Review

25-Hydroxyvitamin D-24-hydroxylase (CYP24A1): Its important role in the degradation of vitamin D

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ABSTRACT

CYP24A1 is the cytochrome P450 component of the 25-hydroxyvitamin D_3-24-hydroxylase enzyme that catalyzes the conversion of 25-hydroxyvitamin D_3 (25-OH-D_3) and 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) into 24-hydroxylated products, which constitute the degradation of the vitamin D molecule. This review focuses on recent data in the CYP24A1 field, including biochemical, physiological and clinical developments. Notable among these are: the first crystal structure for rat CYP24A1; mutagenesis studies which change the regioselectivity of the enzyme; and the finding that natural inactivating mutations of CYP24A1 cause the genetic disease idiopathic infantile hypercalcemia (IIH). The review also discusses the emerging correlation between rising serum phosphate/FGF-23 levels and increased CYP24A1 expression in chronic kidney disease, which in turn underlies accelerated degradation of both serum 25-OH-D_3 and 1,25-$(OH)_2D_3$ in this condition. This review concludes by evaluating the potential clinical utility of blocking this enzyme with CYP24A1 inhibitors in various disease states.

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Introduction

The mitochondrial enzyme, 25-hydroxyvitamin D_3-24-hydroxylase was first described in the early 1970s and initially believed to be involved solely in the renal 24-hydroxylation of 25-OH-D_3 [1]. Work performed over the last 35 years has shown that the enzyme activity is the result of a combination of three components: ferredoxin, ferredoxin reductase and cytochrome P450, only the latter component, referred to as CYP24A1, being specific for this reaction [2,3]. It is now known that CYP24A1 catalyzes the conversion of both 25-OH-D_3 and 1,25-$(OH)_2D_3$ into 24-hydroxylated products targeted for excretion along well-established pathways. We review current knowledge of the structure and function of this protein as well as providing an update on the latest information regarding the physiological and clinical importance of CYP24A1.

CYP24A1: biochemistry and catalytic properties

Though, CYP24A1 was initially referred to as the 25-hydroxyvitamin D_3-24-hydroxylase, work with the recombinant enzyme has shown that it is able to catalyze multiple hydroxylation reactions at carbons C-24 and C-23 of the side chain of both 25-OH-D_3 and its hormonal form, 1,25-$(OH)_2D_3$ [2,3]. Indeed, our view of the role

of CYP24A1 has expanded greatly to suggest that this single P450, alone, is responsible for the 5-step, 24-oxidation pathway from 1,25-$(OH)_2D_3$ to produce calcitroic acid, a known biliary catabolite [4,5], as well as catalyzing a similar pathway which starts with 23-hydroxylation and culminates in the 1,25-$(OH)_2D_3$-26,23-lactone (Fig. 1) [6,7]. In addition, CYP24A1 also efficiently hydroxylates the vitamin D_2 side chain of 25-OH-D_2 and 1,25-$(OH)_2D_2$ to give a more limited series of polyhydroxylated products [8,9]. The 24- and 23-products of the vitamin D_3 side chain appear in a specific order, reinforcing the concept of two distinct pathways initiated by a species-dependent C-24 or a C-23 hydroxylation step. Fig. 2 depicts a partial amino acid sequence alignment of CYP24A1 from 57 species covering bony fish to man and showing an impressive conservation of residues for at least a good part of the protein. Of particular note, is the dichotomy that exists at residue 326 where most species of CYP24A1 have Ala326 and exhibit 24-hydroxylation to calcitroic acid while a number of more primitive organisms have Gly326 and show predominantly 23-hydroxylation to give a 26,23-lactone product. The functional significance of two distinct pathways in different species is unknown [10].

CYP24A1: crystal structure, homology modeling and mutagenesis studies

In 2010, the crystal structure of the rat CYP24A1 was elucidated but in the presence of the detergents Cymal and CHAPS [11]. Although the active site of rat CYP24A1 did not contain its natural substrate, for the most part the crystal structure did confirm the

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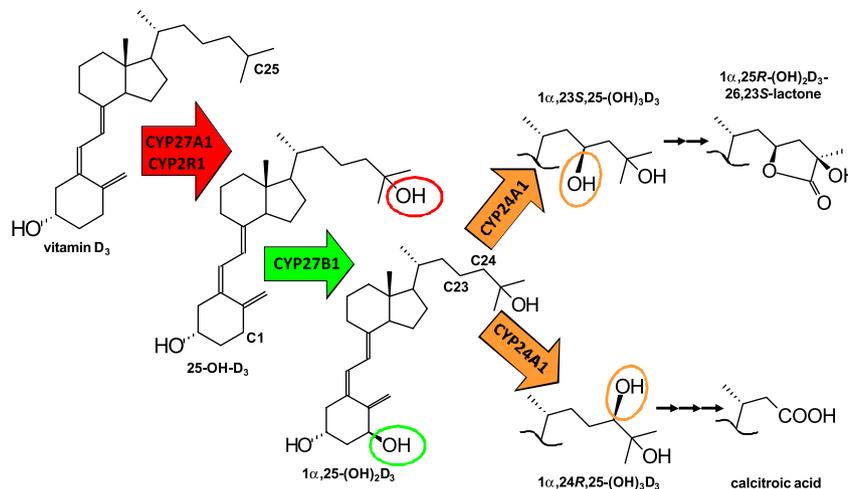E-mail address: gj1@queensu.ca (G. Jones).

Fig. 1. Important steps in the pathway of vitamin D metabolism.

