Biochemical and physiological importance of the CYP26 retinoic acid hydroxylases

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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) it's 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.

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Contents

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 & 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 & 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 & 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 (Fig. 1) (Ong, Kakkad, & MacDonald, 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 & Blaner, 2013). LRAT expression and role in vitamin A storage has also been shown in lung and retina (Amengual, Golczak, Palczewski, & Von Lintig, 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 & 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 &

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 may be 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 & 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 1 μM 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 (Chen et al., 2016; Kawaguchi et al., 2007).

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,
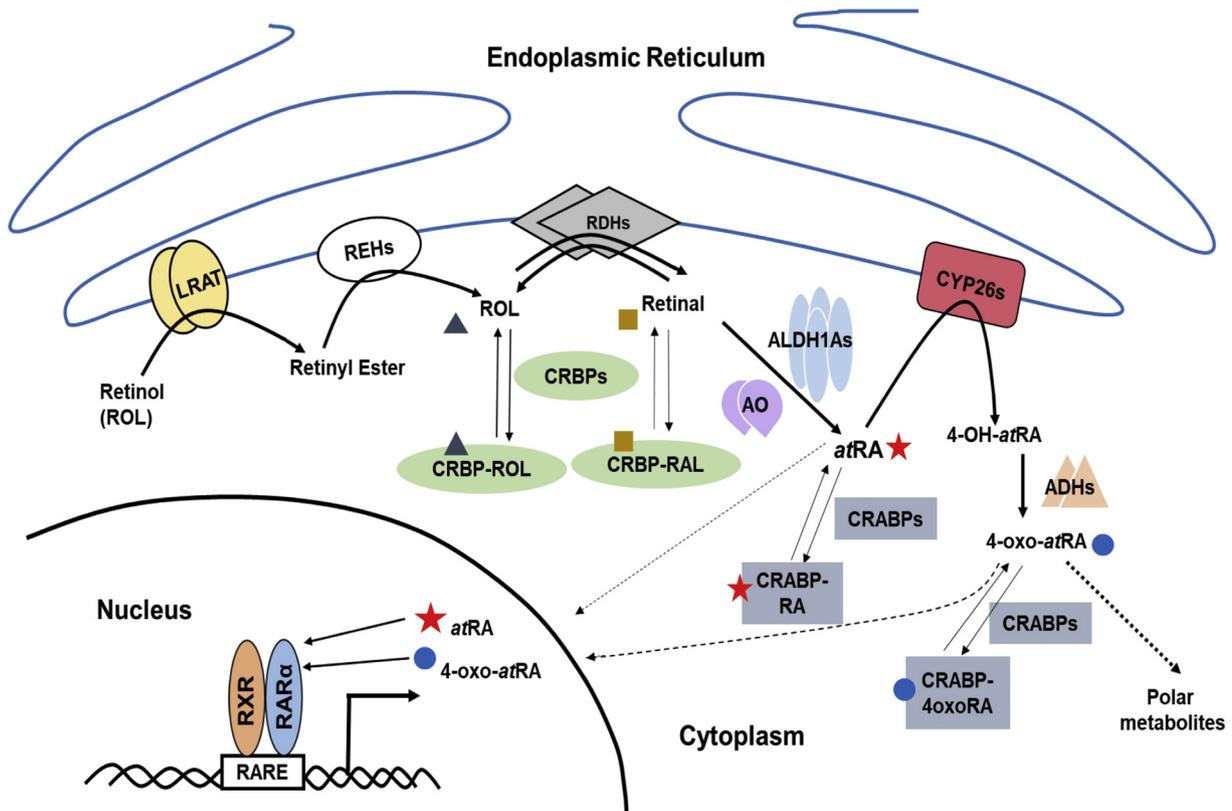
Fig. 1. Metabolic network of vitamin A homeostasis shown with cellular localization of known retinoid metabolizing enzymes. ALDH, aldehyde dehydrogenase; REH, retinyl ester hydrolase; LRAT, lecithin retinol acyltransferase; RDH, retinaldehyde dehydrogenase; AO, aldehyde oxidase; ADH, alcohol dehydrogenase; CRBP, cellular retinol binding protein; CRABP, cellular retinoic acid binding protein. LRAT, RDHs, AO and ADHs function as dimers while ALDH1As function as tetramers and CYP26s as monomers.

