

Faculty of Pharmacy, Uppsala University

LICENTIATE THESES

50

**Vitamin D metabolism in osteoblast-like cells: effects of
drugs on inactivation by CYP24A1**

by

Ahmad Zayny



UPPSALA
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Summary

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This licentiate thesis will be defended on Wednesday, May 30, 2018 at 13:15 in C4:305, BMC, Husargatan 3, Uppsala. The presentation and discussion will be conducted in Swedish.

Abstract

Zayny, A. 2018 Vitamin D metabolism in osteoblast-like cells: effects of drugs on inactivation by CYP24A1

Vitamin D is essential for bone function, and deficiency in active vitamin D hormone can lead to bone disorders. Long-term treatment with glucocorticoids and antiretroviral drugs used to treat HIV infection, results in osteoporosis and increased risk of fractures. Much remains unclear regarding the effects of these compounds in bone cells. In the current study, human osteosarcoma Saos-2 cells and primary human osteoblasts were found to express mRNA for the vitamin D receptor as well as activating and deactivating enzymes in vitamin D₃ metabolism. These bone cells exhibited CYP24A1-mediated 24-hydroxylation, involved in deactivation of the active vitamin form. However, bioactivating vitamin D₃ hydroxylase activities were not detected in either of these cells, indicating that local vitamin D bioactivation is not significant in osteoblasts.

Several glucocorticoids and antiretroviral drugs, including prednisolone, efavirenz and ritonavir, down regulated CYP24A1 mRNA expression. Prednisolone and ritonavir also down regulated CYP24A1-mediated 24-hydroxylase activity in both Saos-2 and primary human osteoblasts.

Also, prednisolone significantly suppressed a human CYP24A1 promoter-luciferase reporter gene in Saos-2 cells co-transfected with the glucocorticoid receptor. Thus, the results of the present study show suppression by glucocorticoids on CYP24A1 mRNA, CYP24A1-mediated metabolism and CYP24A1 promoter activity in human osteoblast-like cells. Interestingly, ritonavir markedly potentiated the induction of CYP24A1 mRNA expression by 1,25-dihydroxyvitamin D₃ suggesting that ritonavir may have different regulatory effects depending on the vitamin D₃ metabolite levels

As part of this study, we examined if glucocorticoids are formed locally in Saos-2 cells. The experiments indicate formation of 11-deoxycortisol, a steroid with glucocorticoid activity, which can bind the glucocorticoid receptor. Our findings showing effects of glucocorticoids and antiretroviral drugs on CYP24A1 expression in human osteoblasts indicate a previously unknown mechanism for effects of glucocorticoids and antiretroviral drugs in human bone, where effects of these drugs may lead to altered levels of active vitamin D₃.

Keywords:

vitamin D, bone, metabolism, osteoblast, osteosarcoma, steroid, glucocorticoid, antiretroviral drug

List of papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

- I Zayny A, Almokhtar M, Wikvall K, Ljunggren Ö, Ubhayasekera K, Bergquist J, Norlin M. Vitamin D metabolism in osteosarcoma Saos-2 cells and primary human osteoblasts: Effects of glucocorticoids on vitamin D₃ 24-hydroxylase. *Submitted*.
- II Zayny A, Wikvall K, Norlin M. Antiretroviral drugs interfere with vitamin D₃ 24-hydroxylase (CYP24A1) expression in Saos-2 cells. *Manuscript*.

Abbreviations

VDR	vitamin D receptor
RXR	retinoid X receptor
RANKL	receptor activator of nuclear factor kappa B ligand
HIV	human immunodeficiency virus
ART	antiretroviral therapy
cARTs	combination treatment with different ARTs
NRTI	nucleoside analog reverse transcriptase inhibitor
NNRTI	non-nucleoside analog reverse transcriptase inhibitor
BMD	bone mineral density
GR	glucocorticoid receptor
GRE	glucocorticoid response elements
hOB	human osteoblasts
FBS	fetal bovine serum
ATCC	American Type Culture Collection
RLU	relative light units

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Introduction

Vitamin D

Vitamin D is one of the endogenous substances that have an important role in the regulation of calcium and phosphorus levels in the blood. In addition to these effects, vitamin D has several non-calcemic functions in the body [1]. The vitamin D receptor (VDR) and the enzymes responsible for bioactivation of vitamin D have been identified in most cell types in the body. This indicates that vitamin D may have very important roles [2]. Vitamin D deficiency has been defined as having a circulating level of 25-hydroxyvitamin D₃ in the blood less than 20 ng/ml (50 nmol/l) while insufficiency corresponds to between 21 and 29 ng/ml (52–72 nmol/l) [3-5]. Vitamin D deficiency was an epidemic in Europe in the late 1800s. Vitamin D was isolated in the 1930s and it was also discovered that vitamin D could be formed from 7-dehydrocholesterol by UV-radiation in the skin. Vitamin D is a fat-soluble vitamin/pro-hormone. Vitamin D can also be obtained from the diet, both from animal (vitamin D₃, cholecalciferol) and plant sources (vitamin D₂, ergocalciferol) (fig. 1). Vitamin D occurs naturally in fatty fish, egg yolk and liver [6–8].