predicted tertiary structure of the protein, as well as the putative active-site residues from previous homology models and mutagenesis studies [10,12–15]. The crystal structure of rat CYP24A1 reveals a canonical cytochrome P450 structure of helices and β-sheets surrounding a prosthetic heme group and a substrate binding cavity. Virtually all of the protein is required to maintain the shape, structure, heme-binding, and function of the enzyme. The crystal structure of the rat CYP24A1 enzyme is shown in Fig. 3 with 1,25-$(OH)_2D_3$ (sticks and spheres; purple) positioned using state-of-the-art docking software into the wide-open cleft that constitutes the substrate binding cavity [11].
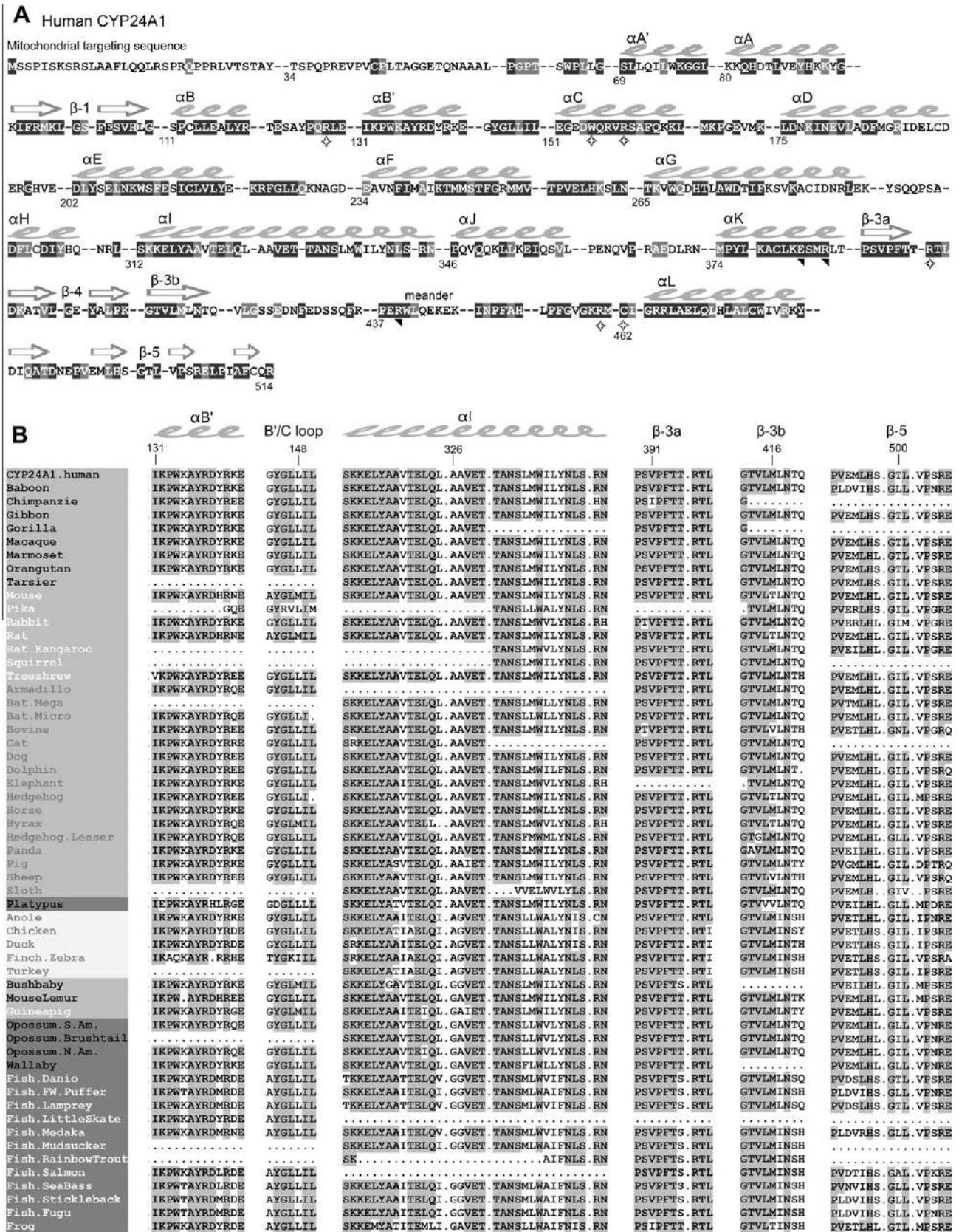
Even before the crystal structure of CYP24A1 was determined, mutagenesis studies were initiated based upon the remarkable conservation of structure across cytochrome P450s. Sakaki and colleagues who had shown that rat CYP24A1 is primarily a C24-hydroxylase, as compared to the human enzyme which is capable of both C24- and C23-hydroxylation [7], performed a follow-up study in which they mutated T416M and I500T in the β3b- and β5-sheets, respectively to try to change the properties of the rat enzyme by substituting amino acids to those found in the human enzyme [12]. They postulated that these residues interact with A-ring and cis-triene moieties of the 1α,25-$(OH)_2D_3$-docked substrate. The T416M and I500T caused significant changes in the C24:C23 hydroxylation ratio from 100:1 to 12.5:1 or 6.3:1, respectively, whereas, in their hands, the wild-type human enzyme had a ratio of 3.7:1 [12]. The observation that these modifications did not completely shift the regioselectivity of the rat enzyme from being a 24-hydroxylase to that of the human ortholog with significant 23-hydroxylase activity suggests that other species-based differences might act individually or in concert with residues at 416 and 500 to explain the observed enzymatic differences between human and rat CYP24A1 forms.

Prosser et al. [10] adopted a somewhat similar approach to study differences between the human and opossum CYP24A1, the latter being representative of the orthologs that are predominantly 23-hydroxylases, with a C24:C23 hydroxylation ratio of 0.04:1. Using the human CYP24A1 as a starting point, they noted 80 amino acid differences between human and opossum CYP24A1 but focused on mutating Ala326 to Gly326 found in the opossum and many other 'primitive' CYP24A1s (see Fig. 2) because this residue occupied a critical position directly above the heme in the I-helix that abuts the side chain of 1α,25-$(OH)_2D_3$ docked within a homology model of hCYP24A1 (see Fig. 3). The single A326G substitution radically changed the metabolic pattern observed for

the resultant enzyme (Fig. 4A and B) by changing the enzyme properties from a 24-hydroxylase with a C24/C23 hydroxylation ratio = 8.1:1 to a 23-hydroxylase with a C24/C23 hydroxylation ratio = 0.12:1, a value that closely resembled opossum CYP24A1 (0.04:1). Thus it appeared that amino acid residue 326 alone was likely responsible for much of the regioselectivity difference observed between human and opossum CYP24A1 orthologs. Further docking studies comparing the positions of 1α,25-$(OH)_2D_3$ docked for optimal C24- versus C23-hydroxylation suggested that the loss of a methyl group from the amino acid at 326 in the I-helix by substituting Gly for Ala, provides extra space for the side chain of 1α,25-$(OH)_2D_3$ to slide deeper into the substrate-binding cavity in order to optimally place C23 as opposed to C24 above the heme (Fig. 4C and D), and committing catabolism through to 1α,25-$(OH)_2D_3$-26,23-lactone. The striking impact of A326G on regioselectivity is logical, given its direct contact with the substrate side-chain directly above the heme – as compared with Ile500 and Met416 located in the distal substrate access channel.

