP26A1* (Topletz et al., 2012).

It should be emphasized that single donor studies may be misleading in characterizing CYP26 expression as *CYP26A1* mRNA expression has been shown to vary nearly 500-fold between donors in the adult human liver (Tay et al., 2010). The variability in the mRNA levels seems to correspond to variability in CYP26A1 protein levels in human liver based on the observed variability in CYP26A1 protein expression in a bank of human liver donors (Tay et al., 2010; Thatcher et al., 2010). The reasons for the variability in CYP26A1 expression between human liver donors has not been explained. In a preliminary analysis of *CYP26A1* mRNA expression in human livers, the mRNA expression of *CYP26A1* was lower in ischemic livers than those that had no ischemia (Tay et al., 2010). No differences in *CYP26A1* mRNA in the human livers were detected in subjects with alcohol consumption nor in subjects with fatty liver (Tay et al., 2010). The lack of effect of nonalcoholic fatty liver disease or NASH on *CYP26A1* mRNA was recently confirmed in a study in a separate bank of human livers (Zhong, Kirkwood, et al., 2019). In the same study human liver vitamin A and concentrations of RA isomers were measured, and surprisingly no correlation was found between *CYP26A1* mRNA and liver *at*RA concentrations (Zhong, Kirkwood, et al., 2019), suggesting that constitutive expression of CYP26A1 is not directly regulated by *at*RA concentrations. Yet, the variability in CYP26A1 expression in the human liver is predicted to contribute to a considerable variability in the overall clearance of *at*RA in the liver as CYP26A1 was predicted to be the main human liver RA hydroxylase (Thatcher et

al., 2010). In some human liver donors the CYP26A1 mediated *at*RA clearance was predicted to be negligible with CYP3A4 contributing to the majority of the clearance while in other donor livers the clearance was very high and mainly mediated by CYP26A1 (Thatcher et al., 2010).

4. Regulation of CYP26 expression

CYP26A1 was originally identified as an *at*RA hydroxylase based on the inducibility of *cyp26a1* mRNA by *at*RA in zebrafish (White et al., 1996). Similarly, the human and mouse orthologs of CYP26A1 were discovered via *at*RA induction experiments (Ray et al., 1997). The mouse *Cyp26a1* was identified from murine embryonic stem cells in which *Cyp26a1* was highly inducible by *at*RA (Ray et al., 1997). Since then, plethora of studies have shown that CYP26A1 is induced by treatment with *at*RA or other RAR binding retinoids in vivo and in vitro in various cell culture systems. The CYP26A1 induction by *at*RA is so robust that the induction of CYP26A1 has been proposed as a marker of RAR mediated retinoid activity (Zolfaghari et al., 2019). The RAR mediated induction of CYP26A1 has been explained via the identification of two retinoic acid response elements (RARE) in the *CYP26A1* promoter that together enhance *CYP26A1* induction (Loudig et al., 2000, 2005; Zhang et al., 2010). One of the RAREs in the *CYP26A1* promoter is similar to the RARE in *RARβ* promoter explaining the shared inducibility by *at*RA of CYP26A1 and RARβ (Loudig et al., 2005). In addition, the collaboration of the two RAREs in the *CYP26A1* promoter in comparison to a single RARE in *RARβ* promoter likely explains the much higher magnitude of induction of *CYP26A1* by *at*RA in comparison to *RARβ* (Tay et al., 2010; Topletz et al., 2015; Zolfaghari et al., 2019). However, the responses of CYP26A1 to *at*RA still appear tissue specific despite the well-defined RAREs and *at*RA responses. For example, in the initial studies of Cyp26a1 induction in mouse brain and liver, *at*RA treatment was found to significantly induce *Cyp26a1* gene expression only in the mouse liver but not in the brain (Ray et al., 1997). In mouse embryonic tissues *Cyp26a1* was shown to be inducible by *at*RA treatment to the mom in embryonic days 9.5-11.5 when the induction and expression of *Cyp26a1* was detected by in situ hybridization (Pennimpede et al., 2010). Interestingly, the pattern of induction in specific tissues was localized to *at*RA-sensitive tissues and the induction of Cyp26a1 had a distinct tissue specific pattern (Pennimpede et al., 2010). It is possible that this tissue specific induction is a result of the tissue specific expression of the different RARs as CYP26A1 induction in liver models was shown to be mediated mainly by RARα (Tay et al., 2010). This finding of the predominant role of RARα in CYP26A1 induction was later reproduced in studies using various promoter activation assays and RAR selective antagonists in HepG2 cells (Zolfaghari et al., 2019). The lack of a contribution of RARγ in CYP26A1 induction in HepG2 cells is likely due to the absence of this nuclear receptor in HepG2 cells (Tay et al., 2010), as RARγ has been shown to efficiently bind and activate the *CYP26A1* promoter RAREs in cell assays (Loudig et al., 2005). An interesting aspect of CYP26A1 induction is that the metabolites generated by CYP26A1, 4-OH-RA and 18-OH-RA and the sequential metabolite 4-oxo-RA that is likely formed by an alcohol dehydrogenase, all also induce *CYP26A1* mRNA expression in liver models (Topletz et al., 2015). This induction can be explained by the

RAR activation by the *at*RA metabolites, and overall suggests that CYP26A1 functions predominantly to degrade active retinoids rather than forming an active metabolite.

In addition to the clear role of exogenously administered *at*RA in inducing CYP26A1 expression, it has also been shown that dietary deficiency of vitamin A in the rat model results in very low *Cyp26a1* expression while *Cyp26a1* is detectable in vitamin A sufficient diet fed rats (Wang et al., 2002). Consistent with the findings of the known induction of CYP26A1 by *at*RA, treating the vitamin A deficient rats with *at*RA significantly induced *Cyp26a1* expression in the liver, lung, intestine and testis. In a follow-up study the authors showed that in rats *Cyp26a1* expression in the liver increased with increased dietary intake of vitamin A as well as with age, so that vitamin A supplemented old rats had the highest liver *Cyp26a1* expression (Yamamoto et al., 2002). Based on these studies the authors concluded that the CYP26A1 expression level in the liver is defined largely by the dietary vitamin A status of the animal. It is interesting that in human livers no correlation between liver vitamin A status and *CYP26A1* mRNA expression level was found (Zhong, Kirkwood, et al., 2019), a finding somewhat in contrast to the work in rats. This could be due to the fact that the liver donors were all from the United States where vitamin A deficiency in humans is very rare, or due to other environmental factors and disease processes in human populations that contribute to regulation of CYP26A1 expression in the liver. One such possible disease process is inflammation. Interestingly, while *at*RA greatly induces CYP26A1 expression in the rat liver, lipopolysaccharide (LPS) treatment in mice as a model of inflammation was shown to antagonize this induction and essentially oppose the induction of CYP26A1 by *at*RA (Zolfaghari et al., 2007). However, in the absence of exogenous *at*RA treatment, LPS had no effect on CYP26A1 expression.

CYP26B1 has also been shown to be inducible by *at*RA in multiple studies. Upon the discovery of CYP26B1, it was shown to be inducible by *at*RA in MCF-7 cells and to a much lesser degree in HeLa and HPK1a-ras cells (White et al., 2000). In vivo in rats, *Cyp26b1* was shown to be inducible in the lungs by *at*RA treatment as well as by treatment with a synthetic RARα agonist AM580 (Wu and Ross, 2010). Notably, in the neonatal rat lung the induction magnitude of *Cyp26b1* was greater than that of *Cyp26a1* by *at*RA treatment (Wu and Ross, 2010), a finding in contrast to the typically lower magnitude of induction of *CYP26B1* gene expression observed in human liver (Zolfaghari et al., 2007; Tay et al., 2010). In the neuronal cell line SH-SY5Y, *CYP26A1* and *CYP26B1* were both induced upon *at*RA treatment but the response in *CYP26B1* gene was more robust and less variable than *CYP26A1* (Stoney et al., 2016). In immortalized lymphoblast cells from human subjects, *CYP26B1* was shown to be highly inducible by *at*RA treatment while *CYP26A1* was not (Wen et al., 2013). This finding is in support of the predominant role of CYP26B1 in the regulation of T-cell differentiation and activation and in modulating immune responses. Similar findings were made in naïve CD4+ mouse T-cells in which RA significantly induced *Cyp26b1* expression but not *Cyp26a1* or *Cyp26c1* (Takeuchi et al., 2011). An interesting aspect of the regulation of Cyp26b1 in T-cells is the finding of the cross-talk of cytokine mediated regulation of *Cyp26b1* expression and *at*RA mediated induction of *Cyp26b1*. In naive CD4+ T-cells TGFβ and IL-12 were shown to significantly downregulate *Cyp26b1* expression even in the presence of *at*RA while IL-4 and TNF-α significantly induced *Cyp26b1* expression in the T-cells (Takeuchi et al., 2011). This is notable as the authors

hypothesized that the attenuation of Cyp26b1 in T-cells by TGFβ may function to protect the *at*RA signaling in T-cells and leading to naïve T-cell differentiation to iT_{reg} cells. On the other hand, one may hypothesize that the induction of Cyp26b1 by IL-4 and TNF-α functions to decrease *at*RA signaling and hence decreases IL-17 production and TH17 cell differentiation.

In contrast to CYP26A1, until now no RARE has been identified in the promoter of *CYP26B1* although CYP26B1 responds to increased *at*RA concentrations. As the expression pattern of CYP26A1 and CYP26B1 is very different, one can hypothesize that different endogenous mechanisms regulate the expression of these two enzymes. One such mechanism could be PPARγ mediated induction of CYP26B1 as synthetic PPARγ agonists were shown to significantly induce *CYP26B1* expression in HepG2 cells and this induction could be attenuated by PPARγ antagonist (Tay et al., 2010). However, it is also likely that various endogenous regulatory pathways that are not linked to RAR mediated gene activation contribute to the regulation of CYP26 expression. In studies of vitamin A deficient quail embryos in which retinoid concentrations cannot be detected, the expression of *Cyp26a1*, *Cyp26b1* and *Cyp26c1* was unaffected in some developing organs but absent in others, suggesting that multiple regulatory mechanisms are at play in modulating the expression patterns of these enzymes in the developing embryo (Reijntjes et al., 2004). In particular the gene expression of the Cyp26 enzymes in neural tissue (*Cyp26a1*) in the head (*Cyp26b1*) and neural crest and hindbrain (*Cyp26c1*) was unaffected by vitamin A deficiency while the gene expression of the Cyp26 enzymes in the trunk, vasculature and heart (*Cyp26b1*), in the tail bud and dorsal neural tube (*Cyp26a1*) and in the rhombomeres (*Cyp26c1*) was dependent of presence of *at*RA (Reijntjes et al., 2004). Taken together these findings suggest that much more work is needed to characterize the regulatory pathways that control CYP26A1 and CYP26B1 gene and protein expression patterns and hence retinoid signaling in different tissues and cell types and in various human pathogenic conditions.

5. Genetic variants in CYP26s and their consequences in humans

CYP26 enzymes are critical for appropriate embryonic development and organogenesis, and therefore, loss-of-function mutations in CYP26A1 or CYP26B1 will likely manifest themselves even in heterozygous individuals with some malformations or developmental abnormalities. One may also predict based on mouse models, that homozygous loss-of-function mutations in CYP26A1 or CYP26B1 will lead to early embryonic death and loss of the pregnancies. Indeed, one case that had homozygous loss of function mutation (Arg363Leu) of CYP26B1 was identified (Table 2) after in utero death (Laue et al., 2011). This case manifested severe craniofacial malformations, skeletal defects and encephalocele as would be expected from the *Cyp26b1* knock out mouse models. Subsequently two siblings from the same family were characterized. Both of these siblings were also homozygous for the loss-of-function mutation (Arg363Leu) of CYP26B1 (Laue et al., 2011) and had occipital encephaloceles and shortened upper and lower limbs. Both of these pregnancies were terminated due to the developmental defects (Laue et al., 2011). The authors sequenced an additional cohort of subjects with malformations of the skull and discovered another CYP26B1 variant, Ser146Pro substitution (Table 2), in an individual with diagnosis of Antley-Bixler syndrome. Via cell transfection assays this mutation was shown

to lack *at*RA metabolism activity and this individual was a homozygous carrier of the CYP26B1 mutation (Laue et al., 2011). The defects observed were consistent with the phenotype described in mice and zebrafish after loss of CYP26B1 activity. In another study, a subject was identified with a homozygous genetic variant in CYP26B1 which predicts a Gly435Ser (Table 2) mutation (Morton et al., 2016). This subject was diagnosed with Antley-Bixler syndrome and showed craniosynsotosis, craniofacial abnormalities and other developmental defects and had survived to adulthood (Morton et al., 2016). However, the functional effect of this CYP26B1 genetic variant was not experimentally characterized and it is possible that this mutation only causes reduced activity rather than complete loss of activity allowing this subject to survive to adulthood with the homozygous mutation.

