Hepatic Activation and Inactivation of Clinically-relevant Vitamin D Analogs and Prodrugs

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Abstract. Like most pharmaceutical agents, vitamin D analogs are subject to hepatic metabolism by a variety of cytochrome P450 (CYP)-based systems. Metabolism can involve activation as well as inactivation of the vitamin D analog and one of the more successful families includes the 1α-hydroxyvitamin D prodrugs (1α-OH-D₂, 1α-OH-D₃, 1α-OH-D₄, 1α-OH-D₅), that all require a step of activation. Some of these prodrugs are in use or clinical trial because they have a therapeutic advantage over calcitriol. However, the nature of the activation of these molecules is poorly understood, particularly with regard to the CYP isoform involved. Various transfected CYPs and hepatic cell lines combined with tandem LC-MS analysis were used to investigate the metabolism of a spectrum of vitamin D analogs, including 1α-OH-Ds and the topical analog, calcipotriol. In the case of the 1α-OH-Ds, evidence was found of multiple sites of side-chain hydroxylation consistent with the generation of more than one active form. The potential involvement of CYP27A and other putative 25-hydroxylases in 1α-OH-D activation was also shown, as well as the potential for CYP24 activation and inactivation. In the case of calcipotriol, the respective roles of non-vitamin D-related CYPs and CYP24 in the catabolism of this anti-psoriatic drug were dissected out using cell lines with or without CYP24 expression, allowing us to demonstrate the potential contribution of CYP24 to "vitamin D resistance". The implications of hepatic metabolism in the context of other facets thought to play a role in the mechanism of action of anticancer and antiproliferative vitamin D analogs are discussed.

The importance of the liver in the activation of vitamin D has long been recognized (1). Over the past decade, the nature of the cytochrome P450 (CYP) species involved in the 25-hydroxylation of vitamins D₂ and D₃ has become much clearer, even if it has not been fully resolved (2). One mitochondrial CYP and four microsomal CYPs have been implicated as potential vitamin D-25-hydroxylases, though it appears that CYP2R1 possesses many of the criteria to be identified as the physiologically-relevant enzyme (3), including a type of human vitamin D deficiency rickets associated with a mutant form of the CYP2R1 enzyme (4). These criteria are worth repeating:

i) Kinetic experiments suggest that there are at least two isoforms in the mitochondrial and endoplasmic reticulum (microsomal) compartments.

ii) The mitochondrial form is a high capacity, low affinity ‘pharmacologically-relevant’ enzyme while the microsomal form is a low capacity, high affinity “physiologically-relevant” enzyme.

iii) Together the two enzymes must explain the in vivo observations that 25-hydroxylase is weakly regulated and leads to side-chain hydroxylation of both vitamins D₂ and D₃.

There are claims that CYP27A1 has many of the properties ascribed to the mitochondrial enzyme (5), though strangely it will 24-hydroxylate vitamin D₂ but will not 25-hydroxylate it at micromolar substrate concentrations. Of the microsomal forms, only CYP2R1 meets all the criteria ascribed to the “physiologically-relevant” enzyme including the ability to 25-hydroxylate vitamin D₂. Comparisons of putative microsomal 25-hydroxylases have been published (6, 7), but no studies have demonstrated the high affinity nature of the enzyme.

Few studies of the hepatic metabolism of vitamin D analogs have been published. The principal exceptions to this statement are studies of the 1α-hydroxyvitamin D prodrugs including 1α-OH-D₃ (One-alpha; Leo Pharma, Denmark); 1α-OH-D₂ (Hectorol; Bone Care International, Madison, WI, USA); 1α-OH-D₄; 1α-OH-D₅ (IIT, Chicago IL, USA) (8). These compounds have proven to be highly

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successful in the treatment of defects of calcium homeostasis and hyperparathyroidism associated with chronic kidney disease (CKD). All of these 1α-hydroxylated analogs are presumed to be active by virtue of being metabolized in vivo to side-chain hydroxylated species analogous to 1α,25-(OH)₂D₃ in order to execute their therapeutic effects (8). The prodrug 1α-OH-D₅ (IIT) has been shown to act as a chemopreventative agent in mammary carcinogenesis models and has been approved for NCI trials in breast cancer patients (9). However, currently no metabolic studies of 1α-OH-D₅ have been published and, thus, in vitro metabolic studies using liver systems would seem to be justified. Furthermore, though in vitro studies of the metabolism of 1α-OH-D₃ and 1α-OH-D₂ have been carried out and have suggested that a variety of mitochondrial and microsomal enzymes can hydroxylate these prodrugs (Figure 1) (5-7), there have been no studies to determine if the newer isoforms (e.g., CYP2R1) can side-chain hydroxylate 1α-hydroxylated prodrugs at physiological substrate concentrations.