Mutations at other sites in human CYP24A1 that have been shown to modulate the regioselectivity of the enzyme include Ile131, Leu148, Met246, and Val391 [13]. In mutagenesis studies of residues over a single turn of the F-helix forming the top of the substrate binding cavity of rat CYP24A1 performed by Annalora and colleagues [14,16], it was shown that mutations at sites facing away from the cavity (Met245, Ser247, Thr248) retained 1α,25-$(OH)_2D_3$ binding affinity similar to the wild-type, whereas mutations at sites Phe249 and Met246 directly protruding into the cavity, impaired substrate binding to different degrees. Based upon the work of Annalora et al. [14,16], CYP24A1 is a 1α,25-$(OH)_2D_3$-binding protein first, and a catabolic enzyme second. All of these residues including Ala326 and Ile500, originally selected on the basis of homology modeling [10,12–15] as putative substrate contact points, have been implicated in forming the CHAPS-containing substrate-binding site in the crystal structure of rat CYP24A1 [11]. A recent report [17] suggests that a V391L mutation in the human CYP24A1 also changes enzymatic properties by introducing 1α-OH-D_3-25-hydroxylase activity absent in the wild-type enzyme and ascribes this to a combination of a change in the position of substrate within the active site and altered substrate binding affinity [10,16].

Taken together, the generation of homology models for CYP24A1 and mutagenesis studies have led to an unprecedented understanding of the amino-acid architecture of the substrate-binding pocket, many aspects of which have been confirmed by
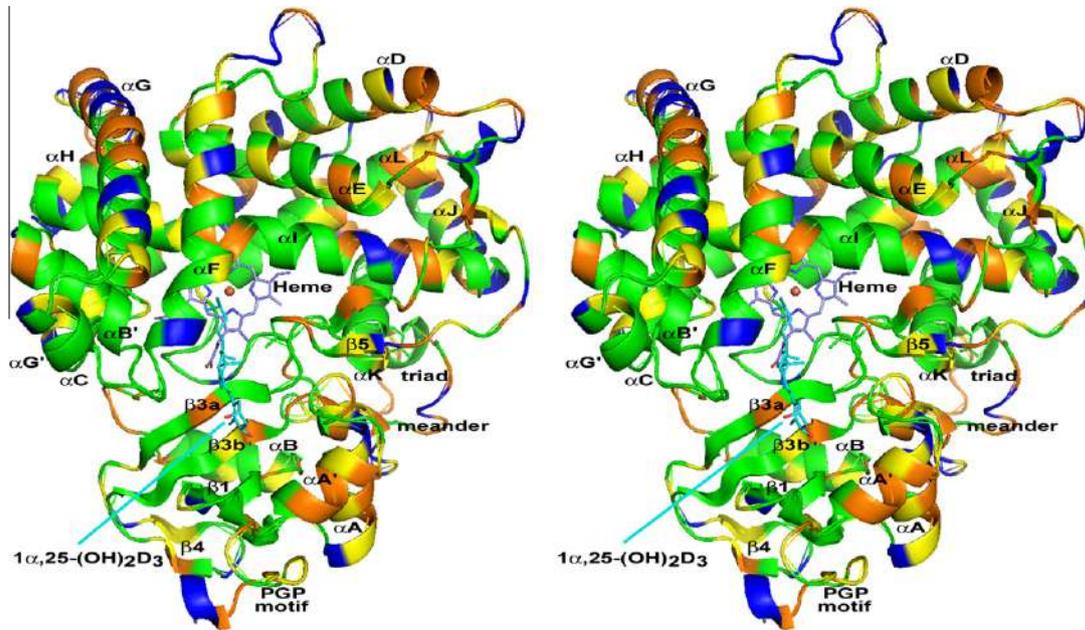
Fig. 2. Sequence alignments of CYP24A1. (A) Full protein sequence and secondary structure of human CYP24A1. Dark shaded residues are >95% conserved in 57 species orthologs. Light shaded residues are >85% conserved. The positions of the ERR triad residues are denoted by black triangles and the positions of heme-binding residues by diamonds. (B) Multi-sequence alignment of selected secondary structures containing substrate contact residues of CYP24A1 from 57 species across the phylogenetic tree. Species are grouped as primates, rodents, other mammals, marsupials, reptiles and birds and fish. Species grouped below turkey contain Gly326 and are all believed to metabolize 1,25-(OH)$_2$D$_3$ via the 23-hydroxylation pathway to 26,23-lactone shown in Fig. 1.

Fig. 3. Crystal Structure of rat CYP24A1. (Taken from Annalora et al. [11]). The structure of rat CYP24A1 has been downloaded from the PDB database and 1,25-(OH)$_2$D$_3$ (cyan) has been docked into the active site above the heme group (purple) using methods described in Refs. [10,13,17]. The CYP24A1 protein structure is colored according to the frequency of conserved residues in the multi-sequence alignment of species orthologs shown in Fig. 2B: green (>95%), yellow (>85%), orange (>60%) and blue (<60%).

the availability of the crystal structure of rat CYP24A1. Our current view of the substrate-binding site is depicted in Fig. 5, where many of the residues that we have discussed are highlighted.

CYP24A1: physiological role

During the same period of time in which the role of CYP24A1 in multi-step hydroxylation of the side-chain of vitamin D was being elucidated, it was also shown that the enzyme is expressed in many, if not all, target cells containing the vitamin D receptor (VDR),[1] including kidney, bone, intestine, etc. and is strongly inducible by vitamin D receptor agonists in such tissues [2]. This led some to propose that the role of CYP24A1 is primarily to limit or attenuate the action of 1,25-(OH)$_2$D$_3$ on target cells after an initial round of transcriptional activation in a negative feedback loop [18] (Fig. 6). The cloning of CYP24A1 in the early 1990s [19] confirmed both the target cell pattern of CYP24A1 expression and its inducibility by its substrate, 1,25-(OH)$_2$D$_3$. Moreover, analysis of the CYP24A1 gene revealed the presence of a strong positive vitamin D response element (VDRE) element in the upstream promoter which mediates this induction at the transcriptional level [20]. This suggested that raising 1,25-(OH)$_2$D$_3$ in target cells could trigger CYP24A1-mediated catabolism and thus protect cells from excess VDR pathway activation. Work with the CYP24A1-null mouse also added support to a catabolic role for CYP24A1, since the clearance of 1,25-(OH)$_2$D$_3$ is dramatically reduced and the plasma half-life of the hormone increases 10-fold from ∼6 to ∼60 h when CYP24A1 is absent [21,22]. Thus, there is abundant evidence that CYP24A1 exists in normal physiology to catabolize 25-OH-D$_3$ to prevent its eventual activation to 1,25-(OH)$_2$D$_3$ and/or degrade the hormone, 1,25-(OH)$_2$D$_3$ within its target cells to terminate its biological activity.