Apparent loss-of-function mutations in CYP26C1 have been identified in humans (Table 2), and even homozygous carriers of loss-of-function CYP26C1 mutations were viable as might be predicted from mouse model (Slavotinek et al., 2013). The CYP26C1 loss-of-function genetic variants were discovered through siblings who had a rare syndrome of focal facial dermal dysplasia Type IV. The siblings carried two different loss-of-function mutations, one in which seven base pairs of the *CYP26C1* gene were duplicated leading to a missense mutation, and second that resulted in Arg478His mutation (Table 2) (Slavotinek et al., 2013). Both of these variants were found to produce a CYP26C1 protein that had no activity towards *at*RA metabolism (Slavotinek et al., 2013). Notably, the *CYP26C1* gene from four other unrelated patients with focal facial dermal dysplasia Type IV were sequenced and three were found to be homozygous for the seven base pair duplication mutation suggesting that loss-of-function mutations in CYP26C1 cause focal facial dermal dysplasia Type IV. In comparison Type II or III facial dermal dysplasia patients did not carry the CYP26C1 mutation. Surprisingly, 0.3% of healthy individuals also carried the CYP26C1 mutation suggesting that this mutation can be non-penetrant (Slavotinek et al., 2013). Nevertheless, this finding of clear causal relationship between the CYP26C1 mutation and focal facial dermal dysplasia Type IV is in agreement with the known role of *at*RA in maintaining epithelia, especially the skin. These studies suggest that better understanding of CYP26C1 function in humans is needed.

Several hemizygous microdeletions of CYP26 enzymes have been identified in humans that led to variety of developmental abnormalities. An 8.3 megabase microdeletion in chromosome 10 led to loss of *CYP26A1* and *CYP26C1* together with 77 other genes (Nilsson et al., 2016). The subject with the microdeletion had advanced bone age and skeletal and dental development along with retinopathy, microcephaly and developmental delay (Nilsson et al., 2016). The accelerated bone development was interpreted to be due to the hemizygous loss of CYP26A1 and CYP26C1 expression and elevated serum total RA (Nilsson et al., 2016). However, it is possible that RBP4, the retinol carrier protein also plays a role in this phenotype as the microdeletion also included RBP4. This was not considered likely though as the subject lacked other characteristic symptoms and phenotype that are expected to be observed in individuals with RBP4 mutations. Another microdeletion of about 300kb in chromosome 10 including *CYP26A1* and *CYP26C1* was identified in twins with optic nerve aplasia and blindness (Meire et al., 2011). Surprisingly, these twins with haploinsufficiency of *CYP26A1* and *CYP26C1* had no other developmental defects except the optic nerve aplasia. These twins were studied at the age of 3 years when their bone

maturation and growth was as expected for age. It is possible that accelerated bone development may develop later in their childhood as observed in the other case. Two cases with a microdeletion in chromosome 2 have also been identified (Wen et al., 2013). Analysis of the overlapping region of the microdeletion in the two individuals showed that both had hemizygous loss of *CYP26B1* together with *EXOC6B* gene (Wen et al., 2013). The two subjects showed cognitive developmental delay and effects on craniofacies development, consistent with diminished function and activity of CYP26B1 (Wen et al., 2013). In addition in one subject, the lymphocytes collected and cultured from this subject were shown not to respond to *at*RA treatment while healthy control lymphocytes showed robust induction of *CYP26B1* in response to *at*RA (Wen et al., 2013). The phenotype in the two cases was believed to be due to the haploinsufficiency of *CYP26B1*.

Multiple genetic variants have been identified in *CYP26A1* (Table 2), of which some result in amino acid changes in the CYP26A1 protein (Deak et al., 2005; Rat et al., 2006; Lee et al., 2007; Wu et al., 2015). In a study that sequenced the *CYP26A1* gene from 92 human participants from different races, 13 genetic variants were identified (Table 2) of which three led to amino acid changes (Lee et al., 2007). These three amino acid changes were designated rs61735552 (R173S, CYP26A1*2), rs1376885914 (F186L, CYP26A1*3) and rs146619916 (C358R, CYP26A1*4) (Lee et al., 2007). Based on the CYP26A1 homology models, none of the amino acid changes identified would be predicted to alter *at*RA binding within the CYP26A1 active site. However, both CYP26A1*3 and CYP26A1*4 variants exhibited significantly lower catalytic rates and metabolite production than the CYP26A1*1 when transfected into COS1 cells (Lee et al., 2007). Interestingly, the formation rate of the different metabolites was also differently affected. 4-oxo-*at*RA and 18-OH-*at*RA formation catalyzed by the CYP26A1*3 protein was decreased to much greater extent than 4-OH-*at*RA formation while the formation of all metabolites catalyzed by the CYP26A1*4 protein appeared to be equally affected. These findings suggest that the F186L variant affects the binding orientation of *at*RA within CYP26A1 active site. No difference in the metabolite formation rates was observed in the CYP26A1*2 protein when compared to CYP26A1*1 in COS cells (Lee et al., 2007). As these genetic variants were each detected in 1-2 individuals (and were not linked) of the 92 participants. Further analyses with larger populations shown in GnomAD database (http://gnomad-old.broadinstitute.org) indicate that the allele frequency of these variants in human populations is less than 0.005. In addition the functional consequences of these genetic variants in their carriers have not been evaluated and the phenotypic consequences and the potential role of these variants in human pathologies remains to be established.

Several studies have explored the potential genetic link between CYP26 genetic variants and neural tube defects. In one study *CYP26A1* gene was sequenced from 40 patients with spina bifida and 40 healthy volunteers (Rat et al., 2006). In this study 7 mutations were identified in the *CYP26A1* gene (Table 2). Of most interest, a genetic variant causing a frameshift and consequently a premature stop codon in *CYP26A1* was identified in a patient with spina bifida. The truncated protein was shown to be inactive via transfection to COS cells. The genetic variant was found to be very rare as it was not present in another 325 individuals (Rat et al., 2006). No other genetic variants with predicted amino acid changes were identified and overall the frequency of the identified sequence variations ranged from

0.6-1.9% in the study. In another study of the role of genetic variants in retinoid metabolizing genes in neural tube defects, a number of variants were identified both in *CYP26A1* and *CYP26B1* (Deak et al., 2005). The genetic variants identified in *CYP26A1* did not cause a change in the amino acid sequence of CYP26A1 protein and only one of them was in the coding region of the protein (Table 2). In comparison, two of the variants identified in the *CYP26B1* gene caused an amino acid change (L264S, G278R) in the CYP26B1 protein (Table 2). However, these variants were only detected in two individuals and their incidence was not different between cases and controls (Deak et al., 2005).

In addition to exploring the role of CYP26 genetic variants in developmental defects, the potential link between genetic variation in *CYP26A1* or *CYP26B1* and various malignancies has also been studied, likely due to the role *at*RA plays in regulating cell cycle and cell proliferation. A genome-wide association study aiming to identify genetic variants that are associated with esophageal squamous cell carcinoma characterized a genetic variant in *CYP26B1* that results in an Arg323Trp change in exon 5 (Table 2) (Chang et al., 2018). This variant was a low frequency variant and was significantly associated (OR 1.82) with esophageal squamous cell carcinoma with an interaction with lifestyle. Smokers and drinkers had a higher OR (2.6) for the risk allele. When the variant was characterized via transfection into KYSE cells, the Arg323Trp mutant was found to have up to 35% higher activity than the wild type enzyme (Chang et al., 2018). Consistent with the presumed role of CYP26B1 in modulating endogenous retinoid homeostasis the authors also discovered that serum *at*RA concentrations were significantly lower in the subjects that were heterozygous for the *CYP26B1* mutation (g.72360331G>A). Another study identified the CYP26B1 L264S variant (Table 2), which also has increased activity, as a candidate single nucleotide polymorphism (SNP) that influences prostate specific antigen levels and as such potentially contributes to prostate cancer (Ge et al., 2014). The associations between known genetic variants in *CYP26A1* and *CYP26B1* and malignant oral disorders was also explored (Wu et al., 2015). The presence of rs4411227 SNP (C/G and C/C) was significantly higher in the group of subjects with oral and pharyngeal cancer despite the lack of change in protein sequence in the presence of this SNP. Similarly, carriers of the rs9309462 (C/T) mutation in *CYP26B1* were significantly more likely to have oral and pharyngeal cancer (Wu et al., 2015). Interestingly, the study also showed that oral cancers had lower expression of *CYP26A1* and *CYP26B1* mRNA than the noncancerous adjacent tissue.

The L264S SNP in CYP26B1 has been evaluated in a number of studies (Krivospitskaya et al., 2012; Fransen et al., 2013; Ge et al., 2014) and the allele frequency is 0.17 based on GnomAD database (http://gnomad-old.broadinstitute.org). This amino acid change has been shown to increase the activity of CYP26B1 via transfection into COS-1 cells and macrophage-like THP-1 cells resulting in decreased *at*RA concentrations (Krivospitskaya et al., 2012). Interestingly, it was found that in the Stockholm Coronary Atherosclerosis Risk Factor subgroup, presence of this *CYP26B1* variant was associated with slightly larger atherosclerotic lesions. The authors also found that *CYP26B1* mRNA was expressed at higher levels in atherosclerotic lesions when compared to atherosclerosis free arteries, and *CYP26B1* expression colocalized with macrophages leading the authors to suggest that CYP26B1 activity may influence development and progression of atherosclerosis (Krivospitskaya et al., 2012). Based on the data, one may hypothesize that *at*RA has a

protective role towards atherosclerosis development. The increase in CYP26B1 activity due to the L264S SNP has also been proposed to affect the development of Crohn's disease and particularly early onset Crohn's disease (Fransen et al., 2013). While the frequency of the wild type allele was higher in the Crohn's disease patients (OR 2.2 confidence interval 1.0-4.7, p=0.03/0.09), no difference in the frequencies of *CYP26B1* genotypes were observed in Ulcerative colitis and Inflammatory Bowel Disease patients when compared to controls. However, the wild-type allele was found to be significantly associated with diagnosis of Chrohn's disease at young age (Fransen et al., 2013). Based on the expression and importance of CYP26B1 in Th17 and iT_{reg} cell activation it is likely that the higher activity L264S CYP26B1 protein depletes *at*RA in the T-cells modulating inflammatory pathways and potentially making carriers less prone to Chron's disease (Fransen et al., 2013). Based on these studies this increased activity CYP26B1 SNP seems to be relatively common and further studies are needed to establish its role in human health and in overall retinoid homeostasis in various tissues.

6. Role of CYP26 Enzymes in the Pharmacokinetics of Exogenous *at*RA and 13-*cis*RA

Retinoids are commonly used in clinical practice for variety of indications primarily in oncology and dermatology (Leyden, 2003; Altucci et al., 2007; Khalil et al., 2017; Smith and Foster, 2018). *at*RA is one of the cornerstones of treatment of APL and is the standard of care in combination with arsenic trioxide in the treatment of patients with APL (Watts and Tallman, 2014; Osman et al., 2018). *at*RA has also been explored for the treatment of various solid tumors but it has not been approved for other indications except APL. 13-*cis*RA has been used both topically and systemically for over 30 years for the treatment of acne (Leyden, 2003) and neuroblastoma (Reynolds et al., 2003; Masetti et al., 2012; Matthay, 2013). 13-*cis*RA has also been recently investigated for the treatment of male infertility with promising results (Amory et al., 2017), but further studies are needed to define its efficacy for this indication. It is noteworthy that increased sperm counts were noted after 13-*cis*RA treatment in the early trials of this retinoid as acne treatment and the finding of increased sperm counts in men receiving retinoid therapy is consistent with the role of RA in spermatogenesis (Amory et al., 2017).

Despite their close structural similarity (*cis-trans* isomers), *at*RA and 13-*cis*RA have very different pharmacokinetics when administered as drugs. In humans, considerable interconversion between *at*RA and 13-*cis*RA is observed, and after treatment with either isomer the circulating concentrations of both isomers are increased significantly (Muindi et al., 2008; Stevison et al.,2019). The oral clearance of *at*RA is about 10- fold greater than that of 13-*cis*RA, based on the 10-fold higher area under the plasma concentration-time curve of 13-*cis*RA in comparison to *at*RA after similar doses of each isomer (Muindi et al., 2008). This much higher apparent oral clearance of *at*RA can likely be explained by the higher intrinsic clearance of *at*RA by CYP26 enzymes in comparison to 13-*cis*RA. Overall the clinical data on the pharmacokinetics of *at*RA and 13-*cis*RA support a predominant role of CYP26A1 in *at*RA clearance (Jing et al., 2017) while other CYP enzymes such as CYP2C8, CYP2B6 and CYP3A4 (Marill et al., 2002) and UGT enzymes likely are the main

contributors to 13-*cis*RA clearance. However, no studies have assessed the hepatic or extrahepatic contribution of CYP26 enzymes to 13-*cis*RA clearance.