Calcipotriol (MC-903) is a vitamin D analog well-studied metabolically in vitro. Sorensen and colleagues (10) established that various liver preparations are able to convert the 24-hydroxylated analog into unsaturated and saturated 24-ketones and this work was extended by Masuda et al. (11), who showed that this metabolic pathway was present in vitamin D-target cells and went further to give 23- and 24- hydroxylated products as well as side-chain-cleaved metabolites. One question not answered in earlier work was the nature of the enzyme(s) responsible for the metabolism of calcipotriol. Since calcipotriol is topically applied, the metabolism of the drug in keratinocytes would seem particularly relevant. To date, since the highly inducible CYP24 represents the only vitamin D-metabolizing CYP identified in vitamin D target cells it seems logical to believe that CYP24 is involved in calcipotriol metabolism, but this hypothesis remains untested and unproven. With the availability of newer tools such as isolated CYPs, newly-synthesized 1α-hydroxylated vitamin Ds and the powerful new LC-MS technology, the metabolic objectives of the current study were to explore:

i) The ability of CYP2R1 in a cell model in situ to metabolize a physiological concentration of [9,11-³H]1α-OH-D₂.

ii) The liver-cell mediated metabolism of 1α-OH-D₅ using newer LC-MS analytical procedures (11).

iii) The putative CYP24-mediated metabolism of calcipotriol.

Materials and Methods

Vitamin D analogs. Synthetic standard 1α-OH-D₃ and 1α,25-(OH)₂D₃ were provided by Leo Pharma (Ballerup, Denmark), 1α-OH-D₂ and [9,11-³H]1α-OH-D₂ (35 Ci/mmol) by Bone Care International and 1α-OH-D₅ by IIT Research Institute (Chicago, IL, USA).
incubated with 10 nM [9,11-3H]1α-OH-D2 for 24 h. In the chromatogram traces depicted, no products were formed in control extracts (parental V79 cells or dead-cell controls) while, after the expression of mouse or human CYP2R1, an additional peak appeared running between 12-13 min in each trace and co-migrating exactly with 1α,25-(OH)2D2. This peak was absent when CYP27A1 was transfected into the same cells (data not shown).

Metabolites of 1α-OH-D5 from HepG2 cells evaluated using LC-MS-based analytical procedures. When 1α-OH-D5 (10 μM) is incubated with HepG2 cells for 24-48 h a number of putative hydroxylated products are produced which elute chromatographically more slowly than the substrate and close to 1α,25-(OH)2D3 on straight-phase HPLC. Compared with control incubations using the same cells and 1α-OH-D3 (10 μM), where there is a demonstrable production of 1α,25-(OH)2D3 and 1α,26-(OH)2D3, the yield of metabolites from 1α-OH-D5 was disappointingly low, suggesting that the rate of activation in the liver was much less efficient for 1α-OH-D5 than for 1α-OH-D3. On reverse-phase HPLC, hydroxylated metabolites of 1α-OH-D5 ran more polar than the parent molecule but less polar than 1α,25-(OH)2D3. On LC-MS (Figure 3), these metabolites all showed spectra consistent with them being mono-hydroxylated products with MH+ ions of m/z 445 (MWt=444; MWt of parent 1α-OH-D5=428). In comparison, the LC-MS of 1α,25-(OH)2D3 derived from 1α-OH-D3 is also contrasted with its parent molecule in Figure 3. Though the mono-hydroxylated products of 1α-OH-D5 are likely to be hydroxylated in the side-chain, LC-MS/MS analysis, and possibly other technologies are required to pinpoint the exact site of this hydroxylation. These studies are on-going, but the current experiments have established that hepatoma cells are able to metabolize the 1α-OH-D5 substrate to hydroxylated products, albeit less efficiently than 1α-OH-D3.