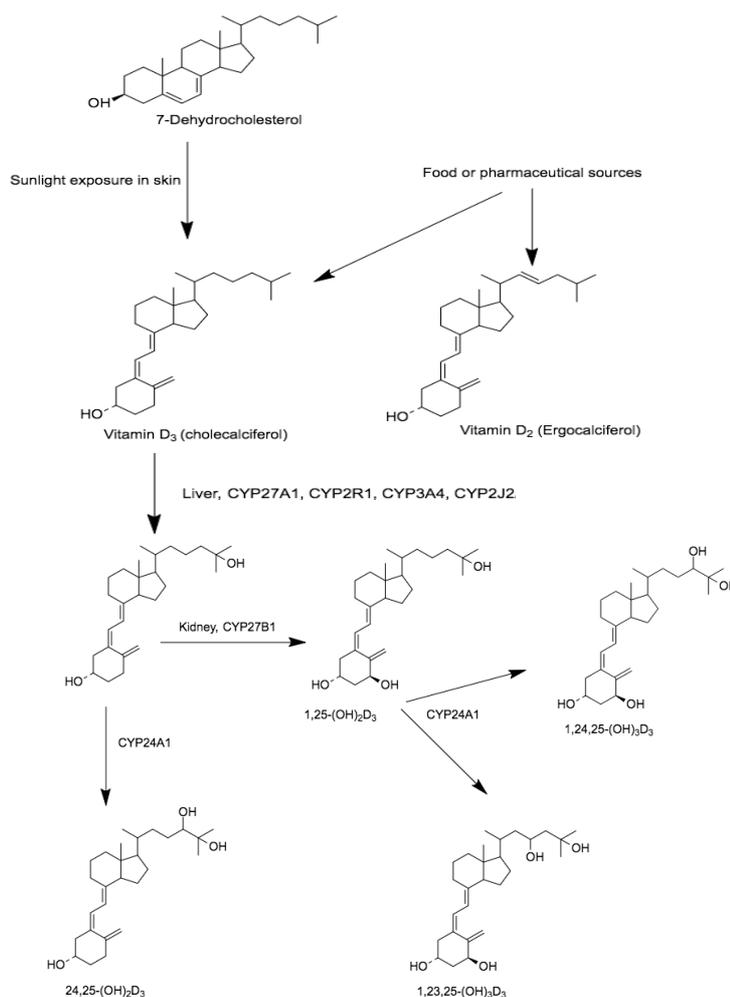


Fig. 1. Overview of enzymes in vitamin D metabolism

Vitamin D metabolism and mechanism of action

Both vitamin D forms (vitamin D₃ and vitamin D₂) must be bioactivated after ingestion via food or through synthesis in the skin. Biological activation of vitamin D occurs through hydroxylation in two steps, first via activation/hydroxylation in the liver, followed by activation/hydroxylation in the kidneys [9]. In the liver, 25-hydroxylation by CYP2R1 and other enzymes (25-hydroxylases) produces 25-hydroxyvitamin D₃ (calcidiol) [10–12] which is the major circulating form of vitamin D [13]. The 25-hydroxyvitamin D₃ metabolite has been reported to down-regulate 25-hydroxylase enzymes [12, 14]. The most active form of vitamin D, 1 α ,25-dihydroxyvitamin D₃ (calcitriol), is formed via 1-hydroxylation in the kidneys by CYP27B1 [15, 16]. Parathyroid hormone and low phosphate levels stimulate CYP27B1 to form more 1 α ,25-dihydroxyvitamin D₃ and high levels of this metabolite inhibit CYP27B1 [17,18]. Inactivation occurs through CYP24A1 (24-hydroxylase) in the kidneys and the CYP24A1-formed products are 24,25-dihydroxyvitamin D₃ or 1,24,25-trihydroxyvitamin D₃ (fig. 1). High levels of 1 α ,25-dihydroxyvitamin D₃ stimulate CYP24A1 expression [14, 19-20].

1 α ,25-Dihydroxyvitamin D₃ is a fat-soluble molecule and therefore requires a transport protein (vitamin D-binding protein) for transport in the blood. Vitamin D effects are mediated via VDR. VDR is a transcription factor that belongs to the steroid hormone nuclear receptor family. This receptor contains three different domains; an N-terminal DNA binding domain with two zinc fingers that bind to DNA elements, a C-terminal ligand binding domain, and a domain binding these two domains together. VDR is found in different tissues of the body [21]. After active vitamin D binds to VDR, VDR undergoes heterodimerization with retinoid X receptor (RXR). When VDR together with RXR binds to VDR elements, coregulatory complexes needed to induce genomic activities are recruited [22]. VDR can be regulated by activators or repressors [23]. Binding of VDR to a specific element in the DNA (vitamin D response element) affects gene transcription resulting in altered amount of mRNA that leaves the nucleus for further translation into the corresponding protein.

Vitamin D functions

Vitamin D is now classified as a steroid hormone because recent research has shown that vitamin D has several hormone-like functions in cells. The overall function of active vitamin D is to maintain calcium and phosphate homeostasis [24, 25]. This is performed by three different actions: The primary action is by increasing absorption of calcium in the intestine when calcium serum levels are low. An increased absorption of calcium occurs mainly in the duodenum but also in the distal segments of the intestine [26]. Before absorption of calcium to the blood it must be bound to a special protein, calcium-binding protein which is produced by the intestinal cells. Increased absorption through intestinal cells is obtained by vitamin D upregulation of calcium transport

channels and calcium binding protein (calbindin D9K) expression. The second action is by minimizing renal excretion of calcium and increasing renal reabsorption of calcium. The last action is by stimulating bone demineralization to maintain calcium serum levels if necessary [27–29].

The active $1\alpha,25$ -dihydroxyvitamin D_3 hormone is very important for bone health [30–32]. In addition to effects on calcium and phosphate homeostasis, other findings on vitamin D showed that this compound inhibits cell proliferation [33], stimulates cell differentiation [34], affects the immune system [35] and influences brain development [36]. Vitamin D affects transcription of genes that increase the risk of autoimmune diseases such as diabetes [37], cardiovascular diseases, diseases of the nervous system such as multiple sclerosis [37] and various types of cancers (breast, prostate) [38, 39].

Bone mineralization

Bone mineralization is a multiphasic process where osteoblast cells are involved in bone formation. Osteoblast cells form small vesicles containing calcium bound to calcium-binding acidic phospholipids and calcium-binding proteins. When the solubility product of phosphate and calcium is exceeded, mineral deposits are formed in the extracellular vesicles. This results in mineralization occurring outside the vesicles and leads to the accumulation of mineral [26, 37, 40].

Bone undergoes continuous remodeling throughout its life. Bone mineralization occurs via osteoblast cells that form bone (anabolic process). Resorption of bone is performed by osteoclast cells that remove bone. There is a connection between the actions of these two cell types but they are not necessarily balanced [26].

Calcitriol is a potent stimulator for osteoclast cell formation. This may be mediated by the VDR-mediated effect of calcitriol in osteoblasts leading to increased expression and release of receptor activator of nuclear factor kappa B ligand (RANKL) from osteoblast cells. RANKL can stimulate osteoclast formation and its function, i.e. to remove bone [41].

As a consequence of vitamin D deficiency there is an indirect decrease in mineralization, which, if present for a long time, can lead to serious diseases, e.g. osteoporosis [31, 42]. Osteoporosis is characterized by a reduction of bone density and a change in bone structure [43-44].