Recent work by St-Arnaud [23] has challenged this uni-dimensional role for CYP24A1 by noting the accelerated healing of bone

fractures in laboratory animals after the administration of 24-hydroxylated metabolites of vitamin D. Work with the CYP24A1-null mouse had shown that the animals exhibit an "intramembranous bone" lesion originally thought to be due to the absence of a bone-specific 24-hydroxylated metabolite but later believed to be explained by excessive blood 1,25-(OH)$_2$D$_3$ levels, since the condition resembles excessive 1,25-(OH)$_2$D$_3$ administration [24]. Also the intramembranous bone lesion appears to be resolved when a double CYP24A1/VDR-null mouse is engineered implying that it requires excessive VDR-mediated gene expression [24]. In the studies of bone fracture healing and in the CYP24A1-null mouse, the potential bone-healing properties of 24-hydroxylated metabolites would be boosted enormously by the demonstration of a 24,25-(OH)$_2$D$_3$ receptor and elucidation of the signal transduction pathway(s) mediating the effect.

While CYP24A1 has been clearly established as the key enzyme responsible for vitamin D catabolism, it has become evident that CYP24A1 works in balance with CYP27B1, which is the cytochrome P450 enzyme responsible for converting 25-OH-D$_3$ to 1,25-(OH)$_2$D$_3$ both in the kidney where its role in vitamin D hormone activation was first established as well as in extra-renal tissues where its specific purpose remains to be elucidated. The emergence of the extra-renal 1α-hydroxylase (CYP27B1) as a mechanism for raising the cellular concentration of 1,25-(OH)$_2$D$_3$ [25,26] has refocused our attention on the crucial role of target-cell CYP24A1 as a fine-tuning mechanism to attenuate and eventually reduce its level after gene expression has been modulated. While the renal CYP24A1 enzyme may function to balance systemic 25-OH-D$_3$ and 1,25-(OH)$_2$D$_3$ levels, target-cell extra-renal enzyme probably acts in conjunction with CYP27B1 to "fine-tune" target tissue exposure to 1,25-(OH)$_2$D$_3$ hormone [27].

CYP24A1: regulation by 1,25-(OH)$_2$D$_3$, PTH and FGF-23

Vitamin D signaling plays a critical role in regulating bone and mineral homeostasis and consequently, enzymes such as CYP24A1 which control vitamin D levels are regulated by hormones which

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[1] *Abbreviations used:* VDR, vitamin D receptor; VDRE, vitamin D response element; PTH, parathyroid hormone; IIH, idiopathic infantile hypercalcemia; XLH, X-linked hypophosphatemic rickets; FGF-23, fibroblast-like growth factor 23.
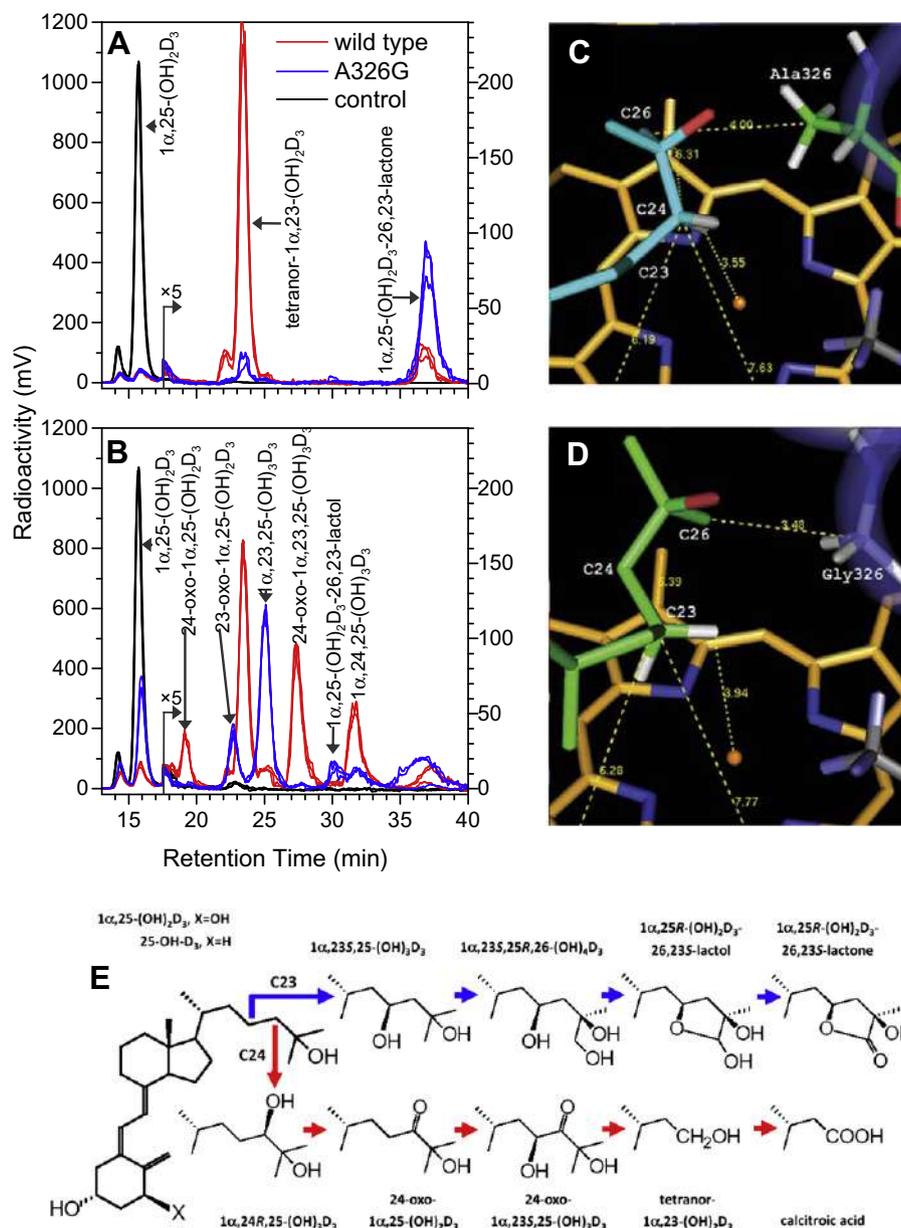
Fig. 4. Mutagenesis studies of human CYP24A1 showing the importance of Ala326. (Taken from Prosser et al. [10]). Human CYP24A1 was mutated to the opossum form at position 326 (A326G). The A326G mutation converted human CYP24A1 from a $1\alpha,25$-$(OH)_2D_3$ C24-hydroxylase into a C23-hydroxylase. (Panels A and B) HPLC chromatograms indicate the predominance of C24 pathway products produced by the wild-type enzyme, as compared with mainly C23 pathway products with the A326G mutant, resembling opossum CYP24A1, at a non-saturating (A; 0.3 µM) and saturating (B; 3.5 µM) substrate concentration. (Panels C and D) The conserved distance of the $1\alpha,25$-$(OH)_2D_3$ side chain relative to Ala326 (C; wild type, docked for C24R-hydroxylation) as compared with Gly326 (D; A326G, docked for C23S-hydroxylation) suggests the A326G creates extra space for the side chain to move deeper into the active site to position C23, as opposed to C24 above the heme for hydroxylation. (Panel E) Detailed intermediates observed in the catabolism of $1\alpha,25$-$(OH)_2D_3$ starting with 24-hydroxylation and ending in calcitroic acid; or starting with 23-hydroxylation and ending with $1\alpha,25$-$(OH)_2D_3$-26,23-lactone.

