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Hydroxylation of 20-hydroxyvitamin D3 by human CYP3A4

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ABSTRACT

20*S*-Hydroxyvitamin D3 [20(OH)D3] is the biologically active major product of the action of CYP11A1 on vitamin D3 and is present in human plasma. 20(OH)D3 displays similar therapeutic properties to 1,25-dihydroxyvitamin D3 [1,25$(OH)_2$D3], but without causing hypercalcaemia and therefore has potential for development as a therapeutic drug. CYP24A1, the kidney mitochondrial P450 involved in inactivation of 1,25$(OH)_2$D3, can hydroxylate 20(OH)D3 at C24 and C25, with the products displaying more potent inhibition of melanoma cell proliferation than 20(OH)D3. CYP3A4 is the major drug-metabolising P450 in liver endoplasmic reticulum and can metabolise other active forms of vitamin D, so we examined its ability to metabolise 20(OH)D3. We found that CYP3A4 metabolises 20(OH)D3 to three major products, 20,24*R*-dihydroxyvitamin D3 [20,24*R*$(OH)_2$D3], 20,24*S*-dihydroxyvitamin D3 [20,24*S*$(OH)_2$D3] and 20,25-dihydroxyvitamin D3 [20,25$(OH)_2$D3]. 20,24*R*$(OH)_2$D3 and 20,24*S*$(OH)_2$D3, but not 20,25$(OH)_2$D3, were further metabolised to trihydroxyvitamin D3 products by CYP3A4 but with low catalytic efficiency. The same three primary products, 20,24*R*$(OH)_2$D3, 20,24*S*$(OH)_2$D3 and 20,25$(OH)_2$D3, were observed for the metabolism of 20(OH)D3 by human liver microsomes, in which CYP3A4 is a major CYP isoform present. Addition of CYP3A family-specific inhibitors, troleandomycin and azamulin, almost completely inhibited production of 20,24*R*$(OH)_2$D3, 20,24*S*$(OH)_2$D3 and 20,25$(OH)_2$D3 by human liver microsomes, further supporting that CYP3A4 plays the major role in 20(OH)D3 metabolism by microsomes. Since both 20,24*R*$(OH)_2$D3 and 20,25$(OH)_2$D3 have previously been shown to display enhanced biological activity in inhibiting melanoma cell proliferation, our results show that CYP3A4 further activates, rather than inactivates, 20(OH)D3.

1. Introduction

20*S*-Hydroxyvitamin D3 [20(OH)D3] is the major product of CYP11A1 action on vitamin D3 [1–4], and acts as a biased agonist on the vitamin D receptor displaying anti-proliferative, anti-inflammatory and pro-differentiative activity [5–11]. Unlike 1,25-dihydroxyvitamin D3 [1,25$(OH)_2$D3], it does not induce hypercalcaemia at high doses (up to 60 μg/kg in rodents) [6,12,13]. 20(OH)D3 exhibits anti-inflammatory and anti-fibrogenic activity in mice *in vivo* [8,14], and reduces DNA damage in skin caused by ultraviolet radiation [8,9,15]. It therefore has potential as a therapeutic agent via topical or systemic administration. 20(OH)D3 is present in human plasma at a concentration of approximately 3 nM, a concentration higher than that required to observe biological effects *in vitro*, suggesting it might exert important physiological effects only partially overlapping with those of 1,25$(OH)_2$D3 [16,17].

The kidney mitochondrial P450, CYP24A1, carries out the inactivation of 1,25$(OH)_2$D3, initially hydroxylating it at C24 and then further oxidising the side chain to give the excretory product, calcitroic acid [18–20]. CYP24A1 also hydroxylates 20(OH)D3 at C24 giving both enantiomers of 20,24-dihydroxyvitamin D3 [20*S*,24*R*$(OH)_2$D3 and 20*S*,24*S*$(OH)_2$D3] as well as hydroxylating it at C25 producing 20,25-dihydroxyvitamin D3 [20,25$(OH)_2$D3] [21–23]. 20,24*R*$(OH)_2$D3 and 20,25$(OH)_2$D3 inhibited colony formation by SKMEL-188 melanoma cells more strongly than the parent 20(OH)D3, indicating they have enhanced anti-proliferative activity [21]. Therefore, CYP24A1 appears to activate rather than inactivate 20(OH)D3 [21].