The oral clearance of *at*RA decreases with increasing doses and this decrease has been explained by classic saturation kinetics of metabolic enzymes, particularly CYP26A1, which leads to increased oral bioavailability and decreased systemic clearance (Jing et al., 2017). In contrast, 13-*cis*RA disposition is believed to be dose linear and free of clear saturation kinetics, again supporting the hypothesis that 13-*cis*RA clearance is not largely dependent on CYP26 enzymes. It should, however, be emphasized that formal dose escalation or single dose-multiple dose comparison studies that would confirm linear kinetics of 13-*cis*RA have not been conducted (*vide infra*). Similarly, a key characteristic of *at*RA kinetics in humans is the autoinduction of *at*RA clearance after repeated dosing (Muindi et al., 1992). This autoinduction is commonly attributed and can be quantitatively modelled and predicted to be due to autoinduction of liver CYP26A1 by *at*RA (Jing et al., 2017). This autoinduction can limit the efficacy of *at*RA therapy and lead to therapy resistance. Therefore several research groups have worked to develop inhibitors of CYP26 or RAMBAs to combat this resistance (Nelson et al., 2013). No RAMBA has been approved for treatment though. In comparison to *at*RA, the clearance of 13-*cis*RA has not been reported to be subject to autoinduction although comparison of pharmacokinetic data obtained from single dose and multiple dose of 13-*cis*RA suggests that 13-*cis*RA does not accumulate after multiple dosing to as great of an extent as would be expected from single dose data (Muindi et al., 2008). In human hepatocytes and in vivo in mice, 13-*cis*RA and its metabolite 4-oxo-13-*cis*RA induced the mRNA expression of *CYP26A1* (and *RARβ*) significantly (Stevison et al., 2019) demonstrating the transcriptional regulation of *RAR* target genes by these two 13-*cis*-retinoids. Based on the *in vitro* and *in vivo* data and the fact that after repeated 13-*cis*RA administration 4-oxo-13-*cis*RA circulates at much higher concentrations than 13-*cis*RA or *at*RA, it is likely that some underlying induction of 13-*cis*RA clearance by CYP26A1 occurs upon multiple dosing of 13-*cis*RA. Such induction could also be implied from the 2-4-fold higher 4-oxo-13-*cis*RA to 13-*cis*RA ratio on multiple dosing when compared to single dose. However, even if CYP26 induction occurs after 13-*cis*RA treatment, this induction does not appear to lead to therapy resistance or contribute to lack of efficacy, possibly due to the high exposure to the active metabolite 4-oxo-13-*cis*RA.

In humans *at*RA has much shorter half-life (~ 1 hour) than 13-*cis*RA (~20 hours) likely due to the higher clearance of *at*RA but also largely due to a higher volume of distribution of 13-*cis*RA in comparison to *at*RA shown in animal species. The different distribution kinetics of *at*RA and 13-*cis*RA are quite surprising as their physicochemical parameters (logP, pKa etc) are not different, neither do the two isomers differ significantly in their plasma protein binding (Thatcher and Isoherranen, 2009). The differences in the distribution volumes would therefore be expected to be either due to the reversible isomerization kinetics that may manifest as distribution phenomena or due to specific binding of *at*RA and 13-*cis*RA to CRABPs and FABPs that may impact tissue binding kinetics.

An unusual characteristic of the kinetics of *at*RA is the disposition of *at*RA as a metabolite of 13-*cis*RA. After administration of 13*cis*RA, the half-life of *at*RA is the same as 13-*cis*RA, and longer than *at*RA half-life after administration of *at*RA as a drug, consistent with what

would be expected by conventional formation-rate limited metabolite kinetics. This longer half-life of *at*RA after 13-*cis*RA administration has also prompted the hypothesis that 13-*cis*RA acts as a prodrug or a drug delivery system for *at*RA. In contrast, unlike one would expect from metabolite kinetic theory, the half-life of 13-*cis*RA after administration of *at*RA is shorter than the half-life of 13-*cis*RA after administration of 13-*cis*RA. The mechanism of this unusual kinetic behavior has remained unexplained but with advances in kinetic modeling and better understanding of the elimination pathways of RA isomers the mechanisms could be explored *in silico*.

7. Conclusions and future directions

After the original cloning of CYP26A1 from zebrafish fins, considerable progress has been made over the past 15 years in characterizing the role of the CYP26 enzymes in the metabolism of endogenous and exogenous *at*RA. The CYP enzymes have now been expressed in recombinant systems and the kinetics of *at*RA oxidation and interactions with CRABPs characterized. At present there is no question that CYP26 enzymes are critical in regulating retinoid homeostasis in various tissues during development and in adult life. Yet, many questions regarding this important family of CYP enzymes remain unanswered. For example, the quantitative role of the CYP26 enzymes in human tissues in clearing *at*RA as well as its isomers is still not well understood, and the consequences of specific inhibition, downregulation or induction of the individual CYP26 enzymes to retinoid homeostasis cannot be easily predicted. Furthermore, the clear role of each of human CYP26 enzyme in specific tissues is still an area open for investigation and promises to provide important data in the future. Further, existing data suggests that genetic variants in these enzymes may play a role in development of human disease and in particular in rare diseases. Hence, more research is needed to define the role of CYP26 genetic variation in humans.

Due to the lack of crystal structures of CYP26 enzymes and the lack of any close homologs of the enzymes in this family, very little is known about their structural characteristics and the reasons why their low sequence homology translates to nearly identical activity as *at*RA hydroxylases. The lack of structural information also limits the potential of designing and synthesizing CYP26 inhibitors for clinical use. Furthermore, none of the functional changes to CYP26 activity due to human genetic polymorphisms have been explained via structures of the CYP26s. Finally, some of the data presented in this review strongly suggest that CYP26 enzymes may also metabolize synthetic retinoids such as tazarotenic acid. However, no systemic studies have been undertaken to determine whether CYP26A1 and CYP26B1 that are expressed in the skin contribute to the concentrations and exposure of synthetic retinoids used topically or systemically. The metabolism of such therapeutic agents at the site of action may impact their efficacy and potentially even contribute to therapy resistance. Overall, further research is needed to characterize the role of CYP26 enzymes in the clearance of xenobiotics and exogenously administered *at*RA and 13-*cis*RA.

Abbreviations:

APL	acute promyelocytic leukemia

***at*RA**	all-trans retinoic acid
CYP	cytochrome P450
13-*cis*RA	13-*cis*-retinoic acid
ALDH	aldehyde dehydrogenase
REH	retinyl ester hydrolase
LRAT	lecithin retinol acyltransferase
RDH	retinaldehyde dehydrogenase
AO	aldehyde oxidase
ADH	alcohol dehydrogenase
CRBP	cellular retinol binding protein
CRABP	cellular retinoic acid binding protein
RAMBA	retinoic acid metabolism blocking agent
RBP	retinol binding protein

Reference List

Abu-Abed S, Dolle P, Metzger D, Beckett B, Chambon P, and Petkovich M (2001) The retinoic acid-metabolizing enzyme, CYP26A1, is essential for normal hindbrain patterning, vertebral identity, and development of posterior structures. Genes Dev 15:226–240. [PubMed: 11157778]

Abu-Abed S, MacLean G, Fraulob V, Chambon P, Petkovich M, and Dolle P (2002) Differential expression of the retinoic acid-metabolizing enzymes CYP26A1 and CYP26B1 during murine organogenesis. Mech Dev 110:173–177. [PubMed: 11744378]

Albalat R, and Cañestro C (2009) Identification of Aldh1a, Cyp26 and RAR orthologs in protostomes pushes back the retinoic acid genetic machinery in evolutionary time to the bilaterian ancestor. Chem Biol Interact 178:188–196. [PubMed: 18926806]

Alder A, Bigler P, Werck-Reichhart D, and Al-Babili S (2009) In vitro characterization of synechocystis cyp120a1 revealed the first nonanimal retinoic acid hydroxylase. FEBS J 276:5416–5431. [PubMed: 19703230]

Alonso S, Jones RJ, and Ghiaur G (2017) Retinoic acid, CYP26, and drug resistance in the stem cell niche Exp Hematol 54:17–25, Elsevier Inc. [PubMed: 28754309]

Altucci L, Leibowitz MD, Ogilvie KM, de Lera AR, and Gronemeyer H (2007) RAR and RXR modulation in cancer and metabolic disease. Nat Rev Drug Discov 6:793–810. [PubMed: 17906642]

Amengual J, Golczak M, Palczewski K, and Von Lintig J (2012) Lecithin: Retinol acyltransferase is critical for cellular uptake of vitamin A from serum retinol-binding protein. J Biol Chem 287:24216–24227. [PubMed: 22637576]

Amory JK, Ostrowski KA, Gannon JR, Berkseth K, Stevison F, Isoherranen N, Muller CH, and Walsh T (2017) Isotretinoin administration improves sperm production in men with infertility from oligoasthenozoospermia: a pilot study. Andrology 5:1115–1123. [PubMed: 28980413]

Arnold SL, Kent T, Hogarth CA, Schlatt S, Prasad B, Haenisch M, Walsh T, Muller CH, Griswold MD, Amory JK, and Isoherranen N (2015) Importance of ALDH1A enzymes in determining human testicular retinoic acid concentrations. J Lipid Res 56:342–357. [PubMed: 25502770]

Arnold SL, Stevison F, and Isoherranen N (2016) Impact of Sample Matrix on Accuracy of Peptide Quantification: Assessment of Calibrator and Internal Standard Selection and Method Validation. Anal Chem 88:746–53. [PubMed: 26606514]

Arnold SLM, Kent T, Hogarth CA, Griswold MD, Amory JK, and Isoherranen N (2015) Pharmacological inhibition of ALDH1A in mice decreases all-trans retinoic acid concentrations in a tissue specific manner Biochem Pharmacol 95:111–192, Elsevier Inc.

Awadalla MKA, Alshammari TM, Eriksson LA, and Saenz-Méndez P (2016) Improved homology model of the human all-trans retinoic acid metabolizing enzyme CYP26A1. Molecules 21:1–13.

Balmer JE, and Blomhoff R (2002) Gene expression regulation by retinoic acid. J Lipid Res 43:1773–1808. [PubMed: 12401878]

Beedle M-T, Stevison F, Zhong G, Topping T, Hogarth C, Isoherranen N, and Griswold MD (2018a) Sources of All-Trans Retinal Oxidation Independent of the Aldehyde Dehydrogenase 1A Isozymes Exist in the Postnatal Testis. Biol Reprod, doi: 10.1093/biolre/ioy200.

Beedle M-T, Stevison F, Zhong G, Topping T, Hogarth C, Isoherranen N, and Griswold MD (2018b) Sources of All-Trans Retinal Oxidation Independent of the Aldehyde Dehydrogenase 1A Isozymes Exist in the Postnatal Testis. Biol Reprod, doi: 10.1093/biolre/ioy200.

Blaner WS, Obunike JC, Kurlandsky SB, Al-Haideri M, Piantedosi R, Deckelbaum RJ, and Goldberg IJ (1994) Lipoprotein lipase hydrolysis of retinyl ester. Possible implications for retinoid uptake by cells. J Biol Chem 269:16559–16565. [PubMed: 8206972]

Blomhoff R, and Blomhoff HK (2006) Overview of retinoid metabolism and function. J Neurobiol 66:606–630. [PubMed: 16688755]

Bowles J, Knight D, Smith C, Wilhelm D, Richman J, Mamiya S, Yashiro K, Chawengsaksophak K, Wilson MJ, Rossant J, Hamada H, and Koopman P (2006) Retinoid Signaling Determines Germ Cell Fate in Mice. Science (80-) 312:596–600.

Bryant JM, Meyer-Ficca ML, Dang VM, Berger SL, and Meyer RG (2013) Separation of spermatogenic cell types using STA-PUT velocity sedimentation. J Vis Exp, doi: 10.3791/50648.

Carvalho JE, Theodosiou M, Chen J, Chevret P, Alvarez S, De Lera AR, Laudet V, Croce JC, and Schubert M (2017) Lineage-specific duplication of amphioxus retinoic acid degrading enzymes (CYP26) resulted in sub-functionalization of patterning and homeostatic roles. BMC Evol Biol 17:1–23, BMC Evolutionary Biology. [PubMed: 28049419]

Chang J, Zhong R, Tian J, Li J, Zhai K, Ke J, Lou J, Chen W, Zhu B, Shen N, Zhang Y, Zhu Y, Gong Y, Yang Y, Zou D, Peng X, Zhang Z, Zhang X, Huang K, Wu T, Wu C, Miao X, and Lin D (2018) Exome-wide analyses identify low-frequency variant in CYP26B1 and additional coding variants associated with esophageal squamous cell carcinoma Nat Genet 50:338–343, Springer US. [PubMed: 29379198]

Chen Y, Clarke OB, Kim J, Stowe S, Kim Y-K, Assur Z, Cavalier M, Godoy-Ruiz R, von Alpen DC, Manzini C, Blaner WS, Frank J, Quadro L, Weber DJ, Shapiro L, Hendrickson WA, and Mancia F (2016) Structure of the STRA6 receptor for retinol uptake. Science (80-)353:aad8266–aad8266.