2016b). For classic retinoid activity and signaling retinol must be biotransformed to the active form all-*trans*-retinoic acid (*at*RA) (Fig. 2). 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 (AO) enzymes in *at*RA synthesis from retinaldehyde, especially in the liver and harderian gland and possibly the testis in mice (Arnold et al., 2015; Beedle et al., 2018a; Terao et al., 2009). However, whether AO 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
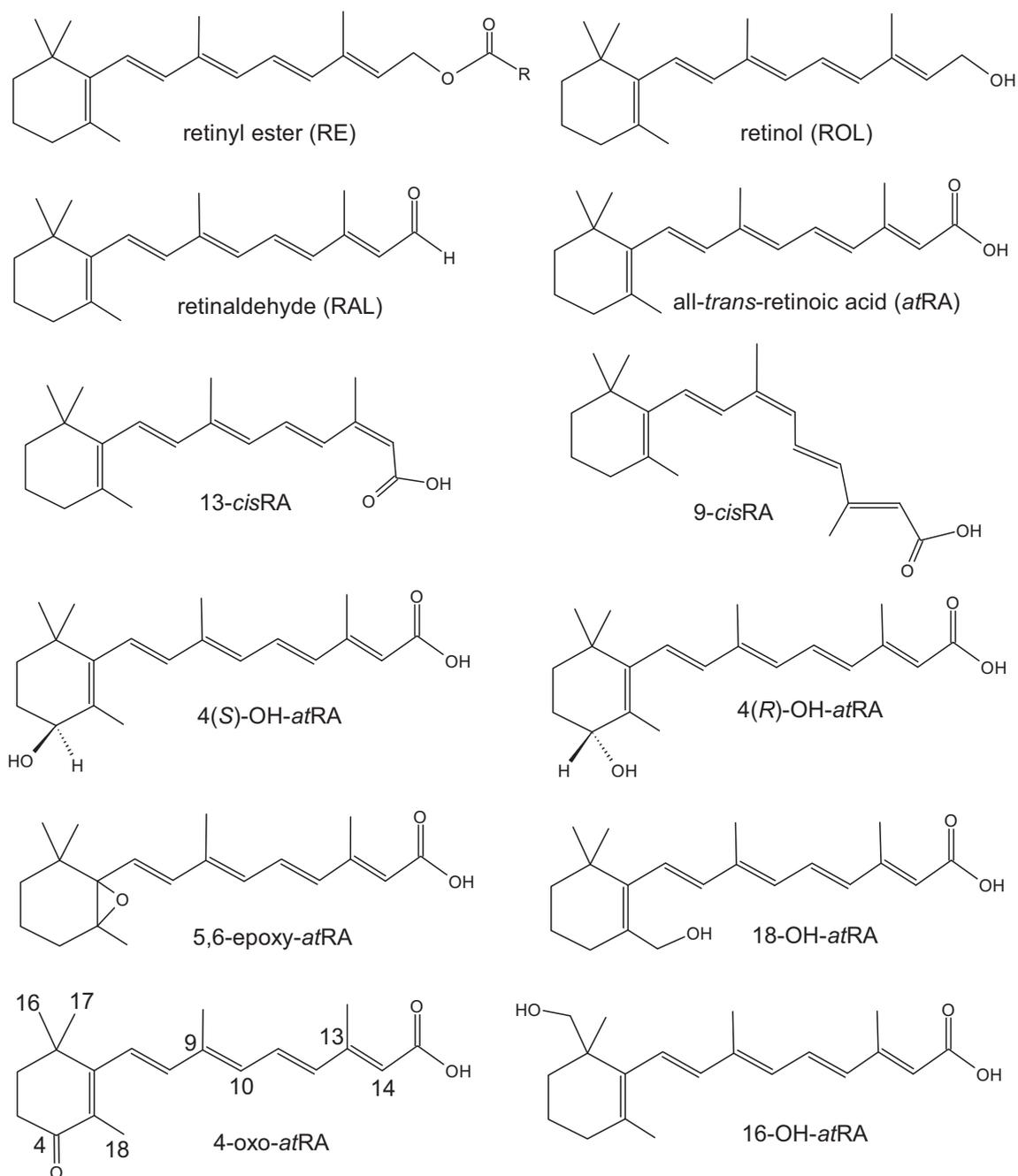

Fig. 2. Chemical structures of the key retinoids. Numbers shown on 4-oxo-*at*RA structure represent positions of carbon atoms in all retinoid chemical structures.

and subsequently halt spermatogenesis (Arnold, Kent, Hogarth, Griswold, et al., 2015; Paik et al., 2014). Based on mouse knock-out studies, of the four ALDH1A enzymes, ALDH1A2 and ALDH1A3 are required during embryonic development for the retinoid signaling while ALDH1A1 knock out mice are viable and relatively healthy (Kumar, Sandell, Trainor, Koentgen, & Duester, 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., 2018), suggesting that other critical atRA 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 (Sladek, 2003) detected levels are generally low (Arnold, Kent, Hogarth, Griswold, 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, Stevison, & Isoherranen, 2016), and the quantitative proteomic approach has been successfully applied to characterizing ALDH1A enzyme expression in the human testis (Arnold, Kent, Hogarth, Schlatt, et al., 2015).