Metabolism of calcipotriol (MC-903) by V79-hCYP24 cells. Calcipotriol metabolism can be readily followed in both hepatoma and keratinocyte cell models, suggesting that this cyclopropane ring-containing analog is subject to the same type of modification at the C-24 position that is observed...
in broken cell systems and in vivo and described previously (10, 11). The studies performed here represent the first attempt to use an individual CYP, expressed in a stable manner in a Chinese hamster lung V79 host cell, to study its potential involvement in the metabolism of calcipotriol. In Figure 4, it is important to note that the host cell showed virtually undetectable metabolism without the introduction of the hCYP24 (Panel A). It is possible that the trace amount of Peak 3 in the chromatogram was incorrectly identified as the metabolite simply co-migrates with the saturated ketone of MC903 depicted in the inset of Figure 4A. This possibility is rendered more plausible considering that there was no evidence of the formation of its precursor, the unsaturated ketone (Peak 2 of Figure 4A). Another trivial explanation is that there was a minor amount of native, inducible CYP24 in the host V79 cells, although this was not observed when the experiments were performed with [1β-3H]1α,25-(OH)2D3. In any event, the ability of V79-4 to generate metabolites was extremely weak. In contrast, V79-hCYP24 cells showed a robust production of a full spectrum of calcipotriol metabolites, observed previously in liver and keratinocyte models (10, 11). While it was believed possible that CYP24 might be responsible for the production of C-24 ketone Peak 3, the subsequent 23-hydroxylated products (Peak 4; actually a mixture of 4a and 4b) and side-chain cleaved products, there was no firm evidence to prove this. Though the data suggest CYP24 may be responsible for the saturation of the Δ22-23 in the side-chain of calcipotriol, these studies cannot exclude the fact that another enzyme is responsible and is unmasked by the production of the unsaturated ketone (Peak 2 of Figure 4B) by CYP24.

Figure 3. LC-MS of specific isolated metabolic products of 1α-OH-D3 and 1α-OH-D5 produced by HepG2 cells along with their respective substrates. MS1 spectra were produced in electrospray positive (ES+) mode using a Micromass Quattro Ultima triple-quadrupole instrument in MS1 mode. Metabolites were generated by incubating 1α-OH-D3 or 1α-OH-D5 with HepG2 cells and the products isolated using preparative LC on Zorbax-SIL. Mass spectra were performed on-line using a reverse-phase LC step. ES+ spectra showed the usual MH+ ions, their dehydration products and various adducts of the metabolites. Asterisks on the 1α-OH-D5 molecule are putative hydroxylation sites targeted by hepatic CYPs.
Discussion

The availability of expression systems which contain specific, cloned cytochrome 450s has opened up the possibility of dissecting out the role of individual enzymes in the metabolism of vitamin D and its analogs. Nowhere is this truer than with the subset of CYPs found in the liver and believed to be primarily involved in activation of the parent molecule and its prodrug analogs, but the approach can also be used for target cell CYPs involved in the metabolism of calcitriol analogs. Here the usefulness of the single CYP approach was demonstrated by showing that CYP2R1 was capable of the 25-hydroxylation of 1α-OH-D2 at nanomolar concentrations and that CYP24 was responsible for some (if not all) of the steps of calcipotriol metabolism. Though, the approaches used entirely different substrate concentrations and, therefore, different modes of detection, the results were equally definitive.

The finding that CYP2R1 will 25-hydroxylate [9,11-3H]1α-OH-D2 at nanomolar concentrations makes this isoform unique and, taken together with the assertion that CYP2R1 is mutated in a patient with a form of hereditary rickets (4), and the distribution and substrate specificity studies produced by Russell’s group (13) and others (7) suggest that it must be the ‘physiologically-relevant’ microsomal form of the 25-hydroxylase. It remains to be seen if others can demonstrate the relevance of CYP2J3 and CYP3A4 to vitamin D physiology, while for the mitochondrial form, CYP27A, the most generous view of its role in vitamin D homeostasis is that it represents a “pharmacologically-relevant” 25-hydroxylase enzyme. Though 1α-OH-D2 (Hectorol) is marketed for treatment of patients with secondary hyperparathyroidism associated with CKD, this analog has potential in the treatment of hyperproliferative conditions. Indeed, one of its activated metabolites, 1α,24-(OH)2D3, formed by the action of CYP27A1 and CYP3A4, has been used to regulate the growth of prostate cancer cells in vivo and in vitro (14). The finding that there are also several enzymes in the microsomal fraction that will activate 1α-OH-D2 to 1α,25-(OH)2D3, and that CYP2R1 is one of them, is valuable new information which opens up the possibility that the prodrug