Chenery A, Burrows K, Antignano F, Underhill TM, Petkovich M, and Zaph C (2013) The Retinoic Acid-Metabolizing Enzyme Cyp26b1 Regulates CD4 T Cell Differentiation and Function. PLoS One 8.

Deak KL, Dickerson ME, Linney E, Enterline DS, George TM, Melvin EC, Graham FL, Siegel DG, Hammock P, Mehltretter L, Bassuk AG, Kessler JA, Gilbert JR, Speer MC, Aben J, Aylsworth A, Powell C, Mackey J, Worley G, Brei T, Buran C, Bodurtha J, Sawin K, Dias MS, Mack P, Meeropol E, Lasarsky N, McLone D, Ito J, Oakes WJ, Walker M, Peterson P, and Iskandar B (2005) Analysis of ALDH1A2, CYP26A1, CYP26B1, CRABP1, and CRABP2 in human neural tube defects suggests a possible association with alleles in ALDH1A2. Birth Defects Res Part A - Clin Mol Teratol 73:868–875. [PubMed: 16237707]

Diaz P, Huang W, Keyari CM, Buttrick B, Price L, Guilloteau N, Tripathy S, Sperandio VG, Fronczek FR, Astruc-Diaz F, and Isoherranen N (2016) Development and Characterization of Novel and Selective Inhibitors of Cytochrome P450 CYP26A1, the Human Liver Retinoic Acid Hydroxylase. J Med Chem 59:2579–2595. [PubMed: 26918322]

Dong D, Ruuska SE, Levinthal DJ, and Noy N (1999) Distinct roles for cellular retinoic acid-binding proteins I and II in regulating signaling by retinoic acid. J Biol Chem 274:23695–23698. [PubMed: 10446126]

Dranse HJ, Sampaio AV, Petkovich M, and Underhill TM (2011) Genetic deletion of Cyp26b1 negatively impacts limb skeletogenesis by inhibiting chondrogenesis. J Cell Sci 124:2723–2734. [PubMed: 21807937]

Duester G (2008) Retinoic acid synthesis and signaling during early organogenesis. Cell 134:921–931. [PubMed: 18805086]

Elmabsout AA, Kumawat A, Saenz-Mendez P, Krivospitskaya O, Savenstrand H, Olofsson PS, Eriksson LA, Strid A, Valen G, Torma H, and Sirsjo A (2012) Cloning and functional studies of a splice variant of CYP26B1 expressed in vascular cells. PLoS One 7:e36839. [PubMed: 22666329]

Emoto Y, Wada H, Okamoto H, Kudo A, and Imai Y (2005) Retinoic acid-metabolizing enzyme Cyp26a1 is essential for determining territories of hindbrain and spinal cord in zebrafish. Dev Biol 278:415–27. [PubMed: 15680360]

Fiorella PD, and Napoli JL (1991) Expression of cellular retinoic acid binding protein (CRABP) in Escherichia coli. Characterization and evidence that holo-CRABP is a substrate in retinoic acid metabolism. J Biol Chem 266:16572–16579. [PubMed: 1653241]

Fiorella PD, and Napoli JL (1994) Microsomal retinoic acid metabolism. Effects of cellular retinoic acid-binding protein (type I) and C18-hydroxylation as an initial step. J Biol Chem 269:10538–10544. [PubMed: 8144640]

Foti RS, Diaz P, and Douguet D (2016) Comparison of the ligand binding site of CYP2C8 with CYP26A1 and CYP26B1: a structural basis for the identification of new inhibitors of the retinoic acid hydroxylases. J Enzyme Inhib Med Chem 6366:1–14.

Foti RS, Isoherranen N, Zelter A, Dickmann LJ, Buttrick BR, Diaz P, and Douguet D (2016) Identification of Tazarotenic Acid as the First Xenobiotic Substrate of Human Retinoic Acid Hydroxylase CYP26A1 and CYP26B1. J Pharmacol Exp Ther 357:281–292. [PubMed: 26937021]

Fransen K, Franzen P, Magnuson A, Elmabsout AA, Nyhlin N, Wickbom A, Curman B, Torkvist L, D'Amato M, Bohr J, Tysk C, Sirsjo A, Halfvarson J, Fransén K, Franzén P, Magnuson A, Elmabsout AA, Nyhlin N, Wickbom A, Curman B, Törkvist L, D'Amato M, Bohr J, Tysk C, Sirsjö A, and Halfvarson J (2013) Polymorphism in the Retinoic Acid Metabolizing Enzyme CYP26B1 and the Development of Crohn's Disease. PLoS One 8:e72739. [PubMed: 23977348]

Fujii H, Sato T, Kaneko S, Gotoh O, Fujii-Kuriyama Y, Osawa K, Kato S, and Hamada H (1997) Metabolic inactivation of retinoic acid by a novel P450 differentially expressed in developing mouse embryos. EMBO J 16:4163–4173. [PubMed: 9250660]

Ge YZ, Xu Z, Xu LW, Yu P, Zhao Y, Xin H, Wu R, Tan SJ, Song Q, Wu JP, Li WC, Zhu JG, and Jia RP (2014) Pathway analysis of genome-wide association study on serum prostate-specific antigen levels Gene 551:86–91, Elsevier B.V. [PubMed: 25168891]

Genchi G, Wang W, Barua A, Bidlack WR, and Olson JA (1996) Formation of β-glucuronides and of β-galacturonides of various retinoids catalyzed by induced and noninduced microsomal UDP-glucuronosyltransferases of rat liver. Biochim Biophys Acta - Gen Subj 1289:284–290.

Ghiaur G, Yegnasubramanian S, Perkins B, Gucwa JL, Gerber JM, and Jones RJ (2013) Regulation of human hematopoietic stem cell self-renewal by the microenvironment's control of retinoic acid signaling. Proc Natl Acad Sci 110:16121–16126. [PubMed: 24043786]

Gomaa MS, Armstrong JL, Bobillon B, Veal GJ, Brancale A, Redfern CPF, and Simons C (2008) Novel azolyl-(phenylmethyl)]aryl/heteroarylamines: Potent CYP26 inhibitors and enhancers of all-trans retinoic acid activity in neuroblastoma cells Bioorg Med Chem 16:8301–8313, Pergamon. [PubMed: 18722776]

Gomaa MS, Yee SW, Milbourne CE, Barbera MC, Simons C, and Brancale A (2006) Homology model of human retinoic acid metabolising enzyme cytochrome P450 26A1 (CYP26A1): active site architecture and ligand binding. J Enzym Inhib Med Chem 21:361–369.

Gu X, Xu F, Song W, Wang X, Hu P, Yang Y, Gao X, and Zhao Q (2006) A novel cytochrome P450, zebrafish Cyp26D1, is involved in metabolism of all-trans retinoic acid. Mol Endocrinol 20:1661–72. [PubMed: 16455818]

Gu X, Xu F, Wang X, Gao X, and Zhao Q (2005) Molecular cloning and expression of a novel CYP26 gene (cyp26d1) during zebrafish early development. Gene Expr Patterns 5:733–739. [PubMed: 15979416]

Hammerling U(2016a) Retinol as electron carrier in redox signaling, a new frontier in vitamin A research. Hepatobiliary Surg Nutr 5:15–28. [PubMed: 26904553]

Hammerling U (2016b) Vitamin A as PKC Co-factor and Regulator of Mitochondrial Energetics, in Sub-cellular biochemistry pp 201–230. [PubMed: 27830506]

Helvig C, Taimi M, Cameron D, Jones G, and Petkovich M (2011) Functional properties and substrate characterization of human CYP26A1, CYP26B1, and CYP26C1 expressed by recombinant baculovirus in insect cells J Pharmacol Toxicol Methods 64:258–263, Elsevier B.V. [PubMed: 21906690]

Hogarth CA, Evans E, Onken J, Kent T, Mitchell D, Petkovich M, and Griswold MD (2015) CYP26 Enzymes Are Necessary Within the Postnatal Seminiferous Epithelium for Normal Murine Spermatogenesis. Biol Reprod 9319. [PubMed: 26040672]

Idres N, Marill J, Flexor MA, and Chabot GG (2002) Activation of retinoic acid receptor-dependent transcription by all-trans-retinoic acid metabolites and isomers. J Biol Chem 277:31491–31498. [PubMed: 12070176]

Jing J, Nelson C, Paik J, Shirasaka Y, Amory JK, and Isoherranen N (2017) Physiologically Based Pharmacokinetic Model of All- *trans* -Retinoic Acid with Application to Cancer Populations and Drug Interactions. J Pharmacol Exp Ther 361:246–258. [PubMed: 28275201]

Karlsson M, Strid A, Sirsjo A, and Eriksson LA (2008) Homology Models and Molecular Modeling of Human Retinoic Acid Metabolizing Enzymes Cytochrome P450 26A1 (CYP26A1) and P450 26B1 (CYP26B1). J Chem Theory Comput 4:1021–1027. [PubMed: 26621242]

Kasimanickam V, and Kasimanickam R(2012) Expression of CYP26b1 and Related Retinoic Acid Signalling Molecules in Young, Peripubertal and Adult Dog Testis. Reprod Domest Anim, doi: 10.1111/j.1439-0531.2012.02125.x.

Kawaguchi R, Yu J, Honda J, Hu J, Whitelegge J, Ping P, Wiita P, Bok D, and Sun H (2007) A membrane receptor for retinol binding protein mediates cellular uptake of vitamin A. Science 315:820–825. [PubMed: 17255476]

Ke N, Baudry J, Makris TM, Schuler MA, and Sligar SG (2005) A retinoic acid binding cytochrome P450: CYP120A1 from Synechocystis sp. PCC 6803. Arch Biochem Biophys 436:110–120. [PubMed: 15752715]

Kedishvili NY (2013) Enzymology of retinoic acid biosynthesis and degradation. J Lipid Res 54:1744–1760. [PubMed: 23630397]

Khalil S, Bardawil T, Stephan C, Darwiche N, Abbas O, Kibbi AG, Nemer G, and Kurban M (2017) Retinoids: a journey from the molecular structures and mechanisms of action to clinical uses in dermatology and adverse effects J Dermatolog Treat 28:684–696, Informa Healthcare USA, Inc. [PubMed: 28318351]

Kipp JL, Golebiowski A, Rodriguez G, Demczuk M, Kilen SM, and Mayo KE (2011) Gene expression profiling reveals Cyp26b1 to be an activin regulated gene involved in ovarian granulosa cell proliferation. Endocrinology 152:303–312. [PubMed: 21084447]

Krivospitskaya O, Elmabsout AA, Sundman E, Soderstrom LA, Ovchinnikova O, Gidlof AC, Scherbak N, Norata GD, Samnegard A, Torma H, Abdel-Halim SM, Jansson JH, Eriksson P, Sirsjo A, and Olofsson PS (2012) A CYP26B1 polymorphism enhances retinoic acid catabolism and may aggravate atherosclerosis. Mol Med 18:712–718. [PubMed: 22415012]

Krüger KA, Blum JW, and Greger DL (2005) Expression of nuclear receptor and target genes in liver and intestine of neonatal calves fed colostrum and vitamin A. J Dairy Sci 88:3971–81. [PubMed: 16230703]

Kudoh T, Wilson SW, and Dawid IB (2002) Distinct roles for Fgf, Wnt and retinoic acid in posteriorizing the neural ectoderm. Development 129:4335–46. [PubMed: 12183385]

Kühnel K, Ke N, Cryle MJ, Sligar SG, Schuler M a., and Schlichting I(2008) Crystal structures of substrate-free and retinoic acid-bound cyanobacterial cytochrome P450 CYP120A1. Biochemistry 47:6552–6559. [PubMed: 18512957]

Kumar S, Sandell LL, Trainor PA, Koentgen F, and Duester G (2012) Alcohol and aldehyde dehydrogenases: retinoid metabolic effects in mouse knockout models. Biochim Biophys Acta 1821:198–205. [PubMed: 21515404]

Kurashima Y, Amiya T, Fujisawa K, Shibata N, Suzuki Y, Kogure Y, Hashimoto E, Otsuka A, Kabashima K, Sato S, Sato T, Kubo M, Akira S, Miyake K, Kunisawa J, and Kiyono H (2014) The Enzyme Cyp26b1 mediates inhibition of mast cell activation by fibroblasts to maintain skin-barrier homeostasis Immunity 40:530–541, Elsevier Inc. [PubMed: 24726878]

Laue K, Janicke M, Plaster N, Sonntag C, and Hammerschmidt M (2008) Restriction of retinoic acid activity by Cyp26b1 is required for proper timing and patterning of osteogenesis during zebrafish development. Development 135:3775–3787. [PubMed: 18927157]

Laue K, Pogoda HM, Daniel PB, Van Haeringen A, Alanay Y, Von Ameln S, Rachwalski M, Morgan T, Gray MJ, Breuning MH, Sawyer GM, Sutherland-Smith AJ, Nikkels PG, Kubisch C, Bloch W, Wollnik B, Hammerschmidt M, and Robertson SP (2011) Craniosynostosis and multiple skeletal anomalies in humans and zebrafish result from a defect in the localized degradation of retinoic acid Am J Hum Genet 89:595–606, The American Society of Human Genetics. [PubMed: 22019272]