Antiretroviral drugs

Human immunodeficiency virus (HIV) is a serious and important public health problem. Since the 1980s, more than 35 million people have died because of the infectious disease (AIDS) caused by this virus. The prevalence of HIV infection in 2015 was 36.7 million and 1.1 million people die every year [45]. The current medication for human immunodeficiency virus (HIV) is antiretroviral

therapy (ART) that do not cure the disease or kill the HIV-virus [46]. WHO recommended antiretroviral combination therapy for all patients with HIV infection [47]. This was due to data based on clinical trials showing the benefits for these patients [48]. Treatment with antiretroviral therapy (ART) results in lower plasma viral load, which results in reduced infections and infection development. Treatment with antiretroviral drugs improves quality of life and prolongs the life of these patients. These patients must be treated with antiretroviral drugs throughout their lives because the drugs are not effective enough to cure this disease.

There are 25 different ARTs that work differently depending on which life cycle stage of the virus that is affected. [49]. Some of these ARTs are no longer used because of their serious side effects and because they are less effective against HIV viruses. Combination treatment with different ARTs (cARTs) provides the most effective treatment because of the use of smaller doses and the decrease in side effects. International guidelines therefore recommend that a first-hand treatment consist of two nucleoside analogue reverse transcriptase inhibitors (NRTI), combined with for example a non-nucleoside analogue reverse transcriptase inhibitor (NNRTI) or a protease inhibitor [45]. Efavirenz is an example among those belonging to NNRTI drugs. Efavirenz is a non-competitive inhibitor of the HIV-1 reverse transcriptase enzyme resulting in decreased enzyme activity [49]. HIV-2 does not respond to efavirenz. The recommended dose of efavirenz is 600 mg/day (3-12 μ M plasma concentration) [50-51].

In a recently infected patient with HIV, initiation of cART with ritonavir-boosted protease inhibitor (PIs) medication is recommended [52]. Ritonavir is a peptidomimetic protease inhibitor of HIV-1 and HIV-2 proteases. Inhibition of protease enzyme results in inhibition of a new virus infection cycle [53]. The recommended dose of antiretroviral drug is 600 mg two times per day (7-13 μ M plasma concentration) [50, 54].

Antiretroviral drugs have non-AIDS-related complications leading to dysfunctions of e.g. the liver, kidneys and bone [55–59]. Previous studies have shown that vitamin D insufficiency as well as therapy with various antiretroviral drugs against HIV infection are associated with low bone mineral density (BMD) [59–64]. Treatment with efavirenz is associated with reduction of bone mineralization [63, 65]. Various clinical trials showed that HIV patients had increased incidence of low BMD and osteoporosis [31, 66].

Glucocorticoids

Glucocorticoids are steroid hormones that have several different effects in our bone cells and elsewhere in the body. Glucocorticoids affect DNA by binding to cytoplasmic glucocorticoid receptor (GR). GR affects different cells differently and is expressed in almost every cell in the

body. GR regulates cell development, metabolism and immune response. GR is a cytosolic multi-protein complex receptor composed of three different domains (N-terminal transactivation domain, a central DNA-binding domain and a C-terminal domain) [67]. When glucocorticoid hormone binds to GR, the receptor undergoes conformational change resulting in the dissociation of the associated proteins. GR is activated and translocated into the cell nucleus [68-69]. GR, which binds directly to glucocorticoids, controls the gene expression of target genes [69].

Endogenous glucocorticoids are necessary for osteoblast function and for bone homeostasis. These endogenous substances are necessary for cell differentiation, proliferation and apoptosis [70]. Normal physiological levels of glucocorticoids are crucial for normal cell development [71]. High levels of glucocorticoids lead to increased bone resorption by affecting the RANKL expression and also by down-regulation of osteoprotegerin. Furthermore, glucocorticoids induce apoptosis in osteoblast cells [72]. Down-regulation of insulin-like growth factors due to glucocorticoid activity leads to an inhibitory effect on bone formation [73].

Glucocorticoids are often used as anti-inflammatory drugs for patients with inflammatory, allergic and autoimmune diseases. Treatment with glucocorticoid analogues for a long time may have serious side effects e.g. osteoporosis or low bone density [74]. Glucocorticoid-induced osteoporosis is the most common form of secondary osteoporosis [75]. Long-term treatment with glucocorticoids results in a substantially increased risk of fractures [15]. Glucocorticoids may have a negative impact on bone cells in several ways and are known to affect several types of bone cells. Reported effects of glucocorticoids in the bone include for instance effects on osteoblast growth and viability as well as interference with the action of cytokines [76-77]. In some studies, but not others, treatment with glucocorticoids have been associated with altered circulating vitamin D levels in patients [78].

Generally, for more specific identification of how drugs affect osteoblasts, it is very important to start studying mechanisms for related side effects of these drugs. By studying mechanisms for how these drugs would interfere with for example vitamin D or with treatment with glucocorticoids and other drugs, treatment could be improved, for example by prophylaxis treatment to avoid serious side effects. An important aspect of side effects is to first study the specific cell type/organ where the side effect occurs [31,79].

Aims and objectives

Currently there is yet much to investigate about the effects of glucocorticoids and antiretroviral drugs on vitamin D metabolism and the influence of these drugs on osteoblast function.

Two main aims were identified:

- To investigate vitamin D metabolism and the effects of glucocorticoids on vitamin D metabolism in human osteosarcoma Saos-2 cells and primary human osteoblasts (hOB)
- To investigate the effects of antiretroviral drugs on vitamin D metabolism in human osteosarcoma Saos-2 cells

Materials and methods

Cultures of primary human osteoblasts (hOB) and Saos-2 cells

Primary cultures of hOB cells were isolated from human bone obtained from the proximal femur in patients undergoing endoprosthetic hip surgery as previously described [80]. The primary hOB cells were then maintained in α -MEM supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics/antimycotics in 5% CO₂ at +37 °C. The medium was changed twice weekly until the cell confluence was 80%. Then the cells were trypsinated and seeded into 60-mm tissue culture dishes for the different experiments.

Saos-2 cells, established from primary osteogenic sarcoma cells from a 11 year old female 1973, were obtained from American Type Culture Collection (ATCC) [81]. These cells are used as a model representing human osteoblastic cells [82]. Saos-2 cells were cultured in McCoy's 5A (modified) medium (high glucose, phenol red, L-glutamine, bacto-peptone), supplemented with 1% antibiotics/antimycotics and 10% FBS. The cells were grown in 5% CO₂ at +37 °C in 100-mm tissue culture dishes. The cells were cultured to 80% confluence for further experiments and studied at passages 13– 30. The cells were seeded and analysed in 6-well plates, 12-well plates or 60-mm tissue cultures dishes. 100-mm tissue plate dishes were used for some experiments on enzymatic activity to increase the number of cells per incubation.