Figure 4. Lipidomic studies of the metabolites of calcipotriol produced by (A) V79-4 cells and (B)V79-hCYP24 over a 48-h incubation period. Cells were incubated with calcipotriol (10 μM) for incubation times from 0-48 h and the lipid extracts run on HPLC using a photo-diode array detector. Chromatograms show A265 traces but putative hCYP24-generated metabolite peaks were checked for their vitamin D chromophore. Only peaks marked #1-4 in the traces possessed the characteristic vitamin D chromophore. Calcipotriol was Peak 1 running at 13.5-14 min and the shoulder just preceding at 13 min was pre-vitamin D. The structures of putative metabolites marked Peaks 2, 3 and 4 are shown in Panel (A).
can be activated and have growth inhibitory activity in extrahepatic sites, since CYP2R1 is known to be more widely distributed than in the liver alone (13).

The other finding that CYP24 was involved in calcipotriol metabolism is novel and relevant to those using calcipotriol for the treatment of psoriasis. Clinical reviews of the use of topical calcipotriol report that approximately 70% of mild to moderate psoriasis patients respond positively to treatment (15). Whether these patients develop a resistance to calcipotriol or whether there are other mechanisms to explain the lack of responsiveness to calcipotriol in 30% of patients remains unclear. It is well known that the CYP24 gene is highly inducible by vitamin D agonists (16), so it is interesting to speculate that tissues exposed to such agents build up a resistance to the actions of the vitamin D analogs by CYP24 induction. Others have produced human tumor staging data to support the idea that CYP24 is a potential tumor suppressor gene which presumably acts by lowering the 1α, 25-(OH)2D3 levels in cancer cells (17). Thus, the demonstration that CYP24 is involved in the catabolism of calcipotriol would suggest that patients who exhibit elevated CYP24 in responding tissues might show short-term improvement or fail to respond altogether. A test of this argument might be the use of a CYP24 inhibitor combined with a vitamin D agonist in “resistant” or non-responsive patients. Such a strategy is not dissimilar to that currently in advanced clinical trials by Cytochroma Inc. (Markham, Canada). The finding that CYP24 might be responsible for the saturation of the Δ22-23 in the side-chain of calcipotriol is reminiscent of our work (18) using 1α,24-(OH)2D3 to produce saturated, side-chain-cleaved metabolites when incubated with the CYP24-containing keratinocyte HPK1A-ras. The success of our studies suggests that V79-CYP24 cell assays (with the V79 control cells) could be used to determine the potential role of CYP24 in the catabolism of all calcitriol analogs in a routine manner, along with the usual battery of hepatic CYP assays commonly employed during pharmacology testing.

The study of 1α-OH-D3 by HepG2 cells provided the first positive evidence that the addition of the C24-C29 ethyl group in the side-chain of 1α-OH-D3 does not block hepatic cell activation of the prodrug. Though the efficiency of 1α-OH-D3 modification was certainly reduced over that observed in the control cultures with 1α-OH-D3, measurable amounts of hydroxylated products were still formed. To date, these products have only been partially identified as mono-hydroxylated metabolites of 1α-OH-D3. A more definitive identification is impossible because of the lack of useful fragments in the MS1 mode. Further information should be available after the same compounds have been subjected to the MS2 mode of LC-MS/MS. It should be noted that, while LC-MS/MS is a valuable and convenient addition to the armamentarium of the vitamin D analyst, it shows a disappointing inability to distinguish between isomers of vitamin D. In this respect, it is markedly inferior to its more laborious but more structurally-informative sister technique of GC-MS (19). In the case such as here, where no synthetic hydroxylated derivatives of 1α-OH-D3 are currently available to be compared with unknown metabolites, the analyst is unable to assess LC retention times and has no definitive fragments to base identification upon. Consequently, the identification is equivocal. Nevertheless, the number (three) of distinct metabolite peaks in the extracts of liver-derived cells is less than the number (seven) of possible hydroxylation sites in the side-chain of 1α-OH-D3 (See Figure 3, lower left panel), our work suggests that the enzymes involved generated a finite number of isomers. Based upon our experience with liver cells and 1α-OH-D3, we would expect one of these products to be 1α,25-(OH)2D3, though 1α,26-(OH)2D3 or 1α,29-(OH)2D3 are other possibilities (20, 21).

Overall, the studies reported here demonstrate the versatility of the CYP complement of the liver to metabolize any synthetic vitamin D analog made by pharmaceutical chemists and indicates the complexity of the task of making effective anticancer vitamin D drugs that cause growth arrest in cancer cells before they are inactivated by CYPs.

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References