Lee CM, Lee BS, Arnold SL, Isoherranen N, and Morgan ET (2014) Nitric oxide and interleukin-1beta stimulate the proteasome-independent degradation of the retinoic acid hydroxylase CYP2C22 in primary rat hepatocytes. J Pharmacol Exp Ther 348:141–152. [PubMed: 24144795]

Lee SJ, Perera L, Coulter SJ, Mohrenweiser HW, Jetten A, and Goldstein JA (2007) The discovery of new coding alleles of human CYP26A1 that are potentially defective in the metabolism of all-trans retinoic acid and their assessment in a recombinant cDNA expression system. Pharmacogenet Genomics 17:169–180. [PubMed: 17460545]

Leyden JJ (2003) A review of the use of combination therapies for the treatment of acne vulgaris J Am Acad Dermatol 49:S200–S210, Mosby. [PubMed: 12963896]

Li H, MacLean G, Cameron D, Clagett-Dame M, and Petkovich M (2009) Cyp26b1 expression in murine Sertoli cells is required to maintain male germ cells in an undifferentiated state during embryogenesis. PLoS One 4:e7501. [PubMed: 19838304]

Loudig O, Babichuk C, White J, Abu-Abed S, Mueller C, and Petkovich M (2000) Cytochrome P450RAI(CYP26) Promoter: A Distinct Composite Retinoic Acid Response Element Underlies the Complex Regulation of Retinoic Acid Metabolism. Mol Endocrinol 14:1483–1497. [PubMed: 10976925]

Loudig O, Maclean GA, Dore NL, Luu L, and Petkovich M (2005) Transcriptional co-operativity between distant retinoic acid response elements in regulation of Cyp26A1 inducibility. Biochem J 392:241–248. [PubMed: 16053444]

Lutz JD, Dixit V, Yeung CK, Dickmann LJ, Zelter A, Thatcher JE, Nelson WL, and Isoherranen N (2009) Expression and functional characterization of cytochrome P450 26A1, a retinoic acid hydroxylase Biochem Pharmacol 77:258–268, Elsevier Inc. [PubMed: 18992717]

MacLean G, Abu-Abed S, Dolle P, Tahayato A, Chambon P, and Petkovich M (2001) Cloning of a novel retinoic-acid metabolizing cytochrome P450, Cyp26B1, and comparative expression analysis with Cyp26A1 during early murine development. Mech Dev 107:195–201. [PubMed: 11520679]

Maclean G, Dollé P, and Petkovich M (2009) Genetic disruption of CYP26B1 severely affects development of neural crest derived head structures, but does not compromise hindbrain patterning. Dev Dyn 238:732–745. [PubMed: 19235731]

MacLean G, Li H, Metzger D, Chambon P, and Petkovich M (2007) Apoptotic extinction of germ cells in testes of Cyp26b1 knockout mice. Endocrinology 148:4560–4567. [PubMed: 17584971]

Maden M (2007) Retinoic acid in the development, regeneration and maintenance of the nervous system. Nat Rev Neurosci 8:755–765. [PubMed: 17882253]

Marill J, Capron CC, Idres N, and Chabot GG (2002) Human cytochrome P450s involved in the metabolism of 9-cis-and 13-cis-retinoic acids. Biochem Pharmacol 63:933–943. [PubMed: 11911845]

Marill J, Cresteil T, Lanotte M, and Chabot GG (2000) Identification of human cytochrome P450s involved in the formation of all-trans-retinoic acid principal metabolites. Mol Pharmacol 58:1341–1348. [PubMed: 11093772]

Masetti R, Biagi C, Zama D, Vendemini F, Martoni A, Morello W, Gasperini P, and Pession A (2012) Retinoids in Pediatric Onco-Hematology: the Model of Acute Promyelocytic Leukemia and Neuroblastoma. Adv Ther 29:747–762. [PubMed: 22941525]

Matthay KK (2013) Targeted isotretinoin in neuroblastoma: Kinetics, genetics, or absorption. Clin Cancer Res 19:311–313. [PubMed: 23209029]

McCaffery PJ, Adams J, Maden M, and Rosa-Molinar E (2003) Too much of a good thing: Retinoic acid as an endogenous regulator of neural differentiation and exogenous teratogen. Eur J Neurosci 18:457–472. [PubMed: 12911743]

Meire F, Delpierre I, Brachet C, Roulez F, Van Nechel C, Depasse F, Christophe C, Menten B, and De Baere E (2011) Nonsyndromic bilateral and unilateral optic nerve aplasia: first familial occurrence and potential implication of CYP26A1 and CYP26C1 genes. Mol Vis 17:2072–9. [PubMed: 21850183]

Millard A, Scanlan DJ, Gallagher C, Marsh A, and Taylor PC (2014) Unexpected evolutionary proximity of eukaryotic and cyanobacterial enzymes responsible for biosynthesis of retinoic acid and its oxidation. Mol Biosyst 10:380–3. [PubMed: 24413378]

Morton JEV, Frentz S, Morgan T, Sutherland-Smith AJ, and Robertson SP (2016) Biallelic mutations in *CYP26B1* : A differential diagnosis for Pfeiffer and Antley-Bixler syndromes. Am J Med Genet Part A 170:2706–2710. [PubMed: 27410456]

Muindi JR, Frankel SR, Huselton C, DeGrazia F, Garland WA, Young CW, and Warrell RP Jr. (1992) Clinical pharmacology of oral all-trans retinoic acid in patients with acute promyelocytic leukemia. Cancer Res 52:2138–2142. [PubMed: 1559217]

Muindi JR, Roth MD, Wise RA, Connett JE, O'Connor GT, Ramsdell JW, Schluger NW, Romkes M, Branch RA, Sciurba FC, and Investigators FS (2008) Pharmacokinetics and metabolism of all-trans- and 13-cis-retinoic acid in pulmonary emphysema patients. J Clin Pharmacol 48:96–107. [PubMed: 18094222]

Napoli JL (1996) Biochemical pathways of retinoid transport, metabolism, and signal transduction. Clin Immunol Immunopathol 80:S52–62. [PubMed: 8811064]

Napoli JL (2017) Cellular retinoid binding-proteins, CRBP, CRABP, FABP5: Effects on retinoid metabolism, function and related diseases Pharmacol Ther 173:19–33, Elsevier Inc. [PubMed: 28132904]

Napoli JL (2012) Physiological insights into all-trans-retinoic acid biosynthesis Biochim Biophys Acta 1821:152–167, Elsevier B.V. [PubMed: 21621639]

Napoli JL, Posch KP, Fiorella PD, and Boerman MH (1991) Physiological occurrence, biosynthesis and metabolism of retinoic acid: evidence for roles of cellular retinol-binding protein (CRBP) and cellular retinoic acid-binding protein (CRABP) in the pathway of retinoic acid homeostasis. Biomed Pharmacother 45:131–143. [PubMed: 1932598]

Nelson CH, Buttrick BR, and Isoherranen N (2013) Therapeutic potential of the inhibition of the retinoic acid hydroxylases CYP26A1 and CYP26B1 by xenobiotics. Curr Top Med Chem 13:1402–1428. [PubMed: 23688132]

Nelson CH, Peng C-C, Lutz JD, Yeung CK, Zelter A, and Isoherranen N (2016) Direct protein-protein interactions and substrate channeling between cellular retinoic acid binding proteins and CYP26B1. FEBS Lett 590:2527–35. [PubMed: 27416800]

Nilsson O, Isoherranen N, Guo M, Lui J, Jee Y, Guttmann-Bauman I, Acerini C, Lee W, Allikmets R, Yanovski J, Dauber A, and Baron J (2016) Accelerated Skeletal Maturation in Disorders of Retinoic Acid Metabolism: A Case Report and Focused Review of the Literature. Horm Metab Res 48:737–744. [PubMed: 27589347]

O'Byrne SM, and Blaner WS (2013) Retinol and retinyl esters: biochemistry and physiology. J Lipid Res 54:1731–1743. [PubMed: 23625372]

Ong DE, Kakkad B, and MacDonald PN (1987) Acyl-CoA-independent esterification of retinol bound to cellular retinol-binding protein (type II) by microsomes from rat small intestine. J Biol Chem 262:2729–2736. [PubMed: 3818619]

Orland MD, Anwar K, Cromley D, Chu C-H, Chen L, Billheimer JT, Hussain MM, and Cheng D (2005) Acyl coenzyme A dependent retinol esterification by acyl coenzyme A:diacylglycerol acyltransferase 1. Biochim Biophys Acta - Mol Cell Biol Lipids 1737:76–82.

Osman AEG, Anderson J, Churpek JE, Christ TN, Curran E, Godley LA, Liu H, Thirman MJ, Odenike T, Stock W, and Larson RA (2018) Treatment of Acute Promyelocytic Leukemia in Adults. J Oncol Pract 14:649–657. [PubMed: 30423270]

Paik J, Haenisch M, Muller CH, Goldstein AS, Arnold S, Isoherranen N, Brabb T, Treuting PM, and Amory JK (2014) Inhibition of Retinoic Acid Biosynthesis by WIN 18,446 Markedly Suppresses Spermatogenesis and Alters Retinoid Metabolism in Mice. J Biol Chem, doi: 10.1074/jbc.M113.540211.

Pautus S, Aboraia AS, Bassett CE, Brancale A, Coogan MP, and Simons C (2009) Design and synthesis of substituted imidazole and triazole *N*-phenylbenzo[*d*]oxazolamine inhibitors of retinoic acid metabolizing enzyme CYP26 J Enzyme Inhib Med Chem 24:487–498, Taylor & Francis. [PubMed: 18608743]

Pennimpede T, Cameron D a, MacLean G a, Li H, Abu-Abed S, and Petkovich M (2010) The role of CYP26 enzymes in defining appropriate retinoic acid exposure during embryogenesis. Birth Defects Res A Clin Mol Teratol 88:883–894. [PubMed: 20842651]

Pirazzi C, Valenti L, Motta BM, Pingitore P, Hedfalk K, Mancina RM, Burza MA, Indiveri C, Ferro Y, Montalcini T, Maglio C, Dongiovanni P, Fargion S, Rametta R, Pujia A, Andersson L, Ghosal S, Levin M, Wiklund O, Iacovino M, Borén J, and Romeo S (2014) PNPLA3 has retinyl-palmitate lipase activity in human hepatic stellate cells. Hum Mol Genet 23:4077–4085. [PubMed: 24670599]

Rat E, Billaut-Laden I, Allorge D, Lo-Guidice JM, Tellier M, Cauffiez C, Jonckheere N, Van Seuningen I, Lhermitte M, Romano A, Guéant JL, and Broly F (2006) Evidence for a functional genetic polymorphism of the human retinoic acid-metabolizing enzyme CYP26A1, an enzyme that may be involved in spina bifida. Birth Defects Res Part A - Clin Mol Teratol 76:491–498. [PubMed: 16933217]

Ray WJ, Bain G, Yao M, and Gottlieb DI (1997) CYP26, a novel mammalian cytochrome P450, is induced by retinoic acid and defines a new family. J Biol Chem 272:18702–18708. [PubMed: 9228041]

Reijntjes S, Gale E, and Maden M (2004) Generating gradients of retinoic acid in the chick embryo: Cyp26C1 expression and a comparative analysis of the Cyp26 enzymes. Dev Dyn 230:509–517. [PubMed: 15188435]

Ren JH, Xiong XQ, Sha Y, Yan MC, Lin B, Wang J, Jing YK, Zhao DM, and Cheng MS (2008) Structure prediction and R115866 binding study of human CYP26A1: homology modelling, fold recognition, molecular docking and MD simulations. Mol Simul 34:337–346.