Treatment of Saos-2 and hOB cells and mRNA expression analysis

The Saos-2 and hOB cells were seeded 10⁵ cells/ml in 6-well or 60-mm tissue plates, in McCoy's 5A medium or α -MEM with supplements, 1 day prior to treatment. The cells were treated with glucocorticoids (10 μ M of cortisol, dexamethasone or prednisolone) and antiretroviral drugs (9 and 14 μ M of efavirenz and ritonavir, respectively) for 24 and 48 h. Control cells were grown in the same medium and the same conditions but were treated with vehicle (ethanol, did not exceed 0,5%) instead of drug. After cell treatment the total RNA was isolated using an RNeasy[®] Mini kit (Qiagen, Sollentuna, Sweden). The RNA concentration used was over 100 ng/mL as measured using Nano-

Drop. RNA purity of the samples was controlled by measuring the ratios 260/280 (≥ 1.6) and 260/230 ratio (≥ 2). RNA samples were stored at $-80\text{ }^{\circ}\text{C}$ for further experiments. Total RNA was converted to cDNA using the protocol as previously described (paper I). The cDNA product was collected and stored at $-20\text{ }^{\circ}\text{C}$ for real-time PCR experiments (quantitative method to measure RNA-levels, qPCR). Cells treated with 25-hydroxyvitamin D₃ and/or glucocorticoids/antiretroviral drugs for 24 and 48 h were compared to vehicle-treated controls. The iQTM SYBR[®] Green Supermix was used for real-time PCR with 200 nM of primers (see paper I, table 1 describing different primers). The quantitation analysis was done by using Bio-Rad CFX manager 3.1 software (Bio-Rad) following the manufacturer's protocol. All mRNA expression levels were normalized to a control (housekeeping) gene, either TBP or GADPH. Melt curve analysis was performed to control that just one PCR product was formed.

Incubations with vitamin D₃ compounds and HPLC/ GC-MS/MS analysis

Enzymatic activity towards vitamin D compounds in Saos-2 and hOB cells was studied by addition of 0.1–5 μM substrate, dissolved in ethanol 99.9%, to the medium and incubation for various time periods (8–96 h) at $37\text{ }^{\circ}\text{C}$. 1α -Hydroxyvitamin D₃ was used as substrate for analysis of 25-hydroxylase activity and 25-hydroxyvitamin D₃ for analysis of 1α -hydroxylase and 24-hydroxylase activities. All incubation experiments were run in duplicate and repeated at least twice. Three different negative controls were used during incubation: cells with ethanol; cells with an 0 h incubation time; and only medium incubated without cells. Most of the experiments were performed with a substrate concentration of 4.8 μM . Directly after incubation, the medium was extracted with ethyl acetate (3:1) and stored in $-20\text{ }^{\circ}\text{C}$ for further analysis by HPLC. The organic phase was evaporated and the samples were dissolved in 100 μL mobile phase (hexane:isopropanol, 85:15). Analysis was carried out using straight phase-HPLC with a 125 \times 4mm silica column and 0.3 mL/min flow rate. UV-detection at 265 nm wavelength was used. Authentic references were used to determine the retention times. The retention time for 24,25-dihydroxyvitamin D₃ was 9 min and for 1,25-dihydroxyvitamin D₃ 17 min. A standard curve with known amounts of authentic compound was used to determine the amounts of 24,25-dihydroxyvitamin D₃. The 24,25-dihydroxyvitamin D₃ metabolite observed in HPLC analysis of incubations with Saos-2 cells was collected and subjected to GC–MS/MS for confirmation of structural identity. In experiments to examine potential 25-hydroxylation and/or 1α -hydroxylation, where no corresponding product was observed in HPLC analysis, we carried out a number of incubations collecting cell media from Saos-2 cells at the following time points; 8, 16, 24, 48, 72, 96 h. The retention time for 1α ,25-dihydroxyvitamin D₃ was determined from injection of authentic reference. Following HPLC, the eluted samples were

examined by GC–MS/MS for detection of potential presence of small amounts of vitamin D metabolites, as described in paper I.

17 α -Hydroxyprogesterone metabolism in Saos-2 cells analysed by TLC

Saos-2 cells was incubated with ³H-labelled 17 α -hydroxyprogesterone (2 μ Ci/ml) and unlabelled 17 α -hydroxyprogesterone (2 μ g/ml), dissolved in ethanol 99.9%, for 48 h at 37 °C. Directly after incubation the medium was transferred to glass tubes and extracted with ethyl acetate (3:1) and stored at -20 °C for further analysis by TLC. Analyses were carried out essentially as described by Lundqvist and Norlin [20] using a mobile phase consisting of chloroform/ethyl acetate 50:50 (v/v). The R_f values for androstenedione, 17 α -hydroxyprogesterone, 11-deoxycortisol, and cortisol in this system were 0.82, 0.68, 0.52, and 0.28, respectively. For further details see paper I.

Transient transfection and luciferase reporter assay studies

In experiments to study the effects of different compounds on CYP24A1 promoter activity, Saos-2 cells were transfected with a human CYP24A1 luciferase reporter construct together with expression vectors for GR or VDR and RXR, using the Lipofectamine 2000 reagent (Invitrogen). One day after the cells were plated the culture medium was replaced with DMEM without antibiotics/antimycotics for 1 hour, followed by transfection with Lipofectamine according to the manufacturers instructions. The cells were transfected with expression vectors for VDR, RXR or GR, together with the luciferase reporter construct containing a CYP24A1 promoter fragment. The transfection mixture also contained a β -galactosidase plasmid to control for transfection efficiency with 200 μ L total volume in each well and was left for 8 h. Thereafter, the cells were treated with vehicle (ethanol), 1 α ,25-dihydroxyvitamin D₃ or prednisolone and was incubated for 48 h. Luciferase activity was measured using a TD-20/20 luminometer (Turner designs). β -Galactosidase activity was measured by incubation with ONPG (o-nitrophenyl- β -galactoside) in 0.1 M sodium phosphate buffer containing β -mercaptoethanol and magnesium chloride and assay of absorbance at 420 nm. Luciferase reporter activity was expressed as relative light units (RLU) divided by β -galactosidase activity (expressed as Abs 420 nm).