are integral to mineral metabolism [2] (see Fig. 6). This is an important consideration in diseases such as chronic kidney disease where key factors such as PTH and FGF-23 increase in a maladaptive response to loss of kidney function. Both PTH and FGF-23 directly affect vitamin D metabolism; overproduction of FGF-23 in particular can change vitamin D status resulting in increased morbidity and mortality associated with vitamin D deficiency.

Parathyroid hormone (PTH) plays an essential role in linking changes in blood calcium to bone function, primarily to avoid wide and potentially toxic fluctuations in blood calcium levels. PTH, once produced in response to low circulating calcium [2,28], stimulates renal CYP27B1 expression thus facilitating the production of

$1,25$-$(OH)_2D_3$, which in turn induces VDR-mediated intestinal calcium absorption to help correct the blood calcium deficit [29,30]. $1,25$-$(OH)_2D_3$-mediated induction of CYP24A1 expression is significantly attenuated by PTH [31–33], due to destabilization and increased degradation of CYP24A1 mRNA [34]. *In vitro* studies demonstrate that PTH-mediated suppression of CYP24A1 mRNA occurs independently of the VDR in kidney cells through the cAMP/PKA signaling pathway [31]. In contrast to CYP24A1 suppression in the kidney, PTH enhances $1,25$-$(OH)_2D_3$-mediated induction of CYP24A1 transcription, mRNA and protein through the cAMP-signaling pathway in osteoblastic cells [35–37]. PTH induction of CYP24A1 in bone cells may prevent aberrant
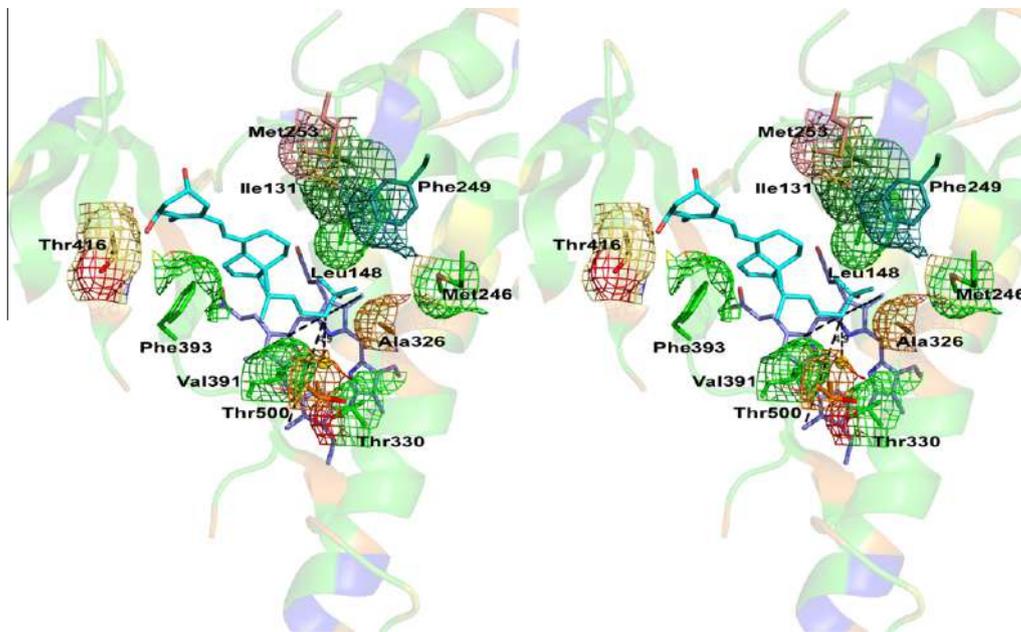
Fig. 5. Model of the active site of CYP24A1 showing key substrate contact residues. Docked $1\alpha,25$-$(OH)_2D_3$ (cyan) sits above the heme group (purple) with several active site residues (in mesh) clustered around the substrate. Helices of CYP24A1 are depicted in the background in pastel shades.
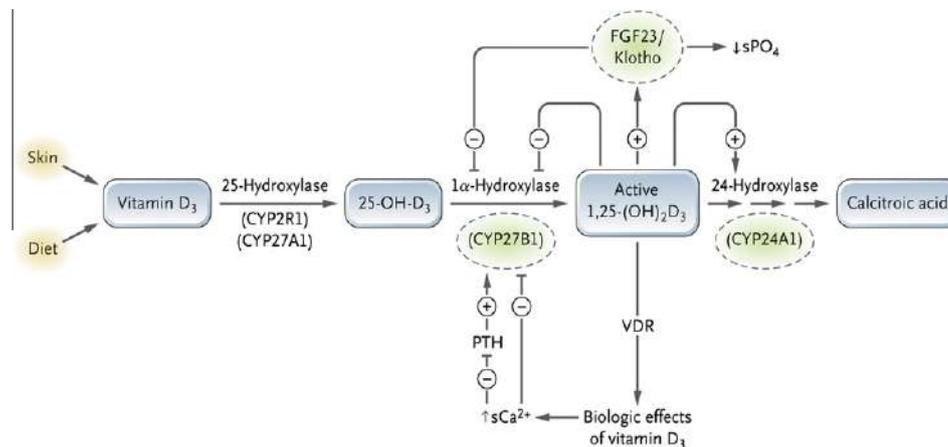