Reynolds CP, Matthay KK, Villablanca JG, and Maurer BJ (2003) Retinoid therapy of high-risk neuroblastoma. Cancer Lett 197:185–192. [PubMed: 12880980]

Rhinn M, and Dolle P (2012) Retinoic acid signalling during development. Development 139:843–858. [PubMed: 22318625]

Roberts AB, Lamb LC, and Sporn MB (1980) Metabolism of all-trans-retinoic acid in hamster liver microsomes: oxidation of 4-hydroxy- to 4-keto-retinoic acid. Arch Biochem Biophys 199:374–383. [PubMed: 7362233]

Ross AC, and Zolfaghari R (2011) Cytochrome P450s in the regulation of cellular retinoic acid metabolism. Annu Rev Nutr 31:65–87. [PubMed: 21529158]

Sakai Y, Meno C, Fujii H, Nishino J, Shiratori H, Saijoh Y, Rossant J, and Hamada H (2001) The retinoic acid-inactivating enzyme CYP26 is essential for establishing an uneven distribution of retinoic acid along the anterio-posterior axis within the mouse embryo. Genes Dev 213–225. [PubMed: 11157777]

Samokyszyn VM, Gall WE, Zawada G, Freyaldenhoven MA, Chen G, Mackenzie PI, Tephly TR, and Radominska-Pandya A (2000) 4-Hydroxyretinoic acid, a novel substrate for human liver microsomal UDP-glucuronosyltransferase(s) and recombinant UGT2B7. J Biol Chem 275:6908–6914. [PubMed: 10702251]

Sass JO, Forster A, Bock KW, and Nau H (1994) Glucuronidation and isomerization of all-trans- and 13-CIS-retinoic acid by liver microsomes of phenobarbital- or 3-methylcholanthrene-treated rats. Biochem Pharmacol 47:485–492. [PubMed: 8117316]

Schmitt MC, and Ong DE (1993) Expression of Cellular Retinol-Binding Protein and Lecithin-Retinol Acyltransferase in Developing Rat Testis1. Biol Reprod 49:972–979. [PubMed: 8286593]

Shimshoni JA, Roberts AG, Scian M, Topletz AR, Blankert SA, Halpert JR, Nelson WL, and Isoherranen N (2012) Stereoselective formation and metabolism of 4-hydroxy-retinoic Acid enantiomers by cytochrome p450 enzymes. J Biol Chem 287:42223–42232. [PubMed: 23071109]

Sladek NE (2003) Human aldehyde dehydrogenases: potential pathological, pharmacological, and toxicological impact. J Biochem Mol Toxicol 17:7–23. [PubMed: 12616643]

Sládek NE (2003) Human aldehyde dehydrogenases: potential pathological, pharmacological, and toxicological impact. J Biochem Mol Toxicol 17:7–23. [PubMed: 12616643]

Slavotinek AM, Mehrotra P, Nazarenko I, Tang PLF, Lao R, Cameron D, Li B, Chu C, Chou C, Marqueling AL, Yahyavi M, Cordoro K, Frieden I, Glaser T, Prescott T, Morren MA, Devriendt K, Kwok PY, Petkovich M, and Desnick RJ (2013) Focal facial dermal dysplasia, type IV, is caused by mutations in CYP26C1. Hum Mol Genet 22:696–703. [PubMed: 23161670]

Smith V, and Foster J (2018) High-Risk Neuroblastoma Treatment Review Child (Basel, Switzerland) 5, Multidisciplinary Digital Publishing Institute (MDPI).

Spoorendonk KM, Peterson-Maduro J, Renn J, Trowe T, Kranenbarg S, Winkler C, and Schulte-Merker S (2008) Retinoic acid and Cyp26b1 are critical regulators of osteogenesis in the axial skeleton. Development 135:3765–3774. [PubMed: 18927155]

Stevison F, Hogarth C, Tripathy S, Kent T, and Isoherranen N (2017) Inhibition of the all trans-retinoic acid hydroxylases CYP26A1 and CYP26B1 results in dynamic, tissue-specific changes in endogenous atRA signaling. Drug Metab Dispos dmd.117.075341.

Stevison F, Kosaka M, Kenny JR, Wong S, Hogarth C, Amory JK, and Isoherranen N (2019) Does *In Vitro* Cytochrome P450 Downregulation Translate to *In Vivo* Drug- Drug Interactions? Preclinical and Clinical Studies With 13- *cis* - Retinoic Acid. Clin Transl Sci cts.12616.

Stoney PN, Fragoso YD, Saeed RB, Ashton A, Goodman T, Simons C, Gomaa MS, Sementilli A, Sementilli L, Ross AW, Morgan PJ, and McCaffery PJ (2016) Expression of the retinoic acid catabolic enzyme CYP26B1 in the human brain to maintain signaling homeostasis Brain Struct Funct 221:3315–3326, Springer Berlin Heidelberg. [PubMed: 26374207]

Sun B, Song S, Hao C-Z, Huang W-X, Liu C-C, Xie H-L, Lin B, Cheng M-S, and Zhao D-M (2015) Molecular recognition of CYP26A1 binding pockets and structure–activity relationship studies for design of potent and selective retinoic acid metabolism blocking agents. J Mol Graph Model 56:10–19. [PubMed: 25541526]

Swindell EC, Thaller C, Sockanathan S, Petkovich M, Jessell TM, and Eichele G (1999) Complementary domains of retinoic acid production and degradation in the early chick embryo. Dev Biol 216:282–96. [PubMed: 10588879]

Tahayato A, Dollé P, and Petkovich M(2003) Cyp26C1 encodes a novel retinoic acid-metabolizing enzyme expressed in the hindbrain, inner ear, first branchial arch and tooth buds during murine development. Gene Expr Patterns 3:449–54. [PubMed: 12915310]

Taimi M, Helvig C, Wisniewski J, Ramshaw H, White J, Amad M, Korczak B, and Petkovich M (2004) A Novel Human Cytochrome P450, CYP26C1, Involved in Metabolism of 9-cis and All-trans Isomers of Retinoic Acid. J Biol Chem 279:77–85. [PubMed: 14532297]

Takeuchi H, Yokota A, Ohoka Y, and Iwata M(2011) Cyp26b1 regulates retinoic acid-dependent signals in T cells and its expression is inhibited by transforming growth factor-beta. PLoS One 6:e16089. [PubMed: 21249211]

Tay S, Dickmann L, Dixit V, and Isoherranen N (2010) A comparison of the roles of peroxisome proliferator-activated receptor and retinoic acid receptor on CYP26 regulation. Mol Pharmacol 77:218–227. [PubMed: 19884280]

Terao M, Kurosaki M, Barzago MM, Fratelli M, Bagnati R, Bastone A, Giudice C, Scanziani E, Mancuso A, Tiveron C, and Garattini E(2009) Role of the Molybdoflavoenzyme Aldehyde Oxidase Homolog 2 in the Biosynthesis of Retinoic Acid: Generation and Characterization of a Knockout Mouse. Mol Cell Biol 29:357–377. [PubMed: 18981221]

Thatcher JE, Buttrick B, Shaffer SA, Shimshoni JA, Goodlett DR, Nelson WL, and Isoherranen N (2011) Substrate specificity and ligand interactions of CYP26A1, the human liver retinoic acid hydroxylase. Mol Pharmacol 80:228–239. [PubMed: 21521770]

Thatcher JE, and Isoherranen N (2009) The role of CYP26 enzymes in retinoic acid clearance. Expert opin drug metab toxicol 5:875–886. [PubMed: 19519282]

Thatcher JE, Zelter A, and Isoherranen N (2010) The relative importance of CYP26A1 in hepatic clearance of all-trans retinoic acid. Biochem Pharmacol 80:903–912. [PubMed: 20513361]

Topletz AR(2013) The Relative Importance of CYP26A1 and CYP26B1 in Mediating Retinoid Homeostasis: Studies on the Formation, Elimination and Biological Activity of All-trans-Retinoic Acid Metabolites, University of Washington.

Topletz AR, Thatcher JE, Zelter A, Lutz JD, Tay S, Nelson WL, and Isoherranen N (2012) Comparison of the function and expression of CYP26A1 and CYP26B1, the two retinoic acid hydroxylases. Biochem Pharmacol 83:149–163. [PubMed: 22020119]

Topletz AR, Tripathy S, Foti RS, Shimshoni JA, Nelson WL, and Isoherranen N (2015) Induction of CYP26A1 by Metabolites of Retinoic Acid : Evidence That CYP26A1 Is an Important Enzyme in the Elimination of Active Retinoids. Mol Pharmacol 87:430–441. [PubMed: 25492813]

Topletz AR, Zhong G, and Isoherranen N (2019) Scaling in vitro activity of CYP3A7 suggests human fetal livers do not clear retinoic acid entering from maternal circulation. Sci Rep 9:4620. [PubMed: 30874620]

Trofimova-Griffin M, and Juchau MR (1998) Expression of Cytochrome P450RAI (CYP26) Human Fetal Hepatic and Cephalic Tissues. Biochem Biophys Res Commun 491:487–491

Trofimova-griffin ME, Brzezinski MR, and Juchau MR (2000) Patterns of CYP26 expression in human prenatal cephalic and hepatic tissues indicate an important role during early brain development. Dev Brain Res 120:7–16. [PubMed: 10727725]

Trofimova-Griffin ME, and Juchau MR (2002) Developmental expression of cytochrome CYP26B1 (P450RAI-2) in human cephalic tissues. Brain Res Dev Brain Res 136:175–8. [PubMed: 12101034]

Uehara M, Yashiro K, Mamiya S, Nishino J, Chambon P, Dolle P, and Sakai Y (2007) CYP26A1 and CYP26C1 cooperatively regulate anterior-posterior patterning of the developing brain and the production of migratory cranial neural crest cells in the mouse. Dev Biol 302:399–411. [PubMed: 17067568]

Vane F, B CJL, R LC, R M, and Doran T (1990) Human biliary metabolites of isotretinoin: identification, quantification, synthesis and biological activity. Xenobiotica 20:193–207. [PubMed: 2333716]

Vernet N, Dennefeld C, Rochette-Egly C, Oulad-Abdelghani M, Chambon P, Ghyselinck NB, and Mark M (2006) Retinoic acid metabolism and signaling pathways in the adult and developing mouse testis. Endocrinology 147:96–110. [PubMed: 16210368]

Wang Y, Zolfaghari R, and Ross AC (2002) Cloning of rat cytochrome P450RAI (CYP26) cDNA and regulation of its gene expression by all-trans-retinoic acid in vivo. Arch Biochem Biophys 401:235–43. [PubMed: 12054474]

Watts JM, and Tallman MS (2014) Acute promyelocytic leukemia: What is the new standard of care? Blood Rev 28:205–212, Elsevier Ltd. [PubMed: 25107311]

Wen J, Lopes F, Soares G, Farrell SA, Nelson C, Qiao Y, Martell S, Badukke C, Bessa C, Ylstra B, Lewis S, Isoherranen N, Maciel P, and Rajcan-Separovic E (2013) Phenotypic and functional consequences of haploinsufficiency of genes from exocyst and retinoic acid pathway due to a recurrent microdeletion of 2p13.2. Orphanet J Rare Dis 8:100. [PubMed: 23837398]

White J a, Boffa MB, Jones B, and Petkovich M (1994) A zebrafish retinoic acid receptor expressed in the regenerating caudal fin. Development 120:1861–72. [PubMed: 7924993]

White JA, Beckett-Jones B, Guo YD, Dilworth FJ, Bonasoro J, Jones G, and Petkovich M (1997) cDNA cloning of human retinoic acid-metabolizing enzyme (hP450RAI) identifies a novel family of cytochromes P450. J Biol Chem 272:18538–18541. [PubMed: 9228017]

White JA, Guo Y, Baetz K, Beckett-jones B, Bonasoro J, Hsu KE, Dilworth FJ, Jones G, and Petkovich M (1996) Identification of the Retinoic Acid-inducible All- trans- retinoic Acid 4-Hydroxylase. J Biol Chem 271:29922–29927. [PubMed: 8939936]

White JA, Ramshaw H, Taimi M, Stangle W, Zhang A, Everingham S, Creighton S, Tam SP, Jones G, and Petkovich M (2000) Identification of the human cytochrome P450, P450RAI-2, which is predominantly expressed in the adult cerebellum and is responsible for all-trans-retinoic acid metabolism. Proc Natl Acad Sci U S A 97:6403–6408. [PubMed: 10823918]

Wongsiriroj N, Jiang H, Piantedosi R, Yang KJZ, Kluwe J, Schwabe RF, Ginsberg H, Goldberg IJ, and Blaner WS (2014) Genetic dissection of retinoid esterification and accumulation in the liver and adipose tissue. J Lipid Res 55:104–114. [PubMed: 24186946]

Wu L, and Ross AC (2010) Acidic retinoids synergize with vitamin A to enhance retinol uptake and STRA6, LRAT, and CYP26B1 expression in neonatal lung. J Lipid Res 51:378–387. [PubMed: 19700416]

Wu S, Chen Y, Shieh T, Chen C, Wang Y, Lee K, Lin Y, Chien P, and Chen P (2015) Association Study between Novel CYP26 Polymorphisms and the Risk of Betel Quid-Related Malignant Oral Disorders. 2015.

Xi J, and Yang Z (2008) Expression of RALDHs (ALDH1As) and CYP26s in human tissues and during the neural differentiation of P19 embryonal carcinoma stem cell. Gene Expr Patterns 8:438–442. [PubMed: 18502188]

Yamamoto Y, Zolfaghari R, and Ross AC (2002) Regulation of CYP26 (cytochrome P450RAI) mRNA expression and retinoic acid metabolism by retinoids and dietary vitamin A in liver of mice and rats. FASEB J 14:2119–2127.