Once atRA 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, Ruuska, Levinthal, & Noy, 1999) or to the cytochrome P450 (CYP) 26 enzymes in the endoplasmic reticulum (Nelson et al., 2016; Zhong, Ortiz, Zelter, Nath, & Isoherranen, 2018). The RARs heterodimerize with retinoid X receptors to regulate the transcription of at least 500 genes although atRA also modulates gene expression via other indirect mechanisms (Balmer & 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, atRA. In addition to atRA, other retinoids such as the metabolites of atRA, 4-OH-RA and 4-oxo-RA, and the isomers 13-cisRA and 9-cisRA bind to RARs and therefore may contribute to overall retinoid signaling depending on their concentrations in different cell types and organisms (Idres, Marill, Flexor, & Chabot, 2002; Topletz et al., 2015).

atRA clearance is mainly mediated by cytochrome P450 enzymes and majority of the clearance of endogenous atRA is believed to be done by CYP26 family enzymes (Ross & Zolfaghari, 2011; Thatcher & Isoherranen, 2009). However, other CYP enzymes such as CYP3A4, CYP3A5, CYP3A7 and CYP2C8 in humans and Cyp2c22 in rodents have been shown to also catalyze atRA hydroxylation although with much lower efficiency than CYP26 enzymes (Lee, Lee, Arnold, Isoherranen, & Morgan, 2014; Marill, Cresteil, Lanotte, & Chabot, 2000; Thatcher, Zelter, & Isoherranen, 2010). As CYP3A enzymes and CYP2C8 are predominantly expressed in the human liver they are unlikely to contribute to atRA clearance in the majority of other atRA 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 atRA 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 of CYP26A1 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 atRA (Topletz, Zhong, & Isoherranen, 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 atRA is the acyl glucuronidation of atRA and subsequent biliary secretion. The uridine glucuronosyltransferase (UGT) 2B7 as well as UGT1A3 have been shown to catalyze the glucuronidation of atRA, although the catalytic activity of UGT1A3 was considerably lower than that of UGT2B7 towards atRA (Samokyszyn et al., 2000). Glucuronidation of atRA has also been observed in rat liver microsomes from uninduced and induced animals, but the UGT isoforms in the rat liver contributing to atRA clearance have not been identified (Genchi, Wang, Barua, Bidlack, & Olson, 1996; Sass, Forster, Bock, & Nau, 1994). Unfortunately, no systematic studies have tested atRA glucuronidation by a panel of human UGT enzymes and as such the number of UGT enzymes that can glucuronidate atRA remains unknown. After administration of atRA, 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, Bugge, Rodriguez, Rosenberger, & Doran, 1990). Since UGT enzymes are expressed in nearly all major organs and tissues in the body it is possible that they contribute significantly to atRA clearance. However, the quantitative importance of UGT enzymes in the clearance of endogenous atRA 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; White et al., 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, Bain, Yao, & Gottlieb, 1997; White et al., 1997) and other species (Fujii et al., 1997; Krüger, Blum, & Greger, 2005; Swindell et al., 1999; Wang, Zolfaghari, & Ross, 2002). The cloning and characterization of CYP26A1 led to the search for other CYP26 enzymes that could metabolize atRA, and cloning of CYP26A1 was followed by the identification of CYP26B1 (P450RAI-2) (MacLean et al., 2001; White et al., 2000; Zhao, Dobbs-McAuliffe, & Linney, 2005) and CYP26C1 (Tahayato, Dollé, & Petkovich, 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., 2006; Gu, Xu, Wang, Gao, & Zhao, 2005).

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 atRA signaling plays in vertebrate development (Carvalho et al., 2017). For example, CYP26 enzymes and the subsequent regulation of atRA gradients in the embryo are critical in the formation of the anterior-posterior body axis. In general, the complement of genes related to atRA- 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 & Cañestro, 2009). Furthermore, using phylogenetic analyses, evolutionary proximities of animal CYP26 enzymes and cyanobacterial CYP120 enzyme were discovered with the proximity likely due to a lateral gene transfer (Millard, Scanlan, Gallagher, Marsh, & Taylor, 2014). CYP120 was in fact the first non-animal atRA hydroxylase identified, and the CYP120 enzyme from Synechocystis has been shown to

metabolize *at*RA in the β-ionone ring similar to CYP26A1 (Alder, Bigler, Werck-Reichhart, & Al-Babili, 2009; Ke, Baudry, Makris, Schuler, & Sligar, 2005; Kühnel et al., 2008).