Statistical analysis

Analysis of statistical significance was performed using two-tailed Student's t-test. A p-value <0.05 was considered statistically significant.

Results

Vitamin D metabolism in primary osteoblasts and Saos-2 cells

Initial experiments with qualitative RT-PCR showed that Saos-2 and hOB cells express mRNA for the vitamin D receptor and enzymes required in vitamin D₃ metabolism (VDR, CYP2R1, CYP27B1 and CYP24A1). Experiments using HPLC indicated that both cell types have the metabolic activity associated with CYP24A1 (can perform 24-hydroxylation). For further characterization of the produced 24,25-dihydroxyvitamin D₃, we collected the metabolite from the HPLC and subjected it to GC-MS/MS. Analysis by HPLC and GC-MS/MS clearly identifies the formed 24,25-dihydroxyvitamin D₃ in Saos-2 cells (paper I, fig 2). The rate of 24-hydroxylation in Saos-2 and hOB cells was 25-50 pmol/mg/h and 3-7 pmol/mg/h, respectively. We observed a time-dependent formation of 24,25-dihydroxyvitamin D₃ by the cells.

Incubations of 25-hydroxyvitamin D₃ and 1 α -hydroxyvitamin D₃ with Saos-2 cells showed no enzymatic activities for 25-hydroxylation and 1 α -hydroxylation in Saos-2 cells, despite the presence of CYP2R1 and CYP27B1 mRNA in the cells.

In order to exclude formation of potential products, in amounts too small to be detected by HPLC, we used GC-MS/MS to identify if there is some metabolite product associated with 1 α -hydroxylase and 25-hydroxylase activities. Our analyses, however, did not detect any 25-hydroxylase or 1 α -hydroxylase activities in Saos-2 cells, even when incubations were analysed by GC-MS/MS.

Effects of glucocorticoids on CYP24A1 mRNA expression and 24,25-dihydroxyvitamin D₃ formation

First we carried out experiments to measure the effects of glucocorticoids on CYP24A1 mRNA expression by quantitative real time RT-PCR. The results showed that glucocorticoids down regulated CYP24A1 mRNA expression in both Saos-2 and hOB cells (fig. 2A). The glucocorticoids that were found to suppress CYP24A1 mRNA levels include two commonly used drugs (prednisolone and dexamethasone) as well as the most potent endogenous glucocorticoid, cortisol. Prednisolone was the most potent compound in down-regulation. Therefore, this glucocorticoid was chosen for further studies. Next experiment was to investigate if this compound inhibits 24-hydroxylase enzyme activity in both cell types. The results showed that prednisolone significantly inhibits formation of 24,25-dihydroxyvitamin D₃ in Saos-2 cells (fig. 2B). A slight inhibition by prednisolone was also observed in hOB cells (fig. 2B).

Assay of 25-hydroxyvitamin D₃ 24-hydroxylation requires addition of 25-hydroxyvitamin D₃ to the cultures to drive the metabolic reaction forward. Such a condition might induce the 24-hydroxylase levels. Therefore, we measured effects of prednisolone on CYP24A1 mRNA expression both in the

presence and absence of 25-hydroxyvitamin D₃. The results showed that prednisolone has an inhibitory effect on mRNA levels both in the presence and absence of 25-hydroxyvitamin D₃. We observed somewhat increased levels of CYP24A1 mRNA in cultures treated with 25-hydroxyvitamin D₃ compared to cells treated with vehicle (see paper I, fig 6).

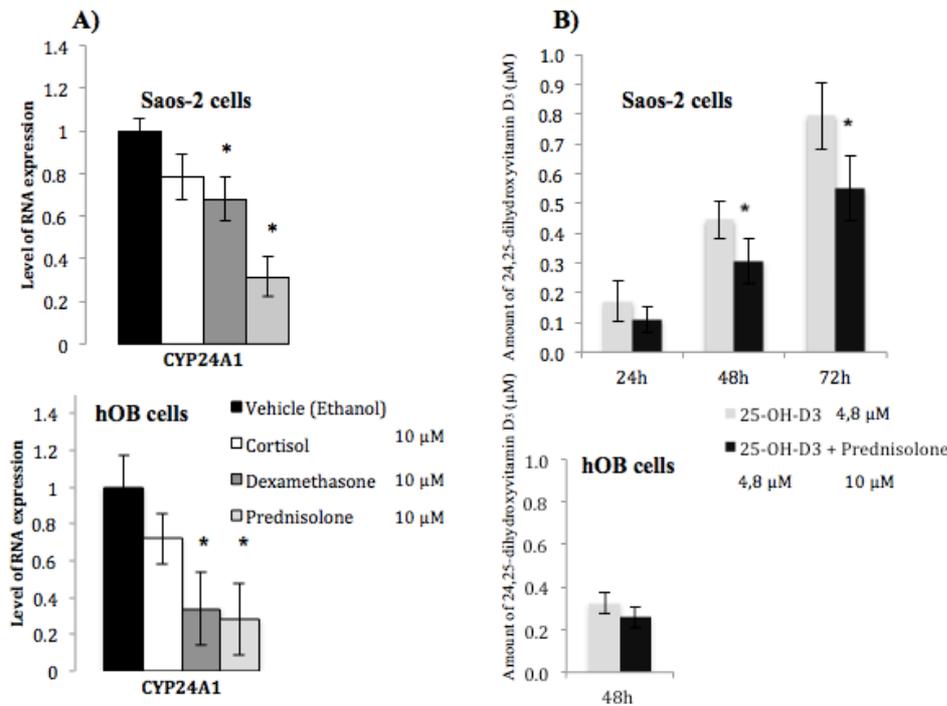


Fig. 2. Treatment with glucocorticoids decreases CYP24A1 mRNA levels (A) and inhibits formation of 24,25-dihydroxyvitamin D₃ (B) in Saos-2 and hOB cells. For more information, see Paper I.