Yashiro K, Zhao X, Uehara M, Yamashita K, Nishijima M, Nishino J, Saijoh Y, Sakai Y, and Hamada H (2004) Regulation of retinoic acid distribution is required for proximodistal patterning and outgrowth of the developing mouse limb. Dev Cell 6:411–422. [PubMed: 15030763]

Zhang Y, Zolfaghari R, and Ross AC (2010) Multiple retinoic acid response elements cooperate to enhance the inducibility of CYP26A1 gene expression in liver. Gene 464:32–43. [PubMed: 20682464]

Zhao Q, Dobbs-McAuliffe B, and Linney E (2005) Expression of cyp26b1 during zebrafish early development. Gene Expr Patterns 5:363–369. [PubMed: 15661642]

Zhong G, Hogarth C, Snyder J, Palau L, Topping T, Huang W, Czuba L, Lafrance J, Ghiaur G, and Isoherranen N(2019) The retinoic acid hydroxylase Cyp26a1 has minor effects on postnatal vitamin A homeostasis, but is required for exogenous *at* RA clearance. J Biol Chem jbc.RA119.009023.

Zhong G, Kirkwood J, Won K-J, Tjota N, Jeong H-Y, and Isoherranen N (2019) Characterization of Vitamin A Metabolome in Human Livers with and without NAFLD. J Pharmacol Exp Ther jpet. 119.258517.

Zhong G, Ortiz D, Zelter A, Nath A, and Isoherranen N (2018) CYP26C1 Is a Hydroxylase of Multiple Active Retinoids and Interacts with Cellular Retinoic Acid Binding Proteins. Mol Pharmacol 93:489–503. [PubMed: 29476041]

Zolfaghari R, Cifelli CJ, Lieu SO, Chen Q, Li N, and Ross AC (2007) Lipopolysaccharide opposes the induction of CYP26A1 and CYP26B1 gene expression by retinoic acid in the rat liver in vivo. Am J Physiol Liver Physiol 292:G1029–G1036.

Zolfaghari R, Mattie FJ, Wei C-H, Chisholm DR, Whiting A, and Ross AC (2019) CYP26A1 gene promoter is a useful tool for reporting RAR-mediated retinoid activity. Anal Biochem 577:98–109. [PubMed: 31039331]

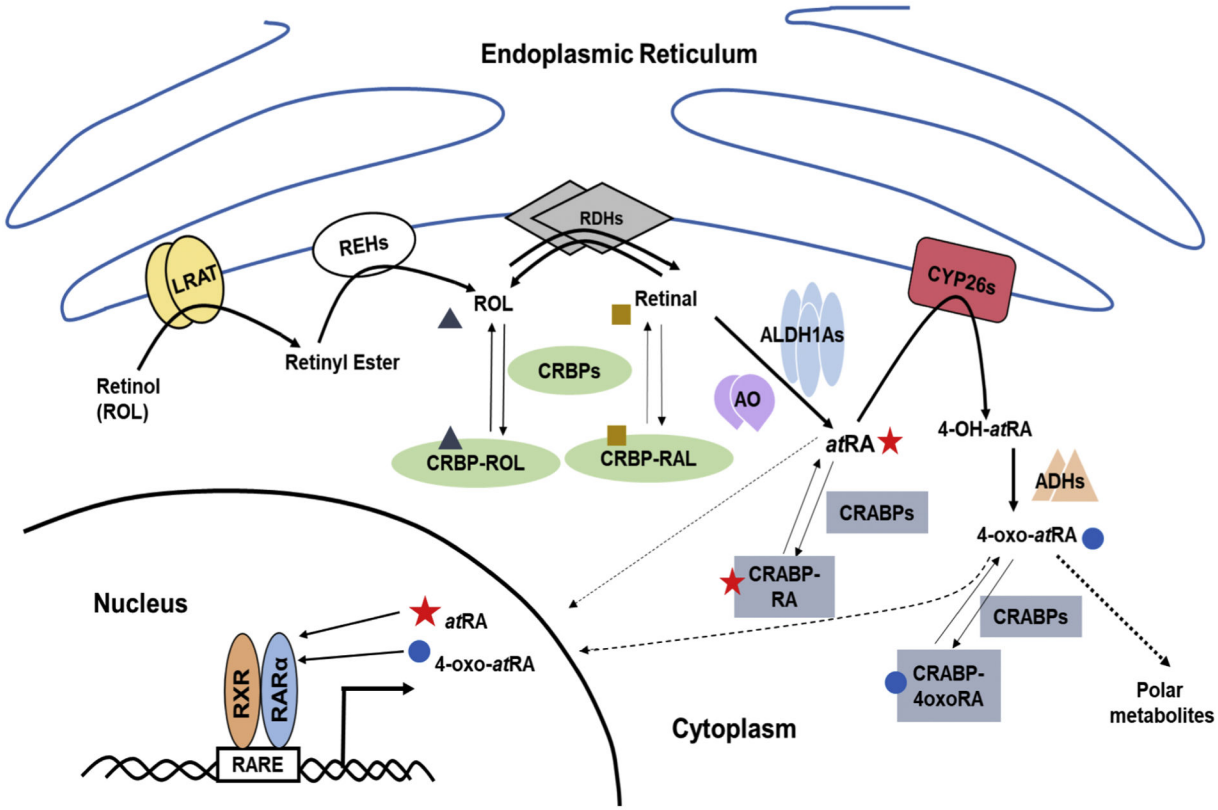

Figure 1.

Metabolic network of vitamin A homeostasis shown with cellular localization of known retinoid metabolizing enzymes. ALDH, aldehyde dehydrogenase; REH, retinyl ester hydrolase; LRAT, lecithin retinol acyltransferase; RDH, retinaldehyde dehydrogenase; AO, aldehyde oxidase; ADH, alcohol dehydrogenase; CRBP, cellular retinol binding protein; CRABP, cellular retinoic acid binding protein. LRAT, RDHs, AO and RDHs function as dimers while ALDH1As function as tetramers and CYP26s as monomers.

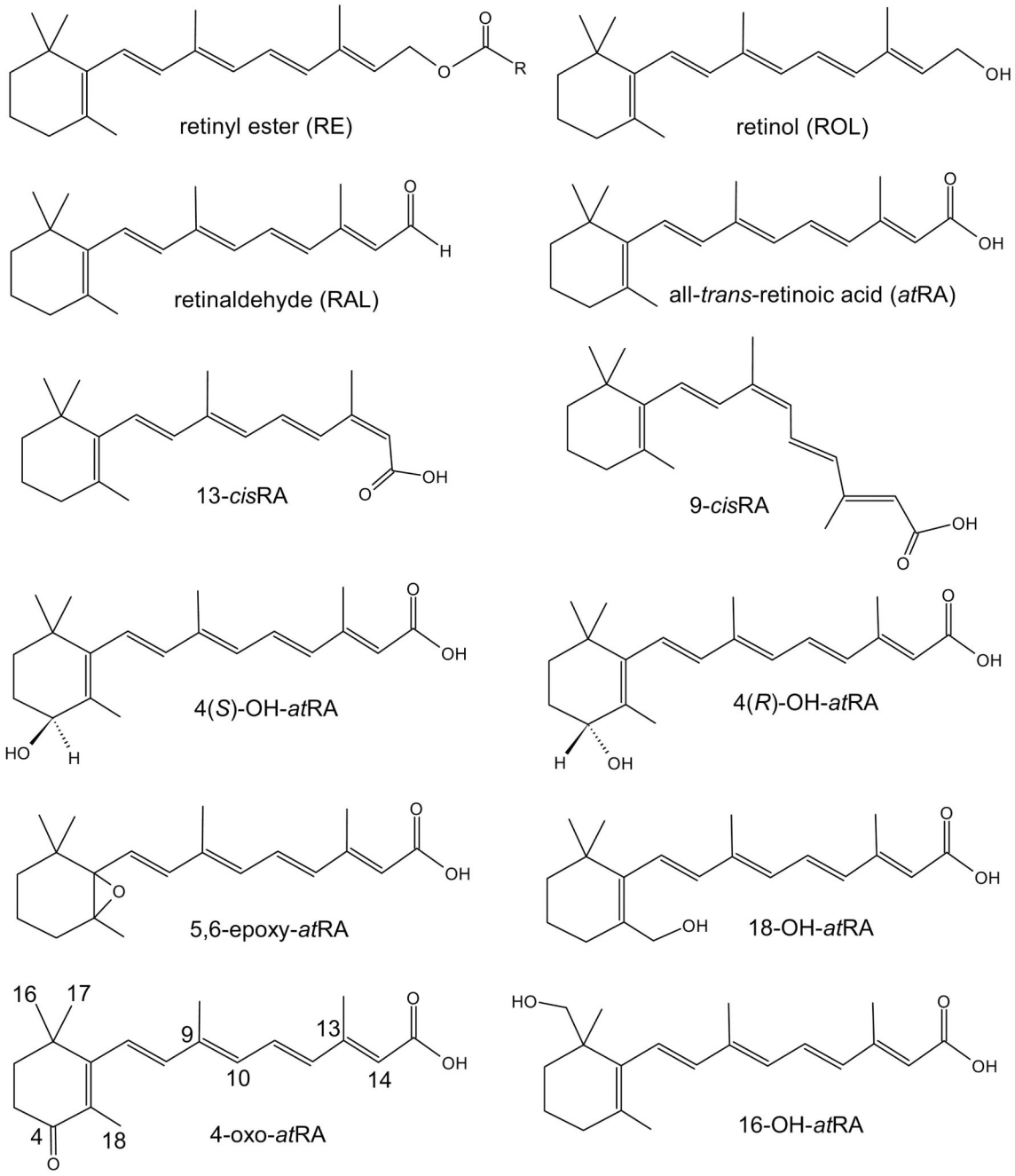

Figure 2.

chemical structures of the key retinoids. Numbers shown on 4-oxo-*at*RA structure represent positions of carbon atoms in all retinoid chemical structures.

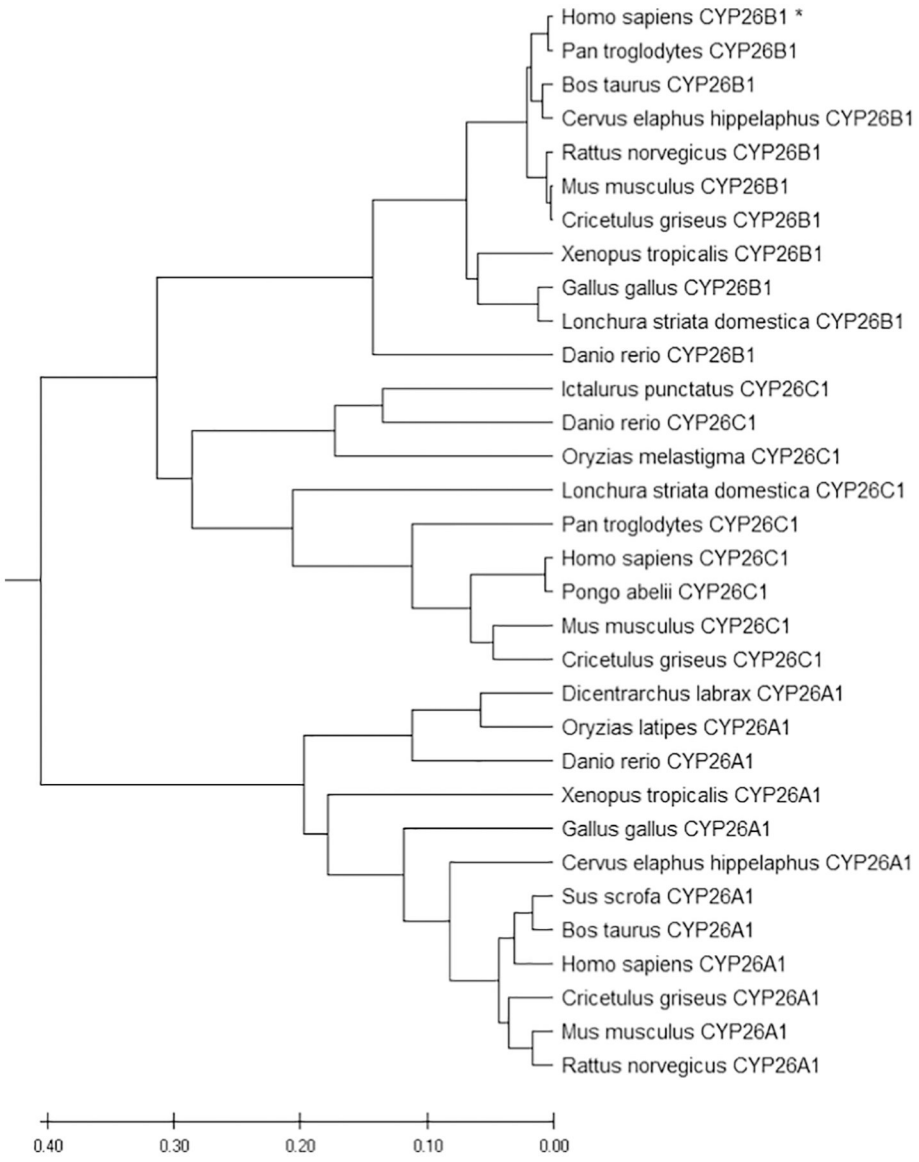

Figure 3.