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 & 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) (Taimi et al., 2004; White et al., 2000). 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
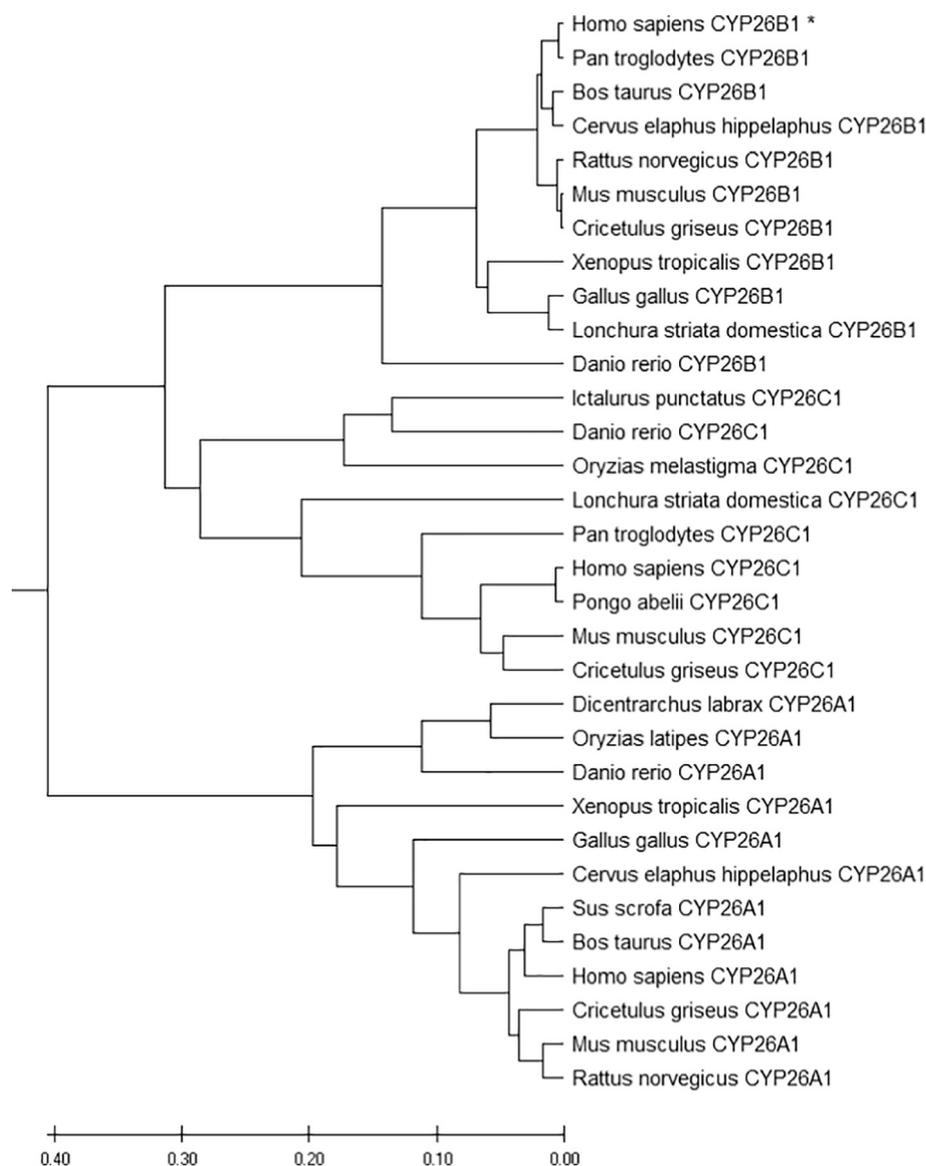
Fig. 3. CYP26 enzyme phylogenetic tree constructed with protein sequences. All CYP26 protein sequences were obtained from NCBI protein database except that human CYP26B1 (*) protein sequence is from published work (Foti, Isoherranen, et al., 2016). Evolutionary analyses were conducted in MEGA X (Kumar et al., 2012). 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.