Fig. 6. Physiological role of CYP24A1 in calcium and phosphate homeostasis. (Taken from Schlingmann et al. [66]). The position of CYP24A1 in the degradation of 25-OH-D$_3$ and $1\alpha,25$-$(OH)_2D_3$ is shown in juxtaposition to the other important enzymes CYP2R1 and CYP27B1 and other hormonal factors which regulate these enzymes.

elevations in $1,25$-$(OH)_2D_3$ and resultant bone formation abnormalities [24], whereas in kidney, suppression of CYP24A1 by PTH would be expected to result in a net increase in systemic $1,25$-$(OH)_2D_3$ and subsequent levels of calcium in circulation.

As with PTH, FGF-23 also plays a central role in the regulation of mineral homeostasis affecting both expression of genes regulating serum phosphate, as well as those controlling vitamin D metabolism [38–40]. Induction of FGF-23 expression in osteocytes and osteoblasts follows rising serum phosphate levels; subsequently, FGF-23 reduces renal phosphate reabsorption by inhibiting Na/Pi co-transporter activity [41,42] and indirectly suppresses intestinal phosphate absorption by suppressing renal expression of CYP27B1 thus lowering blood $1,25$-$(OH)_2D_3$ [42–44]. FGF-23 also controls $1,25$-$(OH)_2D_3$ levels by inducing expression of CYP24A1 mRNA in the kidney [43–47]. This effect of FGF-23 in kidney may be partially dependent on the VDR, however, this remains controversial [44,47]. FGF-23 induction of CYP24A1 has also been demonstrated

in renal proximal tubule cells, albeit to a lesser extent than that observed *in vivo* [43]. While these findings confirm that FGF-23 plays an essential role in the regulation of CYP24A1, the mechanisms underlying this elevation remain relatively unknown yet important to ascertain to better understand the relationship between FGF-23 and vitamin D status in diseases, such as hypophosphatemia, CKD and cancer.

CYP24A1: pharmacological role

In addition to its involvement in the catabolism of $1,25$-$(OH)_2D_3$, CYP24A1 plays a crucial role in the clearance of various vitamin D analogs, especially those used in the treatment of CKD around the world: 19-nor-$1,25$-$(OH)_2D_2$ (paricalcitol or Zemplar), 1α-OH-D$_2$ (doxercalciferol or Hectorol) and 22-oxa-$1,25$-$(OH)_2D_2$ (OCT or Maxacalcitol) [48,8,49]. All of these vitamin D analogs

are vulnerable to metabolism at C-24 and/or C-23 and are thus inactivated in the target cells by CYP24A1. This is not to exclude metabolism in liver or specific target tissues by other cytochrome P450s such as CYP3A4, which has been shown to degrade 1,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_2$ [50,51] at pharmacological concentrations, but target cell catabolism is clearly the domain of CYP24A1 at physiological concentrations. Interestingly, CYP24A1 has also been shown to act on modified forms of the vitamin D side chain, catalyzing the oxidation of fluorine-blocked analogs such as 24-F_2-1,25-$(OH)_2D_3$ and F_6-1,25-$(OH)_2D_3$ [52,53]; hydroxylation of the anti-cancer drug, EB1089 [54]; and side-chain cleavage of the anti-psoriatic drug, calcipotriol [55].

The pharmacological involvement of CYP24A1 has a number of implications. Most important of these is that it offers a mechanism to explain the drug resistance ascribed towards vitamin D analogs after frequent administration in clinical applications, whether these are in nephrological, dermatological or cancer treatment applications [56,57]. Secondly, the lack of action of CYP24A1 towards certain "metabolism-resistant" analogs (e.g. EB1089) probably explains their extended half-life in the body, higher potency and narrower therapeutic window, as compared to 1,25-$(OH)_2D_3$ [58]. Lastly, it points to CYP24A1 being a major drug target, since CYP24A1 inhibitors would offer the potential to change the target-cell concentrations of endogenously-synthesized 1,25-$(OH)_2D_3$ or exogenously co-administered vitamin D analogs [59,60].

CYP24A1: human polymorphisms and genome-wide linkage studies

Mining of several genomic databases reveals that a number of polymorphisms of CYP24A1 have been identified in recent years and the list is growing rapidly (Fig. 7). Though little is known of the effects of these polymorphisms on CYP24A1 enzyme activity, inactivating mutations would be expected to give rise to a hypercalcemic phenotype, and since these had not been reported, it was presumed that these polymorphisms must be innocuous. However, the recent reports of human inactivating mutations (see section below), suggest that we should re-examine these polymorphisms and look more broadly for more within the general population. Furthermore, a recent genome-wide study of the determinants of serum 25-OH-D in 30,000 individuals also suggests that this would be a fruitful exercise, since it identified four relevant genes including CYP24A1, the others being the vitamin D-transport protein, DBP; the putative 25-hydroxylase enzyme, CYP2R1; and the vitamin D provitamin substrate regulator, 7-dehydrocholes-terol reductase [61] as the main modulators of circulating 25-OH-D.

CYP24A1: pathological role and implications in disease

The attenuation of vitamin D signaling by CYP24A1 has been implicated in a number of diseases including metabolic bone disease, chronic kidney disease and several types of cancer [59,60].

CYP24A1 and genetically-linked idiopathic infantile hypercalcemia

Hypercalcemic conditions are not uncommon in the pediatric literature but they appear to be a heterogeneous group of diseases including: Williams–Beuren syndrome and idiopathic infantile hypercalcemia (IIH); all characterized by transient hypercalcemia and other features. Of these, only IIH has unknown etiology and until recently, had no gene locus assigned to it [62,63]. Fifty years ago, a group headed by the famous US physician Harold Harrison [64] proposed that:

> "….IIH is a metabolic defect in the degradation mechanism of vitamin D such that excessive accumulation of the vitamin could occur"

though of course, vitamin D metabolism, and in particular, the principal catabolic enzyme, CYP24A1 were unknown at the time. In 1979, Weisman proposed that IIH may be caused by defective vitamin D esterification, though the importance of vitamin D esters in the metabolism of vitamin D is minor and more likely any role of vitamin D esters is in the storage of excessive supplies of vitamin D [65]. Recently, we along with a group of German pediatricians have been studying CYP24A1 gene polymorphisms/mutations in a cohort of nine families of German, Turkish and Russian IIH patients and showed that inactivating mutations of CYP24A1 may be the main underlying cause of the disease [66] (Fig. 7). This work reinforced the important conclusion drawn from the CYP24A1-null mouse studies that CYP24A1 is primarily a catabolic enzyme.