Studies on endogenous glucocorticoid production

Based on the finding that cortisol was one of the glucocorticoids that inhibited CYP24A1, we examined if osteoblast-like cells are able to produce this or other glucocorticoids using TLC analysis. Experiments were performed to assay potential CYP21A2- and CYP11B1-mediated activities that are involved in glucocorticoid biosynthesis. Experiments using radiolabelled 17 α -hydroxyprogesterone showed formation of a compound with the same R_f value as 11-deoxycortisol, a steroid with known glucocorticoid activity. Incubations with 3 μ g of 17 α -hydroxyprogesterone showed a rate of about 4% conversion into 11-deoxycortisol after 48 h of incubation (paper I, fig. 7). We did not detect any formation of cortisol in these cell cultures.

Studies on the CYP24A1 promoter

To further examine the effects of glucocorticoids on CYP24A1 expression, we carried out studies with a human CYP24A1 promoter-luciferase reporter gene. From studies in other cell types, it is known that 1 α ,25-dihydroxyvitamin D₃ up-regulates the CYP24A1 gene expression to maintain

appropriate cellular levels of active vitamin D₃ [83-84]. We first examined if this 1 α ,25-dihydroxyvitamin D₃-regulating system is functional in Saos-2 cells. Cells were transfected with a human CYP24A1 luciferase reporter vector and vectors expressing VDR and RXR and cultured in the presence or absence of 1,25-dihydroxyvitamin D₃. The luciferase activity was analysed and resulted in substantial stimulation of the CYP24A1 promoter-luciferase construct in Saos-2 cells (fig. 3A).

Next experiment was to study effects by prednisolone on the CYP24A1 promoter activity. Cells were transfected with the human CYP24A1 luciferase reporter vector and a vector expressing GR and cultured in the presence or absence of prednisolone. The results showed significant suppression of the CYP24A1 promoter-luciferase reporter activity in the presence of prednisolone, about 50% suppression (fig. 3B).

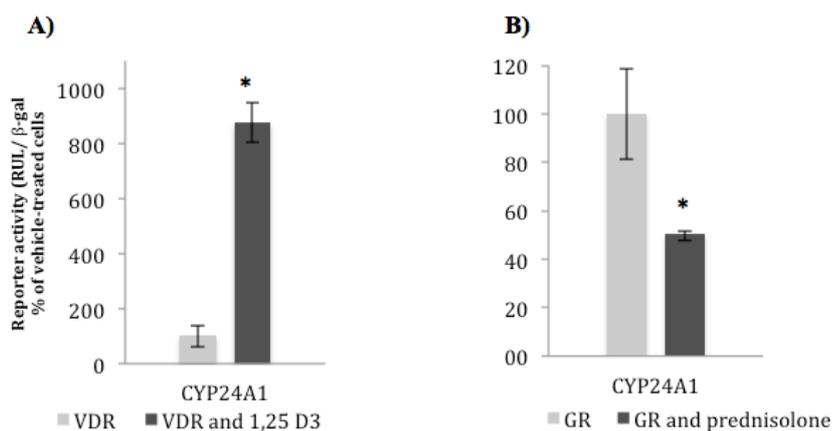


Fig. 3. (A) Effect of 10 nM 1,25-dihydroxyvitamin D₃ (1,25 D₃) on a CYP24A1 promoter-luciferase reporter gene in Saos-2 cells overexpressing VDR/RXR. (B) Effect of 10 μM prednisolone on a CYP24A1 promoter-luciferase reporter gene in Saos-2 cells overexpressing GR. For more information, see Paper I.

As far as we know, response elements for GR (GRE) in the human CYP24A1 promoter have not been reported. A search for putative response elements using the ALGGEN PROMO program/virtual laboratory predicted several possible GRE-like sequences in the human CYP24A1 promoter. Some are located within the promoter fragment carried by the promoter-luciferase reporter vector.

Effects of antiretroviral (ART) drugs on CYP24A1 expression

Earlier studies reported an association between HIV patients using antiretroviral drugs and vitamin D₃ deficiency. We first examined mRNA levels of the deactivating enzyme CYP24A1 in cells treated with these drugs. Saos-2 cells were treated with efavirenz and ritonavir to investigate effects on mRNA levels using real time-PCR. mRNA expression of CYP24A1 was down regulated

significantly, about 40%, by treatment with both drugs compared to the vehicle (ethanol) (fig 4A). The next step was to examine if these antiretroviral drugs also have an effect on the metabolic formation of 24,25-dihydroxyvitamin D₃, i.e. whether mRNA down regulation would also lead to a decreased CYP24A1 enzyme activity. Ritonavir showed somewhat more potent effects on down-regulation of CYP24A1 mRNA expression (see paper II). Therefore, ritonavir was chosen to investigate effects on the conversion of 24-hydroxyvitamin D₃ into 24,25-dihydroxyvitamin D₃ in experiments using HPLC analysis as previously described (paper I, Materials and methods).

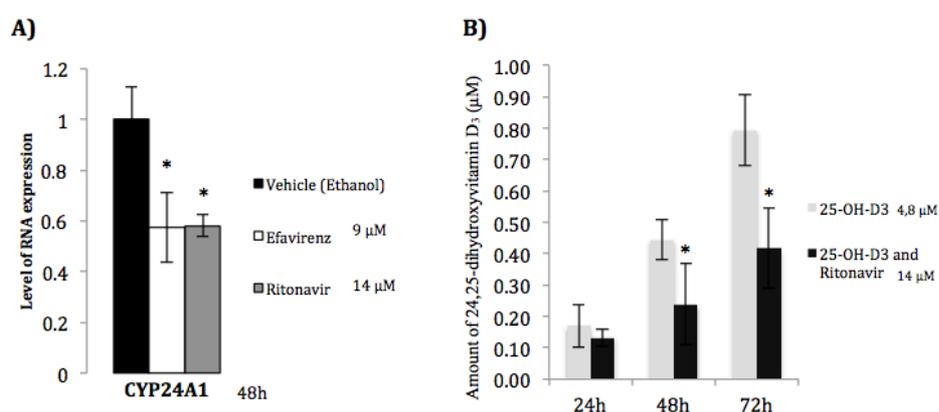


Fig. 4. Treatment of Saos-2 cells with efavirenz and ritonavir decreases CYP24A1 mRNA levels (A) and decreases 24,25-dihydroxyvitamin D₃ formation (B). For more information, see Paper II.

Saos-2 cells were incubated for various time periods with the CYP24A1 substrate 25-hydroxyvitamin D₃ in the absence and presence of ritonavir. The experiment showed that ritonavir has a significant time-dependent inhibitory effect on 24,25-dihydroxyvitamin D₃ formation (Fig 4B).