Another potential site for the metabolism of 20(OH)D3 is the liver, which is the major tissue involved in drug metabolism in the body. Here, the initial 25-hydroxylation step in the activation of vitamin D3 is carried out by either microsomal CYP2R1, or mitochondrial CYP27A1 [24]. CYP27A1 also hydroxylates 20(OH)D3 producing 20,25$(OH)_2$D3 and 20,26-dihydroxyvitamin D3

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[20,26(OH)$_2$D3] [25]. Human liver microsomes do not contain CYP24A1 or CYP27A1 since these are both mitochondrial P450s [24,26], but do contain CYP3A4, the most abundantly expressed P450 enzyme in the human liver and small intestine [27,28]. Besides its well-known role in drug metabolism, CYP3A4 can metabolise various vitamin D analogues. It acts as a vitamin D 24- and 25-hydroxylase on substrates such as 1α-hydroxyvitamin D2, 1α-hydroxyvitamin D3 and vitamin D2, but does not act on vitamin D3 [29,30]. The ability of CYP3A4 to hydroxylate 1,25(OH)$_2$D3 has been compared with that of CYP24A1, revealing that both enzymes hydroxylate 1,25(OH)$_2$D3 at the same carbon atoms, C23 and C24, but with opposite product stereoselectivity [31]. In addition to this ability to hydroxylate the vitamin D side chain, CYP3A4 has been reported to hydroxylate the A-ring of 25-hydroxyvitamin D3 [25(OH)D3], producing 4α,- and 4β,25-dihydroxyvitamin D3, with these products being detected in the plasma [32].

Mouse liver microsomes can act on 20(OH)D3 and like CYP24A1 in the kidney, produce the biologically active products, 20,24(OH)$_2$D3 and 20,25(OH)$_2$D3 [33]. Mouse liver microsomes do not express CYP3A4, but do express six other family 3A isoforms [34]. Whether any of these are responsible for 20(OH)D3 metabolism is unknown. Since CYP3A4 is the most abundantly expressed P450 in human liver microsomes and can act on 25(OH)D3 and 1,25(OH)$_2$D3, we investigated its ability to metabolise 20(OH)D3 using both recombinantly expressed enzyme (supersomes) and human liver microsomes.

2. Materials and methods

2.1. Materials

A mixed gender 50-donor pool of human liver microsomes, and supersomes made from baculovirus-infected insect cells that express recombinant human CYP3A4 with P450 reductase and cytochrome b_5, were purchased from Corning (Corning, NY). NADPH was purchased from Merck (Darmstadt, Germany). Glucose-6-phosphate and azamulin were from Sigma (NSW, Australia), glucose-6-phosphate dehydrogenase was from Roche (NSW, Australia) and 2-hydroxypropyl-β-cyclodextrin (HP-β-CD) was from Cerestar (Hammond, IN). Troleandomycin was from Enzo Life Sciences (Farmingdale, NY). All solvents used were of HPLC grade, and were purchased from Merck (Darmstadt, Germany). 20(OH)D3 was synthesised enzymatically by the action of recombinant bovine CYP11A1 on vitamin D3 and was purified by TLC and reverse-phase HPLC [2,4]. 20,25(OH)$_2$D3, 20,24*R*(OH)$_2$D3 and 20,24*S*(OH)$_2$D3 were produced by the action of recombinant rat CYP24A1 on 20(OH)D3 [21,22]. 20,26(OH)$_2$D3 was produced from the action of recombinant CYP27A1 on 20(OH)D3 [25]. The structures of these metabolites including the stereochemistry have been determined previously by NMR [21–23]. Concentrations of hydroxyvitamin D standards were determined from their absorbance at 263 nm using an extinction coefficient of 18,000 M^{-1} cm^{-1} [35].