Fig. 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 (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 (positiv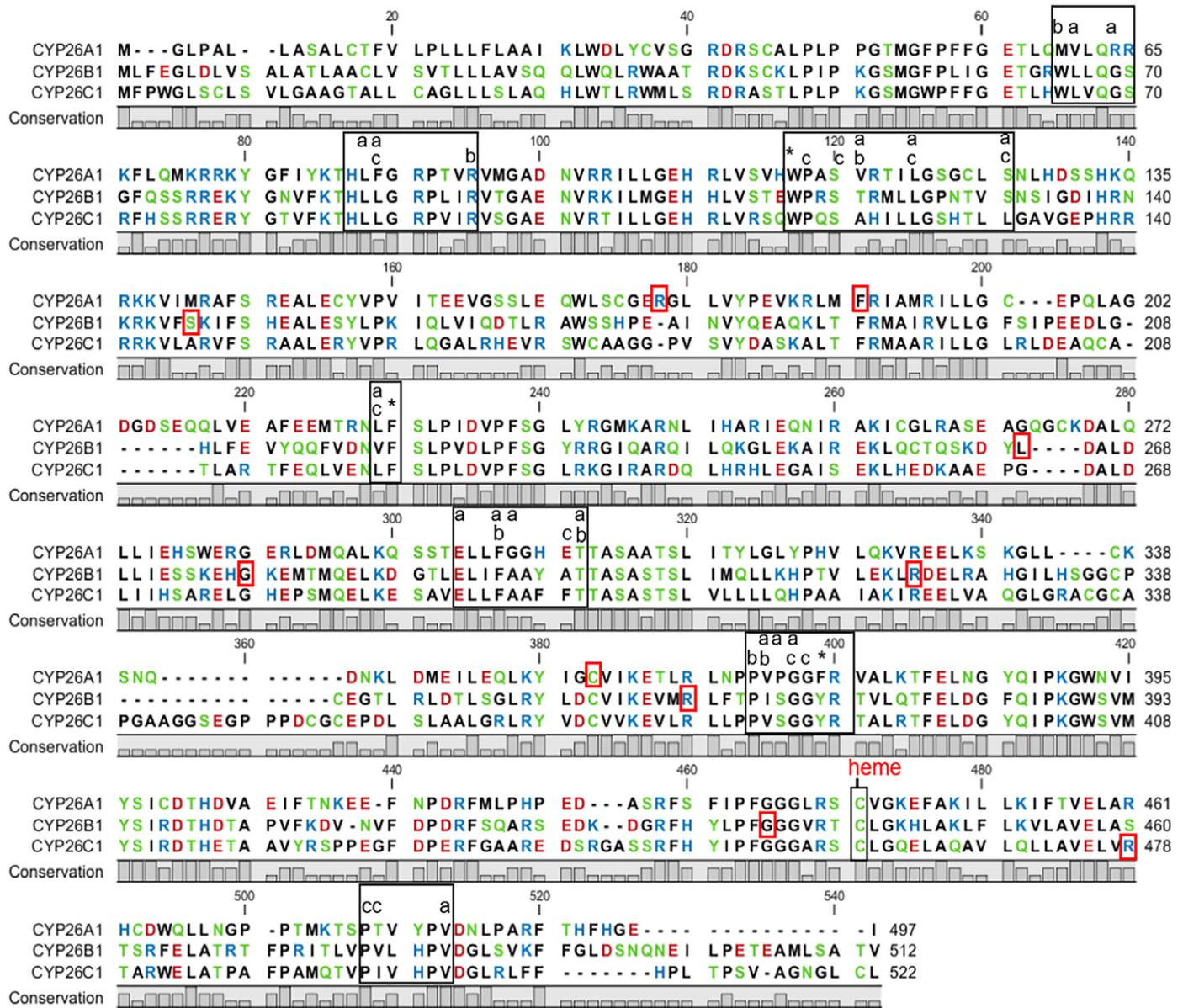ely charged) as blue. Bar graphs indicate the degree of amino acid conservation. Black boxes indicate sequence regions involved in *at*RA binding based on homology models as indicated in the text. Amino acids proposed to interact with *at*RA are indicated by symbols a for CYP26A1, b for CYP26B1, c for CYP26C1 and * for all three CYP26 enzymes. Amino acids which can change due to SNPs (Table 1) are marked by red boxes.

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 (Fig. 2). The metabolite profiles of human CYP26 enzymes were first explored in transiently transfected cells with RA isomers as substrates (Taimi et al., 2004; White et al., 1997, 2000). 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 atRA 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; Lutz et al., 2009; Topletz et al., 2012; White et al., 1997; Zhong et al., 2018). Of these metabolites the 18-OH-RA and 16-OH-RA (Fig. 2) 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; Shimshoni et al., 2012; Thatcher et al., 2011; Topletz et al., 2012; Zhong et al., 2018). Of note, the formation of 4-oxo-atRA is oftentimes observed in CYP26-expressing systems (e.g. transiently transfected cells and liver microsomes). However, the formation of 4-oxo-atRA likely requires an alcohol dehydrogenase enzyme in tissue and cell systems for the second oxidization step after initial 4-hydroxylation. Studies in HepG2 cells and microsomes have shown that 4-oxo-atRA formation from 4-OH-atRA, 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, Lamb, & Sporn, 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 atRA in human liver but not in mice (Zhong 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 atRA 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 atRA hydroxylases, and together with the fact that CYP26 enzymes are generally inducible by atRA, the high substrate affinity is considered as the main evidence that CYP26 enzymes are biologically significant atRA hydroxylases. Other atRA metabolizing CYP enzymes (e.g. CYP2C8, CYP3A4, CYP3A5 and CYP3A7) show K$_m$ values in the micromolar range for atRA (Thatcher et al., 2010), demonstrating that atRA 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 atRA indicated by the highest Cl$_{int}$, while CYP26B1 appears to be a high affinity low capacity atRA hydroxylase and exhibits the lowest Cl$_{int}$ of atRA among the three CYP26s (Table 1). The metabolite patterns formed by the CYP26 enzymes also differ slightly between the three family members. CYP26A1 hydroxylates atRA 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

Table 1
Summary of human CYP26 enzyme kinetic parameters.