Effects of 25-hydroxyvitamin D₃ and 1,25-dihydroxyvitamin D₃ on CYP24A1 mRNA expression in the absence and presence of efavirenz or ritonavir

Experiments to assay 25-hydroxyvitamin D₃ 24-hydroxylation require addition of 25-hydroxyvitamin D₃ as a substrate to the cultures (cf fig 4B). The presence of 25-hydroxyvitamin D₃ might induce the 24-hydroxylase levels and modulate the effects of efavirenz and ritonavir on mRNA expression. In order to further study this, an additional set of real time-PCR experiments was performed. The results showed a significant down-regulating effect on CYP24A1 mRNA expression when Saos-2 cells were treated with a combination of the substrate 25-hydroxyvitamin D₃ and ritonavir. A slight stimulation of CYP24A1 mRNA expression by 25-hydroxyvitamin D₃ compared to vehicle was observed in the absence of drug (fig 5A).

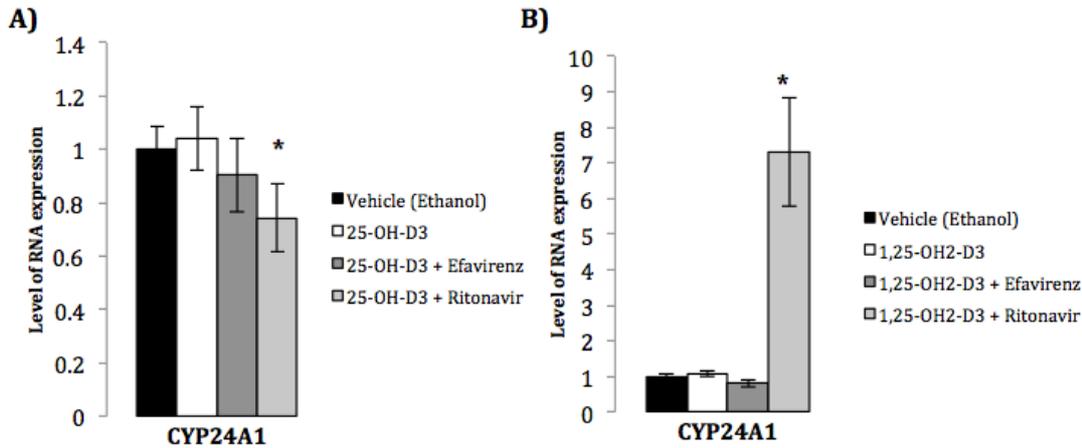


Fig. 5. Treatment with 25-OH-D₃ together with efavirenz or ritonavir decreases CYP24A1 mRNA levels in Saos-2 cells (A). Treatment with 1,25-OH₂-D₃ together with ritonavir increases CYP24A1 mRNA levels in Saos-2 cells. For more information, see Paper II.

It is known that 1 α ,25-dihydroxyvitamin D₃ induces expression of CYP24A1 in several different cell types in order to stimulate degradation of the most active form of vitamin D₃, 1 α ,25-dihydroxyvitamin D₃ [85]. In the present study, 1 α ,25-dihydroxyvitamin D₃ was added to Saos-2 cells in the absence and presence of ritonavir or efavirenz, respectively. Treatment with 1 α ,25-dihydroxyvitamin D₃ induced CYP24A1 mRNA expression (paper II, fig. 5A). Ritonavir significantly potentiated this induction. Thus, co-treatment with 1,25-dihydroxyvitamin D₃ and ritonavir induced the CYP24A1 mRNA expression, about 7-fold (fig 5B). On the other hand, combination treatment with 1 α ,25-dihydroxyvitamin D₃ and efavirenz did not show a significant effect on the mRNA levels of CYP24A1 (see also paper II, fig 5A).

Discussion

Previous studies have shown a strong association between some drugs and diseases such as osteomalacia and osteoporosis related to vitamin D deficiency. These include glucocorticoids and drugs in treatment of HIV. In the present studies, we examined metabolic activities of importance for the levels of active vitamin D in bone cells. Our results reveal strong inhibitory effects of glucocorticoids and antiretroviral drugs on CYP24A1 expression in human osteosarcoma Saos-2 cells, a cell line commonly used as an osteoblast model. Similar results on effects of glucocorticoids in primary human osteoblasts were also found.

Cortisol is the most potent endogenous glucocorticoid. Glucocorticoids perform a number of important regulatory actions on metabolism in various cells of the body [86]. Some inflammatory diseases are treated with exogenous glucocorticoids, for example prednisolone and dexamethasone. Antiretroviral and glucocorticoid drugs cause serious side effects that affect bone density and increase the risk of osteoporosis in patients, particularly in elderly patients [43, 77]. Many of the

reported side effects of glucocorticoid and antiretroviral therapy appear to involve effects on osteoblasts [63, 65-66, 76, 87-88]. The results reported here suggest that one of the mechanisms for effects of these compounds in bone may be effects on the levels of active vitamin D. Since the active form of vitamin D is very potent, it is important that the levels of this compound are properly regulated in various tissues and cells. Vitamin D status can be regulated through hormones, e.g. parathyroid hormone in the kidneys [17-18, 89]. As previously described in the introduction, vitamin D is primarily activated by kidneys and liver, but recent research has also shown that bioactivation and metabolism can occur locally in other tissues and cell types [19, 22, 90-92].

The current study does not indicate local bioactivation into $1\alpha,25$ -dihydroxyvitamin D_3 in human osteoblast cells or Saos-2 cells (cell model for osteoblast cells). The results of this study may indicate that osteoblasts may be dependent on delivery of bioactivated vitamin D_3 from the blood because we could not find metabolic formation of 25- or 1α -hydroxylated metabolites in osteoblast cells even though mRNA for bioactivating enzymes was found to be expressed. The sensitive GC-MS/MS method was used to confirm these results. Cheng et al. [10] reported a similar finding in a study on CYP2R1 mRNA expression in the mouse. mRNA for CYP2R1 was found to be as abundant or even more abundant in testis than in liver, in spite of the fact that the liver is the major site for CYP2R1-mediated vitamin D_3 25-hydroxylation [10, 93]. Not all mRNAs present in the testis are translated into active protein [94] and our results indicate that this is true also for the bone cells examined in this study.