2.2. Metabolism by CYP3A4 supersomes

Substrates (see Results for concentrations) solubilised in 0.45% HP-β-CD [3,36] were incubated with CYP3A4 supersomes, at a final CYP3A4 concentration of 60 nM in buffer comprising 50 mM K_2HPO_4 (pH 7.4), 3.3 mM $MgSO_4$, 500 μM NADPH, 2 mM glucose-6-phosphate and 2 U/mL glucose-6-phosphate dehydrogenase. The typical incubation volume was 0.5 mL. Tubes containing all components except supersomes were preincubated for 8 min at 37 °C, then the reaction started by the addition of the CYP3A4 supersomes. Following incubation at 37 °C (see Results for times), reactions were stopped by the addition of 2.5 vols of ice-cold dichloromethane. Tubes were centrifuged (670 × *g* for 10 min) and the lower organic phase retained. This extraction was repeated twice more, each time with 2.5 vols dichloromethane. The extracted secosteroids were dried under nitrogen gas at 30 °C and dissolved in the solvent required for HPLC analysis (see below), and stored at −20 °C.

2.3. Metabolism of 20(OH)D3 by human liver microsomes

The procedure used was similar to the one described previously for mouse liver microsomes [33]. Human liver microsomes (1.5 mg/mL) were incubated with 20(OH)D3 solubilised in 0.45% HP-β-CD, in buffer comprising 0.25 M sucrose, 50 mM Hepes (pH 7.4), 20 mM KCl, 5 mM $MgSO_4$, 0.2 mM EDTA, 500 μM NADPH, 2 mM glucose-6-phosphate and 2 U/mL glucose-6-phosphate dehydrogenase. Tubes were preincubated for 5 min at 37 °C with all components except the microsomes which were used to start the reaction. Following incubation at 37 °C, reactions were stopped with 2.5 vols ice-cold dichloromethane and extracted as described in Section 2.2.

2.4. Reverse-phase HPLC and mass spectrometry

The 20(OH)D3 metabolites produced from the action of CYP3A4 supersomes or human liver microsomes were analysed using a Perkin Elmer HPLC system (Biocompatible Binary Pump 250 or Flexar Binary Pump Series 200) with a UV detector set at 265 nm, equipped with a C18 column (Grace Alltima, 25 cm × 4.6 mm, particle size 5 μm). Metabolites were initially analysed using a gradient of 45–100% acetonitrile in water for 30 min, followed by 100% acetonitrile for 35 min, at a flow rate of 0.5 mL/min. As this system was unable to achieve full separation of some metabolites, a second solvent system was used which comprised a gradient of 64–100% methanol in water for 20 min, followed by 100% methanol for 25 min or 30 min, at a flow rate of 0.5 mL/min. Quantitation of products was based on the major products having an unaltered conjugated triene structure like 20(OH)D3, as shown by their UV spectrum with a peak at 263 nm (Fig. S2). Therefore, each product gives a proportional response from the UV detector based on their concentration. The amount of each major product could then be calculated from the initial concentration of substrate and the percentage of substrate converted, based on peak integration [3]. The kinetic parameters were obtained by fitting the Michaelis-Menten equation to experimental data using Kaleidagraph 4.0 (Synergy Software). For mass spectrometry, metabolites were purified and collected using both acetonitrile-water and methanol-water solvent systems [33,37]. The purified metabolites (2 nmol) were reconstituted in 30 μL of 70% methanol + 0.1% formic acid, with 20 μL being injected into the LC/MS. These assays employed electrospray ionization and were performed as a service by UWA Center for Metabolomics, as described previously [37].

2.5. Incubation of microsomes and supersomes with CYP3A4 inhibitors

Incubations were carried out with 20(OH)D3 (10 or 50 μM) solubilised in 0.45% HP-β-CD and buffers as described for CYP3A4 supersomes or human liver microsomes (Sections 2.2 or 2.3, respectively). The CYP3A4 inhibitor, troleandomycin [38] was added from an ethanol stock to a final concentration of 250 μM, azamulin [39] was added from a methanol stock to 1 or 10 μM and isoniazid [40] was added from a methanol stock to 1 mM, prior to preincubation [29,30]. Reactions were started by the addition of either CYP3A4 supersomes or human liver microsomes and tubes incubated for 20 or 30 min, respectively, at 37 °C. Human liver microsomes were also preincubated with troleandomycin (10 μM)