Substrate	k$_{cat}$ (min^{-1})	K$_m$ (nM)	Cl$_{int}$ (μl/min/pmol)	Measurement	Reference
CYP26A1					
atRA	11.4	9.3	1200	Substrate depletion	Lutz et al., 2009
	3.5	21.4	164	4OH-atRA formation	
	9.5 ± 2.2	50.1 ± 32.4	190	4OH-atRA formation	Topletz et al., 2012
	5.0 ± 0.5	18.9 ± 13.4	102	18OH-atRA formation	
9-cisRA		134 ± 30		4OH-, 16OH-atRA formation	Thatcher et al., 2011
4(S)-OH-atRA		5.2 ± 1.3	1450 ± 150	Substrate depletion	Topletz dissertation; Shimshoni et al., 2012
4(R)-OH-atRA		11 ± 5.2	740 ± 12	Substrate depletion	
4-oxo-atRA		63.0	90 ± 9.0	Substrate depletion	Topletz et al., 2015
18-OH-atRA		35.8	230 ± 43	Substrate depletion	
CYP26B1					
atRA	0.81 ± 0.2	18.8 ± 18.7	43	4OH-atRA formation	Topletz et al., 2012
	0.45 ± 0.02	64.6 ± 10.3	7.0	4OH-atRA formation	Nelson et al., 2016
9-cisRA	3.6	555	6.5	4OH-9cisRA formation	Diaz et al., 2016
4(S)-OH-atRA		4.0 ± 2.5	46 ± 18	Substrate depletion	Topletz, 2013; Shimshoni et al., 2012
4(R)-OH-atRA		31 ± 19	104 ± 19	Substrate depletion	
4-oxo-atRA		29 ± 6.6	15 ± 4.0	Substrate depletion	
18-OH-atRA		4.8 ± 6.3	33 ± 10	Substrate depletion	
CRABPI-atRA	0.17 ± 0.006	21.7 ± 2.9	7.8	4OH-atRA formation	Nelson et al., 2016
CRABPII-atRA	0.28 ± 0.01	24.3 ± 4.2	11.5	4OH-atRA formation	
CYP26C1					
atRA	5.9 ± 1.9	50.4 ± 6.2	120 ± 49	4OH-atRA formation	Zhong et al., 2018
13-cisRA	7.7 ± 2.9	38.2 ± 4.9	208 ± 101	4OH-13cisRA formation	
9-cisRA	4.7 ± 1.2	9.7 ± 2.6	504 ± 140	4OH-9cisRA formation	
9-cisRA		5.9 ± 0.3		16OH-9cisRA formation	
4-oxo-atRA		15.7 ± 4.3	153 ± 20	Substrate depletion	
		10.2 ± 2.2		4oxo-16OH-atRA formation	
CRABPI-atRA	2.1 ± 0.2	50.8 ± 8.0	41 ± 2	4OH-atRA formation	
CRABPII-atRA	2.1 ± 0.8	34.1 ± 7.1	66 ± 37	4OH-atRA formation	
CRABPI-4oxoatRA		3.5 ± 0.5		4oxo-16OH-atRA formation	
CRABPII-4oxoatRA		5.4 ± 1.5		4oxo-16OH-atRA formation	

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

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 (4S)-OH-RA (Fig. 2) while CYP26C1 converts atRA preferably to (4R)-OH-atRA (Shimshoni et al., 2012; Zhong et al., 2018). CYP26B1 forms both (4S)-OH-atRA and (4R)-OH-atRA, with a 4R/4S ratio of 1:1–1:2 (Topletz, 2013). The metabolite profile and stereospecificity of the 4-hydroxylation of atRA 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 atRA (Helvig, Taimi, Cameron, Jones, & Petkovich, 2011). All three CYP26 enzymes also metabolize 13cis-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 (Diaz et al., 2016; Lutz et al., 2009; Thatcher et al., 2011; Zhong et al., 2018). Of the three enzymes, CYP26C1 has the highest affinity towards 9-cisRA and CYP26A1 and CYP26B1 showed approximately 13- and 55-fold higher K_m values than CYP26C1 towards 9-cisRA, respectively (Table 1). Somewhat surprisingly, the Cl_{int} of 9-cisRA by CYP26C1 was nearly as high as that of atRA by CYP26A1 while the Cl_{int} towards atRA by CYP26C1 was only about 10% of that by CYP26A1. This finding prompted the hypothesis that high clearance of 9-cisRA 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-cisRA and 9-cisRA as substrates, human CYP26A1 metabolized 13-cisRA to both (4S)- and (4R)-OH-13cisRA (ratio 1:1), and 9-cisRA to (4S)-OH-9cisRA (Zhong et al., 2018). CYP26C1 converted 13-cisRA to both (4S)- and (4R)-OH-13-cisRA (ratio 1:1), and 9-cisRA to (4S)-OH-9-cisRA (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-atRA (Table 1) and the sequential metabolism of atRA is characteristic for these enzymes. The K_m values of 4-OH-atRA and 18-OH-atRA with CYP26A1 and CYP26B1 are all in the nanomolar range, indicating similar affinity of these substrates as atRA (Shimshoni et al., 2012; Topletz et al., 2015). In contrast, CYP26C1 was most efficient in clearing 4-oxo-atRA showing 10-fold higher Cl_{int} towards this substrate than the other two CYP26 enzymes (Zhong et al., 2018). Both CYP26A1 and CYP26B1 preferred (4S)-OH-atRA as a substrate over (4R)-OH-atRA (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 atRA and 4-oxo-atRA both have high affinities to cellular retinoic acid binding proteins (CRABPs), CRABP I and II (K_d < 20 nM; (Fiorella & Napoli, 1991, 1994). In addition, before the CYP26 enzymes were identified, it was shown that the CRABPs interact with the microsomal enzymes that metabolize atRA in rat testis microsomes and impact the metabolite formation and atRA clearance (Fiorella & Napoli, 1991,