Contrary to the current study, there are other studies that indicate that there may be a possible local bioactivation to form $1\alpha,25$ -dihydroxyvitamin D_3 in osteoblast cells [19, 90, 92]. The reason for this discrepancy remains to be established. It may be noted that the metabolite detection methods are different in the respective studies. In the previous studies, immunological methods such as radioimmunoassay were used to analyse $1\alpha,25$ -dihydroxyvitamin D_3 formation [19, 90], whereas HPLC and sensitive GC-MS/MS detection were used in the current study. The experimental conditions like cell culturing also may differ between the current study and the other studies.

It is clear from the current studies that CYP24A1 mRNA and 24-hydroxylase activity, forming $24,25$ -dihydroxy vitamin D_3 , are present in primary osteoblasts and Saos-2 cells. The CYP24A1 hydroxylase enzyme activity leads to inactivation and elimination of vitamin D_3 [18, 21, 84]. Local inhibitory effects on this enzyme should therefore lead to an increased effect of vitamin D in the cell and may strongly affect the cellular functions. It is known from previous research that the action of active vitamin D affects many cell responses. Actions of active vitamin D include induction of many transcription factors that affect bone formation like osteocalcin, sialoprotein-1 and RANKL in

osteoblasts and other cells [95]. Also, active vitamin D is one of the factors which regulates osteoblast growth, bone resorption and bone mineralization [10]. This study shows that several glucocorticoids and the antiretroviral drugs efavirenz and ritonavir have the ability to inhibit CYP24A1 on both the mRNA and enzyme activity level in osteoblasts. Further, the current study shows glucocorticoid-mediated suppression of the CYP24A1 promoter activity in osteoblast-like cells, supporting the results on mRNA and enzyme activity. This inhibition may have negative effects on bone health.

Previous studies have shown that there are several therapies and diseases associated with development of osteoporosis, including the use of different drugs such as antiretrovirals and glucocorticoids [51, 52, 96–99]. Previous studies have shown that patients with HIV infections have a high risk of hip fractures compared to uninfected patients [59, 100-101]. Several studies indicate that the HIV-infection itself as well as the antiretroviral treatment used to treat the infection may increase the risk of osteoporosis [52, 100–103]. For instance the incidence of bone diseases (osteopenia and osteoporosis) is reported to increase in HIV patients treated with protease inhibitors compared to HIV patients not receiving treatment [61, 102, 104]. The odds ratio for HIV patients with protease inhibitors treatment is reported to be about two times greater for osteoporosis compared to untreated HIV patients [61, 102, 104]. The effects of antiretroviral drugs on bone cells are most likely multifactorial. More studies are needed to fully understand the effects of antiretroviral drugs on endogenous systems including vitamin D bioactivation and deactivation. For instance the mechanisms for how these drugs affect receptor signaling in cells of the bone and other tissues should be evaluated. It should be noted that treatment of HIV patients with antiretroviral drugs is usually a long-term treatment and studies with short-term treatment for 24 or 48 h in bone cell cultures in vitro may not adequately reflect the physiological conditions. Cells may behave differently depending on time, but also on dosage [105].

Prednisolone was the most potent of the compounds tested in decreasing the levels of CYP24A1 mRNA. Our results in this study indicate that different glucocorticoids (cortisol, dexamethasone and prednisolone) and antiretroviral drugs (ritonavir and efavirenz) may have varying inhibitory effects on CYP24A1 expression, depending on their chemical structures. Different structures in drugs from the same drug group may result in different potential effects. Prednisolone showed stronger down-regulation compared to the endogenous glucocorticoid (cortisol). The observations on cortisol-mediated down-regulation may reflect a normal physiological regulation of vitamin D levels in osteoblasts since cortisol is known to affect cells by many different mechanisms. The present investigation, using experiments with radiolabelled 17α -hydroxyprogesterone and TLC analysis, did not show detectable formation of cortisol in Saos-2 cells. Our results did, however, indicate

formation of 11-deoxycortisol, a steroid metabolite that does have glucocorticoid activity and can bind to the glucocorticoid receptor with a lower affinity compared to cortisol [106].

Some studies have reported increased CYP24A1 expression by glucocorticoids in different cell types including osteoblasts [107–109] which are contrary to our findings in this study. The reason for this discrepancy is not clear. The different results may, at least in part, depend on potential differences in the species studied, since cells or tissues of rat or mouse origin were used in most of the previous studies. Although more studies would be needed to clarify this point, it may be speculated that effects of glucocorticoids on CYP24A1 expression could vary between species.

An interesting finding in the current study was that ritonavir markedly potentiated the induction of CYP24A1 mRNA expression by $1\alpha,25$ -dihydroxyvitamin D₃ in Saos-2 cells. Treatment with ritonavir together with $1\alpha,25$ -dihydroxyvitamin D₃ strongly upregulated mRNA gene expression for CYP24A1. This was not observed with efavirenz. These somewhat surprising findings indicate that ritonavir may have different regulatory effects depending on the vitamin D₃ metabolite levels. It is well known from previous research that high levels of $1\alpha,25$ dihydroxyvitamin D₃ lead to induction of CYP24A1 in order to inactivate vitamin D₃. Ritonavir might potentiate this effect very strongly (fig. 5B), and in this case would lead to reduced levels of vitamin D₃. Taken together, the results of the present studies suggest that antiretroviral drugs may adversely affect bone by interference with the vitamin D system in osteoblasts. More studies are needed to evaluate the nature of these adverse effects during different conditions.

Concluding remarks

In summary, the results presented here contribute to our understanding of vitamin D metabolism and effects of glucocorticoids and antiretroviral drugs in the bone. The antiretroviral and glucocorticoid drugs studied had significant effects on metabolic action of importance for the levels of active vitamin D in osteoblasts. Our data do not support previous studies suggesting local vitamin D bioactivation in osteoblasts. These cells might therefore be dependent of $1\alpha,25$ -dihydroxyvitamin D₃ supplied from the blood. Our results showing suppression by glucocorticoids on CYP24A1 expression in osteoblasts suggest a previously unknown mechanism for effects of glucocorticoids in human bone, where these compounds may act by increasing the levels of active vitamin D. Also the antiretroviral drugs efavirenz and ritonavir showed significant effects on CYP24A1, which may lead to altered levels of active vitamin D₃ in the bone. With more understanding of the actions of these drugs, future treatment for these patients could be improved and serious side effects in the patient groups who use antiretroviral and glucocorticoid drugs could be minimized.

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