CYP26 enzyme phylogenetic tree constructed with protein sequences. All CYP26 protein sequences were obtained from NCBI protein data base except that human CYP26B1 (*) protein sequence is from published works (Foti, Isoherranen, et al., 2016). Evolutionary analyses were conducted in MEGA X (Kumar *et al.*, 2018). The evolutionary history was inferred using the UPGMA method (Sneath and Sokal, 1973) and the evolutionary distances were computed using the Poisson correction method (Zuckerkandl and Pauling, 1965). CYP26A1 GeneBank accession numbers: NP_000774.2, NP_031837.2, NP_569092.2, RLQ71010.1, AKO62658.1, AAI49356.1, OWK07689.1, NP_571221.2, AHY95171.1, NP_001265772.1, NP_001001129.1, AAI71087.1; CYP26B1 GeneBank accession numbers: AAH59246.1 NP_851601.1, AAQ82596.1, NP_001179722.1, XP_015141554.1, RLQ56656.1, OWK10279.1 NP_001072655.1, PNI38749.1, OWK56765.1; CYP26C1

GeneBank accession numbers: NP_899230.2, OWK58156.1, AAI51107.1, AGN04289.1, AHH39274.1, PNI82309.1, AAI29132.1, RLQ71011.1, PNJ89455.1.

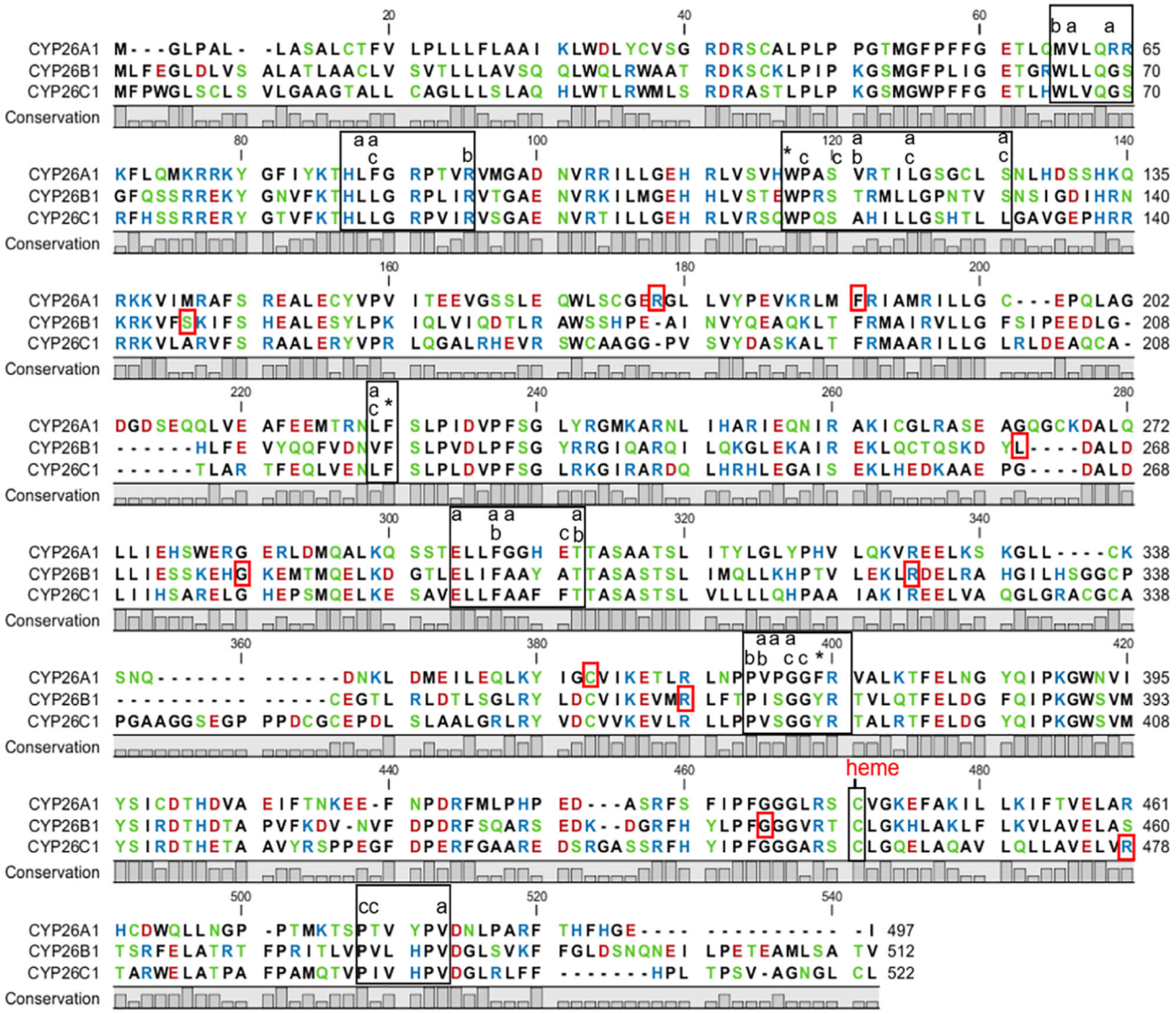

Figure 4.

Protein sequence alignment of human CYP26 enzymes. CYP26A1 (NP_000774.2) and CYP26C1 (NP_899230.2) sequences are from NCBI protein database while CYP26B1 sequence is as previously published work (Foti, Isoherranen, et al., 2016). Hydrophobic residues are shown in black color, hydrophilic residues as green, acidic residues (negatively charged) as red and basic residues (positively charged) as blue. Bar graphs indicate the degree of amino acid conservation. Black boxes indicate sequence regions involved in *at*RA binding based on homology models as indicated in the text. Amino acids proposed to interact with atRA are indicated by symbols a for CYP26A1, b for CYP26B1, c for CYP26C1 and * for all three CYP26 enzymes. Amino acids which can change due to SNPs (Table 1) are marked by red boxes.

Table 1.

Summary of human CYP26 enzyme kinetic parameters

Substrate	k_{cat} (mm^{-1})	K_m (nM)	Cl_{int} (μl/min/pmol)	Measurement	Reference
CYP26A1					
*at*RA	11.4	9.3	1200	Substrate depletion	Lutz et al., 2009
	3.5	21.4	164	4OH-*at*RA formation	
	9.5 ± 2.2	50.1 ± 32.4	190	4OH-*at*RA formation	Topletz et al., 2012
	5.0 ± 0.5	18.9 ± 13.4	102	18OH-*at*RA formation	
9-*cis*RA		134 ± 30		4OH-, 16OH-*at*RA formation	Thatcher et al., 2011
4(*S*)-OH-*at*RA		5.2 ± 1.3	1450 ± 150	Substrate depletion	Topletz dissertation; Shimshoni 2012
4(*R*)-OH-*at*RA		11 ±5.2	740 ± 12	Substrate depletion	
4-oxo-*at*RA		63.0	90 ±9.0	Substrate depletion	Topletz et al., 2015
18-OH-*at*RA		35.8	230 ±43	Substrate depletion	
CYP26B1					
*at*RA	0.81 ± 0.2	18.8 ± 18.7	43	4OH-*at*RA formation	Topletz et al., 2012
	0.45 ± 0.02	64.6 ± 10.3	7.0	4OH-*at*RA formation	Nelson et al., 2016
9-*cis*RA	3.6	555	6.5	4OH-9*cis*RA formation	Diaz et al., 2016
4(*S*)-OH-*at*RA		4.0 ± 2.5	46 ± 18	Substrate depletion	Topletz dissertation; Shimshoni 2012
4(*R*)-OH-*at*RA		31 ± 19	104 ± 19	Substrate depletion	
4-oxo-*at*RA		29 ± 6.6	15 ± 4.0	Substrate depletion	
18-OH-*at*RA		4.8 ± 6.3	33 ± 10	Substrate depletion	
CRABPI-*at*RA	0.17 ± 0.006	21.7 ± 2.9	7.8	4OH-*at*RA formation	Nelson et al., 2016
CRABPII-*at*RA	0.28 ± 0.01	24.3 ± 4.2	11.5	4OH-*at*RA formation	
CYP26C1					
*at*RA	5.9 ± 1.9	50.4 ± 6.2	120 ± 49	4OH-*at*RA formation	Zhong et al., 2018
13-*cis*RA	7.7 ± 2.9	38.2 ± 4.9	208 ± 101	4OH-13*cis*RA formation	
9-*cis*RA	4.7 ± 1.2	9.7 ± 2.6	504 ± 140	4OH-9*cis*RA formation	
9-*cis*RA		5.9 ± 0.3		16OH-9*cis*RA formation	
4-oxo-*at*RA		15.7 ± 4.3	153 ± 20	Substrate depletion	
		10.2 ± 2.2		4oxo-16OH-*at*RA formation	
CRABPI-*at*RA	2.1 ± 0.2	50.8 ± 8.0	41 ± 2	4OH-*at*RA formation	
CRABPII-*at*RA	2.1 ± 0.8	34.1 ± 7.1	66 ± 37	4OH-*at*RA formation	
CRABPI-4oxo*at*RA		3.5 ± 0.5		4oxo-16OH-*at*RA formation	
CRABPII-4oxo*at*RA		5.4 ± 1.5		4oxo-16OH-*at*RA formation	

Note: data are shown as mean ± S.D. or mean values.

Table 2.

Summary of human CYP26 genetic variants reported in the literature. rs numbers are listed where available

Sequence Accession Number	Site	Sequence Variation	rs number	Amino acid change/frame shift	Allele Frequency[b]	References
CYP26A1						
NT_030059.12	Promoter	g.−59G>A		-		Lee et al., 2007
	Promoter	g.−36G>A		-		
	IVS1	g.199G>A		-		
	IVS1	g.330G>A		-		
	IVS1	g.369G>A		-		
	Exon 3	g.947C>A	rs61735552	R173S[a]	0.004	
	Exon 3	g.988C>A	rs1376885914	F186L[a]	4.0e-6	
	IVS4	g.1380G>A		-		
	IVS4	g.1381G>A		-		
	IVS4	g.1884G>A		-		
	Exon 6	g.2682T>C	rs146619916	C358R[a]	1.3e-4	
	IVS6	g.2901G>A		-		
	Exon 7	g.3190G>A		-		
AL358613	5'-flanking region	g.−280G>A		-		Rat et al., 2006
	IVS1	g.199G>C		-		
	IVS1	g.220T>C		-		
	Exon 3	g.877C>G		-		
	IVS4	g.1380_81AG> GA		-		
	Exon 7	g.3067T>C		-		
	Exon 7	g.3116delT		frame shift		
NM_000783	IVS1	g.369C>T		-		Deak et al., 2005
	Exon 2	c.246T>C		-		
	IVS2	g.664T>C		-		
	IVS5	g.1380A>G		-		
	IVS5	g.1381G>A		-		
NC_000010.11		g.93071756C>G	rs4411227	-	0.266	Wu et al., 2015
CYP26B1						
NM_019885	Exon 4	c.791 T>C	rs2241057	L264S[a]	0.170	Deak et al., 2005; Ge et al., 2014; Fransen et al., 2013; Krivospitskaya et al., 2012
NM_019885	Exon 4	c.832 G>A	rs371681434	G278R[a]	6.0e-5	Deak et al.,2005;
	IVS4	g.13099G>T	rs2241058	-	0.165	
	3' UTR	c.1583T>C	rs2241059	-	0.174	
	3' UTR	c.2620C>T	rs10181341	-	0.065	
	3' UTR	c.3440C>G	rs3768647	-	0.180	

Sequence Accession Number	Site	Sequence Variation	rs number	Amino acid change/frame shift	Allele Frequency[b]	References
	3' UTR	c.3559G>A				
	3' UTR	c.3925-3926insTGTGCG				
	3' UTR	c.4268A C	rs887842	-	0.362	
NM_019885		c.1303G>A	rs1211950654	G435S[a]	4.0e-6	Morton et al., 2016
NM_019885.2		c.436T>C	rs281875232	S146P[a]		Laue et al., 2011
		c.1088G>T	rs281875231	R363L[a]		
(NC_000002.11		g.72360331G>A	rs138478634	R323W[a]	0.001	Chang et al., 2018
NC_000002.12		g.72129066A>G	rs887844	-	0.481	Wu et al., 2015
		g.72130326G>C	rs3768647	-	0.180	
		g.72145857T>C	rs9309462	-	0.086	
CYP26C1						
NM_183374.2	Exon 4	c.844_851dupCCATGCA	rs565866662	frame shift	0.002	Slavotinek et al., 2013
	Exon 6	c.1433A>G	rs756334431	R478H[a]	8.5e-5	

Note: Sequence variation numbering was based on the corresponding gene sequence obtained from NCBI accession number with the A of the start codon being +1, and negative numbers representing the upstream region.

[a] amino acids are marked by red boxes in Fig 4.

[b] allele frequency was obtained from GnomAD database.