CYP24A1 and genetically-linked hypophosphatemia

The link between vitamin D metabolism and hypophosphatemic conditions was first established in the hypophosphatemic mouse model (Hyp), a murine homologue of X-linked hypophosphatemic rickets (XLH) in humans, initially characterized by Eicher et al. [67]. More recently, it has been shown that deletion in the 3′ region
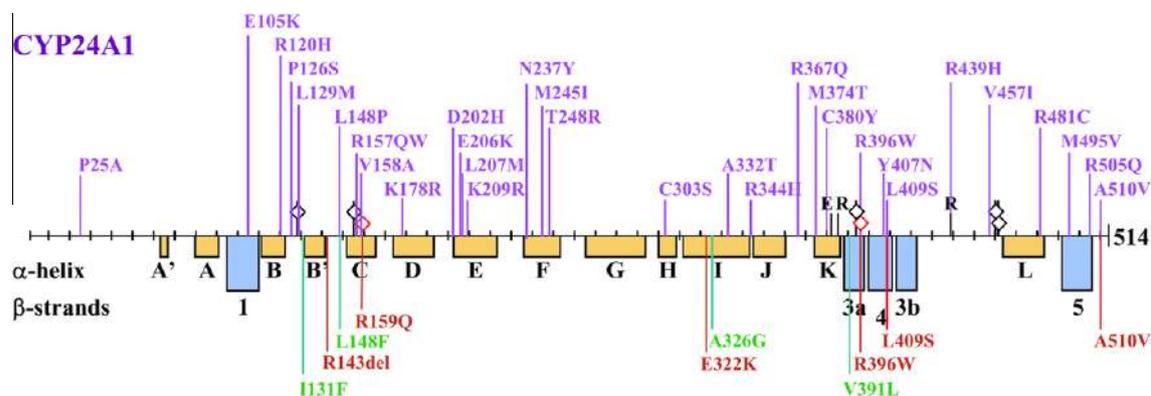

Fig. 7. Polymorphisms, naturally-occurring inactivating mutations and mutagenized residues in human CYP24A1. The figure depicts domains in the human CYP24A1 structure. SNP data base polymorphisms are shown in purple; naturally-occurring inactivating mutations which result in IIH are shown in red; and artificially-mutated residues that change regioselectivity are shown in green.

of the Phex gene [68,69] is associated with hypophosphatemia and impaired bone metabolism arising, in part, from defective renal reabsorption of inorganic phosphate at the brush border membrane [67,70,71]. Similar to human patients with XLH, hypophosphatemic mice exhibit normal levels of 1,25-$(OH)_2D_3$ in spite of significant hypophosphatemia, a condition normally associated with elevated concentrations of 1,25-$(OH)_2D_3$ in animals and humans [72,73]. Moreover, serum levels of 1,25-$(OH)_2D_3$ decrease or remain constant with further phosphate deprivation in *Hyp* mice and patients with XLH [74,75], respectively, whereas phosphate supplementation markedly increases 1,25-$(OH)_2D_3$ levels [74,75]. In normal mice, serum levels of 1,25-$(OH)_2D_3$ increase with phosphate deprivation, while remaining unchanged with supplementation [72,74].

Abnormal regulation of vitamin D metabolism indicated by the paradoxical response of 1,25-$(OH)_2D_3$ to dietary phosphate manipulation observed in mutant mice and XLH patients, is partly attributed to low CYP27B1 activity and possible changes in mRNA expression in the proximal convoluted tubule [76–79], as well as a blunted response to regulators of CYP27B1 activity, including hypophosphatemia [72,76] and PTH [80,81]. However, levels of 1,25-$(OH)_2D_3$ in serum are also correlated with the rate of renal catabolism of 1,25-$(OH)_2D_3$ in *Hyp* mice as evidence by an increased production of CYP24A1-dependent metabolites 1,24,25-$(OH)_3D_3$, 24-oxo-1,25-$(OH)_2D_3$ and 24-oxo-1,23,25-$(OH)_3D_3$ [82–84]. Accelerated catabolism of 1,25-$(OH)_2D_3$ in *Hyp* mice has been ascribed to an increase in CYP24A1 enzyme activity in renal mitochondria [84], as well as elevated levels of CYP24A1 mRNA and protein expression in the kidney proximal tubules [85].

CYP24A1: involvement in chronic kidney disease

This subject has been extensively reviewed by Petkovich and colleagues [59,60]. Vitamin D deficiency is commonly observed in patients with CKD and is causally related to secondary hyperparathyroidism, a disorder characterized by elevated serum intact PTH levels, parathyroid gland hyperplasia and imbalances in bone and mineral metabolism [86,87]. Declining renal mass and concomitant loss of renal CYP27B1 capacity in CKD is commonly associated with reductions in circulating levels of both 1,25-$(OH)_2D$ and 25-OH-D [88,89]. However, observations of low serum 1,25-$(OH)_2D$ have not been consistently linked with decreases in renal CYP27B1 expression, since levels of CYP27B1 mRNA may in some cases remain unchanged in CKD patients deficient in 1,25-$(OH)_2D$ [90]. Moreover, diminishing CYP27B1 expression levels cannot directly account for the progressive loss of serum 25-OH-D commonly observed in CKD. These findings suggest that additional intrinsic mechanisms may underlie declining vitamin D metabolites, 25-OH-D and 1,25-$(OH)_2D$, in renal disease.

In vitamin D target tissues, including the kidney, it is well established that CYP24A1 is inversely correlated with vitamin D status [2,91,92]. Using the adenine uremic rat model, recent studies have demonstrated a decline in serum 25-OH-D_3 and 1,25-$(OH)_2D_3$ levels in uremic animals [93], as well as a marked elevation CYP24A1 mRNA and protein expression in the uremic kidney in the absence of a concomitant decline in CYP27B1. While CYP24A1 mRNA levels were low or non-detectable in the kidney of vitamin D deficient animals, expression of CYP24A1 mRNA remained markedly elevated in uremic kidney tissue despite animals being rendered vitamin D deficient. These findings suggest that factors other than vitamin D determine the expression levels of CYP24A1 in the uremic state. A similar pattern of renal enzyme expression was recently reported in promycin aminonucleoside nephrosis rats which exhibit proteinuria and podocyte injury [94]. In agreement with animal models of CKD, aberrant expression of CYP24A1 was also recently reported in CKD patients with acute renal inflammation [90], as well as in renal tissue biopsied from patients with diabetic nephropathy [93]. Although CYP24A1 elevation appears to be strongly correlated with kidney injury, whether induced by hypophosphatemia, diabetes, or exposure to kidney damaging agents, the consequences of elevated CYP24A1 are not known.