1994; Napoli, Posch, Fiorella, & Boerman, 1991). Based on current knowledge of the enzymes expressed in the testis and hydroxylating atRA, 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-atRA (holo-CRABP) to accept atRA as a substrate via substrate channeling, but also that apo-CRABP inhibits the activity of CYP26B1 (Nelson et al., 2016). As atRA binding with the CRABPs is tight and essentially in the presence of CRABPs no free atRA 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 atRA were either lower or similar to free atRA, an observation that indicates a much lower K_m value for free atRA 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 atRA channeling, the experiments with recombinant CYP26C1 suggest that CRABPs may also deliver atRA metabolites for further oxidation. When 4-oxo-atRA was bound with CRABPI or CRABPII the K_m and k_{cat} values were lower when compared with free 4-oxo-atRA suggesting similar protein-protein interactions as with atRA (Table 1) (Zhong et al., 2018). In contrast, CRABPI and CRABPII had no impact of 9cisRA 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, Buttrick, & Isoherranen, 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) (Diaz et al., 2016; Foti, Diaz, & Douguet, 2016; Thatcher et al., 2011; 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 atRA based on CYP26 homology models are shown in Fig. 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 atRA 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, Strid, Sirsjo, & Eriksson, 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 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 (Awadalla, Alshammari, Eriksson, & Saenz-Méndez, 2016; Ren et al., 2008; Shimshoni et al., 2012; Sun et al., 2015). 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 co-crystallized 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 et 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 (Awadalla et al., 2016; Foti, Isoherranen, et al., 2016; Gomaa et al., 2006; Karlsson et al., 2008; Ren et al., 2008; Shimshoni et al., 2012). 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 (Awadalla et al., 2016; Foti, Isoherranen, et al., 2016; Karlsson et al., 2008). 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 et al., 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 atRA from the heme iron and the binding energy predicted a reduced catabolic activity of the splice variant towards atRA (Saenz-Méndez 2012). This prediction is consistent with the experimental data (Elmabsout et 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 atRA, 13-cisRA, 9-cisRA 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 atRA to orient differently in the CYP26C1 active site in comparison to atRA bound to CYP120A1.

The homology models of the CYP26 enzymes have provided important information and hypotheses of the structure-function of CYP26 enzymes. However, systematic 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

atRA 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 (Duester, 2008; Maden, 2007; McCaffery, Adams, Maden, & Rosa-Molinar, 2003; Rhinn & 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 atRA signaling during embryogenesis have been previously reviewed (Pennimpede et al., 2010; Ross & 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 atRA 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 hindbrain 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) (Dranse, Sampaio, Petkovich, & Underhill, 2011; Maclean, Dollé,

& Petkovich, 2009; Pennimpede et al., 2010; Yashiro et al., 2004). 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, Wada, Okamoto, Kudo, & Imai, 2005; Laue, Janicke, Plaster, Sonntag, & Hammerschmidt, 2008; Spoorendonk et al., 2008). Specifically, the studies in zebrafish have focused on the link between atRA concentration as a posteriorizing signal during body axis establishment and cyp26 expression (Kudoh, Wilson, & Dawid, 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 atRA 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 atRA 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 atRA. 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 atRA 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 appear to be expressed in distinct sites in gestational stage specific manner (MacLean et al., 2001), and the Cyp26 enzymes are 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, Gale, & Maden, 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 atRA synthesized by the mesonephros. Strikingly, Cyp26b1 expression was male-specific by E12.5 in the mice (Bowles et al., 2006) suggesting that Cyp26b1 degrades atRA 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, MacLean, Cameron, Clagett-Dame, & Petkovich, 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 atRA 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, Li, Metzger, Chambon, & Petkovich, 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 atRA concentrations or in spermatogenesis was observed (Zhong 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 & 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 atRA, a factor inducing proliferation of this cell type.