CYP24A1: involvement in pathogenesis and treatment of hyperproliferative disorders

The initial demonstration that 1α,25-$(OH)_2D_3$ is an anti-proliferative, pro-differentiating agent for certain cell types *in vivo* and many cell lines *in vitro* [95], coupled with the fact that cancer cell studies have showed decreased CYP27B1 and increased CYP24A1 expression in prostatic, colonic and breast cell lines as they progress towards a more tumorigenic phenotype [96–100] has caused some researchers to speculate that cancer progression involves dysfunctional vitamin D metabolism [100]. But the hypotheses that vitamin D deficiency contributes to cancer incidence or that supplemental vitamin D_3 might prevent cancer are difficult to test because of the duration of clinical trials or the multiple confounding factors that accompany vitamin D deficiency. The VDR-knockout mouse which lacks vitamin D-mediated signaling altogether is more susceptible to chemically-induced cancers arguing that vitamin D plays a role in cancer prevention [101]. Although elevated CYP24A1 expression and reduced CYP24A1 gene silencing has been reported in specific tumors [102–104], proof that it is a causative agent in cancer development is still lacking. Nevertheless, there are many claims that CYP24A1 is a candidate oncogene [105–107].

While the link between CYP24A1 and cancer development is tentative, the importance of CYP24A1 in cancer treatment is well established. Vitamin D analogs are widely used in hyperproliferative disorders such as psoriasis and cancer [2]. As pointed out earlier in this review, the effectiveness of vitamin D analogs appears to be limited by CYP24A1-induction in target cells (e.g. keratinocytes), causing a type of drug resistance; many vitamin D analogs derive a potency advantage over 1,25-$(OH)_2D_3$ insofar as they are modified at carbons C23 and C24 to resist metabolism by CYP24A1; and certain tumor cells amplify the CYP24A1 gene in order to gain a selection advantage over surrounding normal cells.

CYP24A1 inhibitors

The possibility that increased CYP24A1 expression may be an underlying cause of vitamin D deficiency and progression of disease states suggests that this enzyme might be a potential therapeutic target. Over the past decade, a number of inhibitors have been developed in the treatment of diseases associated with elevation of vitamin D catabolism including

(a) General azole-based CYP24 inhibitors, such as ketoconazole and liarazole, which bind heme at the catalytic core of the protein [57,91].
(b) Genistein (4,5,7-trihydroxyisoflavone) is a natural product, plant-derived isoflavanoid present in soya products [108–110].
(c) A family of imidazole derivatives targeting vitamin D-related CYPs including VID-400 VID400 possesses a 40-fold increased selectivity for CYP24A1 and enhances vitamin D signaling in keratinocytes [111].
(d) Highly specific-CYP24A1 specific inhibitors. These include CTA091 (MK-24(S)-S(O)(NH)-Ph-1), a non-azole type 24-(S)-NH phenyl sulfoximine D-ring side chain vitamin D analog, which is potent and highly selective CYP24A1 inhibitor [112]. This compound does not bind to the VDR or activate

VDR-mediated transcription, and therefore is classified as a "pure" CYP24A1 inhibitor. It inhibits CYP24A1 with an IC_{50} in the low nanomolar range ($\sim$7.5 nM) and does not appreciably inhibit CYP27B1 or CYP27A1 [112]. Another series of CYP24A1 inhibitors that have VDR agonist properties can be considered mixed-CYP24A1 inhibitor/VDR agonists. The sulfone GHP-GH-16,23-diene-25S02-1 (CTA018) binds VDR and is a potent activator of VDR-mediated transcription but is not readily catabolized by the target-cell CYP24A1. CTA018 inhibits CYP24A1 with an IC_{50} 27 ± 6 nM, about 10 times more potent than the non-selective CYP24 inhibitor ketoconazole (253 ± 20 nM). It remains to be seen whether any of these CYP24A1 inhibitors will be used clinically to block CYP24A1 in disease states. Two other families of CYP24A1 inhibitors were recently synthesized [113,114].

Perspectives

This is an exciting time for research on CYP24A1. The elucidation of the crystal structure of the rat enzyme [11] opens the door to determining the precise positioning of vitamin D substrates in the substrate-binding pocket. This in turn will allow us to better understand the mechanism of multiple hydroxylations and will facilitate the development of a second generation of CYP24A1 inhibitors using rational drug design [54].

Some of the first generation of CYP24A1 inhibitors [112] have reached an advanced stage of drug development in the therapy of renal, dermatological and oncologic conditions. We now recognize that CYP24A1 over-expression can result from either 1,25-$(OH)_2D_3$ analog use [56,57]; or phosphate retention in chronic kidney disease [59,60] both of which are important, potentially-surmountable, clinical problems. Consequently, the success (or failure) of CYP24A1 inhibitors in treating these indications will determine some of the continued clinical interest in this increasingly-fashionable enzyme.

Undoubtedly, the recent discovery of inactivating CYP24A1 mutations in IIH patients [66] will also drive clinical interest in CYP24A1 research. As was pointed out earlier in this review, the number of CYP24A1 polymorphisms in the genomic databases currently stands at around 50 and is growing rapidly. One would expect that some of these polymorphisms may be correlated with mild and more severe diseases in the hypercalcemic constellation including IIH but it remains to be seen whether CYP24A1 dysregulation can be connected with other disease states, e.g. nephrolithiasis. There is no doubt that the CYP24A1-knockout mouse [21–24] still has much more to reveal about the roles of CYP24A1 in vivo. More large genome-wide studies, in addition to the Wang et al. study of the determinants of circulating 25-OH-D [61], are likely to implicate CYP24A1 activity as an important factor in mineral homeostasis.

In conclusion, though it appears that our knowledge of CYP24A1 has blossomed in recent times and now ranges from the molecular level to the clinic, there are still many questions about this enigmatic and under-appreciated enzyme that still need to be answered.

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