atRA 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 atRA 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 atRA levels in the hippocampus. This increase in atRA 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 atRA 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 atRA (Zhong, Hogarth, et al., 2019). These findings are consistent with the assessment of changes in tissue specific atRA concentrations in mice after talarozole treatment (Stevison, Hogarth, Tripathy, Kent, & Isoherranen, 2017). When Cyp26a1 and Cyp26b1 were inhibited with talarozole in adult mice, following a single dose administration of talarozole, atRA concentrations increased transiently in the liver, testis and serum but the magnitude of increase in atRA 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 atRA concentrations in these tissues. Yet, atRA 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 atRA concentrations and atRA clearance in the skin (Kurashima et al., 2014). As Cyp26b1 was expressed in the skin fibroblasts it was shown to regulate the atRA 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, Yokota, Ohoka, & Iwata, 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$_H$17 cells both had Cyp26b1 with T$_H$17 cells having significantly higher *Cyp26b1* mRNA expression than iT$_{reg}$ cells. This difference in *Cyp26b1* expression was linked to the role of *at*RA signaling in differentiation of Naïve CD4$^+$ T-cells. In addition, deletion of *Cyp26b1* in the T-cells led to a greater frequency of IL17 producing T$_H$17-cells and in CD4$^+$CD25$^+$Foxp3$^+$iT$_{reg}$ cells (Chenery et al., 2013). These findings suggest that increased *at*RA concentrations stimulate IL17 production by T$_H$17 cells and iTreg responses, and Cyp26b1 acts to degrade this *at*RA and hence limits T$_H$17 and iT$_{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 & 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 & 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, Brzezinski, & Juchau, 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 & 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 & 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 & 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 & 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 & 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 & 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 (Tay, Dickmann, Dixit, & Isoherranen, 2010; Topletz et al., 2012; Xi & Yang, 2008). 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 & 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, Jones, & Ghiaur, 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 & 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 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 that 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; Loudig, Maclean, Dore, Luu, & Petkovich, 2005; Zhang, Zolfaghari, & Ross, 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 *at*RA metabolites, and links the induction of CYP26A1 with its catalytic activity towards these same retinoids.

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, Zolfaghari, &

Ross, 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 & Ross, 2010). Notably, in the neonatal rat lung the induction magnitude of *Cyp26b1* was greater than that of *Cyp26a1* by *at*RA treatment (Wu & Ross, 2010), a finding in contrast to the typically lower magnitude of induction of *CYP26B1* gene expression observed in human liver (Tay et al., 2010; Zolfaghari et al., 2007). 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 TNFα 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 TNFα 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 patterns of CYP26A1 and CYP26B1 are 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, Frentz, Morgan, Sutherland-Smith, & Robertson, 2016). This subject was diagnosed with Antley-Bixler syndrome and showed craniosynstosis, craniofacial abnormalities and other developmental defects (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

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.2682 T > 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.220 T > C		–		
	Exon 3	g.877C > G		–		
	IVS4	g.1380_81AG > GA		–		
	Exon 7	g.3067 T > C		–		
	Exon 7	g.3116delT		frame shift		
NM_000783	IVS1	g.369C > T		–		Deak et al., 2005
	Exon 2	c.246 T > C		–		
	IVS2	g.664 T > 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.1583 T > C	rs2241059	–	0.174	
	3' UTR	c.2620C > T	rs10181341	–	0.065	
	3' UTR	c.3440C > G	rs3768647	–	0.180	
	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.436 T > 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.72145857 T > 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.

causal relationship between the CYP26C1 mutation and focal facial dermal dysplasia Type IV is in agreement with the known role of atRA 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 300 kb 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 atRA treatment while healthy control lymphocytes showed robust induction of CYP26B1 in response to atRA (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; Lee et al., 2007; Rat et al., 2006; 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 (F186 L, 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 atRA 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-atRA and 18-OH-atRA formation catalyzed by the CYP26A1*3 protein was decreased to much greater extent than 4-OH-atRA formation while the formation of all metabolites catalyzed by the CYP26A1*4 protein appeared to be equally affected. These findings suggest that the F186 L variant affects the binding orientation of atRA 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). In this study, 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, however, that the allele frequency of these variants in human populations is <0.005. 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 atRA 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 atRA 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 (Fransen et al., 2013; Ge et al., 2014; Krivospitskaya et al., 2012) 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 atRA 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 atRA 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 atRA 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 the 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 (Altucci, Leibowitz, Ogilvie, de Lera, & Gronemeyer, 2007; Khalil et al., 2017; Leyden, 2003; Smith & 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 (Osman et al., 2018; Watts & Tallman, 2014). *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 (Masetti et al., 2012; Matthay, 2013; Reynolds, Matthay, Villablanca, & Maurer, 2003). 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, Capron, Idres, & Chabot, 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 h) than 13-*cis*RA (~20 h) 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 & 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 variations 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 of and exposure to 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 atRA and 13-cisRA.

Acknowledgments

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