

# Inhibitory effects of imatinib on vitamin D<sub>3</sub> synthesis in human keratinocytes

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**Abstract.** Chronic myeloid leukemia (CML) is a myeloproliferative disease characterized by the presence of the *BCR-ABL1* fusion gene, a constitutively active, oncogenic tyrosine kinase that is responsible for the clinical features of CML. Tyrosine kinase inhibitors, such as imatinib, have markedly altered the treatment of CML. However, tyrosine kinase inhibitors are associated with side effects on bone metabolism, in adult and pediatric patients. Vitamin D<sub>3</sub> is involved in the complex cycle of bone remodeling, therefore the present study aimed to investigate the influence of imatinib on vitamin D<sub>3</sub> metabolism in the HaCaT human keratinocyte cell line, using commercially available enzyme assays. Imatinib was shown to significantly reduce the production of calcidiol and calcitriol. Based on interaction studies of imatinib with the cytochrome P450 (CYP450) inhibitors VID400 and ketoconazole, it is proposed that imatinib may interfere with the vitamin D<sub>3</sub> cascade due to its metabolism by CYP27B1, which is involved in vitamin D<sub>3</sub> metabolism.

## Introduction

Chronic myeloid leukemia (CML) is characterized by the presence of the Philadelphia (Ph<sup>+</sup>) chromosome [t(9;22) q34;q11] (1). This chromosome harbors the constitutively active, oncogenic tyrosine kinase (TK) Breakpoint Cluster Region-Abelson murine leukemia viral proto-oncogene 1 (*BCR-ABL1*), which is responsible for leukemic cell transformation (2-4). Imatinib mesylate (Glivec®/Gleevec®, Novartis, Basel, Switzerland) is a potent and selective inhib-

itor of *BCR-ABL1*. It was initially licensed in 2001 (5-10), and has since rapidly become the standard front-line treatment for CML, leading to high response rates (11). However, imatinib shows off-target effects on TKs other than *BCR-ABL1*, such as platelet-derived growth factor and colony-stimulating factor 1 receptor, which are involved in the bone remodeling cycle (12). Previous studies have revealed that prolonged imatinib treatment in adult CML patients may cause hypophosphatemia and altered bone mineralization (13-15), whereas pediatric CML patients develop growth retardation in ≤70% of cases (16,17).

Growth delay due to long-term imatinib intake is increasingly observed (11,12,16,18,19), and is more prominent in patients who began treatment with imatinib at prepubertal age (12). In addition, pediatric patients exhibit reduced serum levels of 25-hydroxyvitamin D<sub>3</sub> (25-OH-VD<sub>3</sub>; calcidiol) and 1,25-dihydroxyvitamin D<sub>3</sub> (1,25-(OH)<sub>2</sub>-VD<sub>3</sub>; calcitriol) (20) whilst on imatinib treatment. In humans, vitamin D<sub>3</sub> (VD<sub>3</sub>) is synthesized by keratinocytes in the skin, by UVB-induced photolysis of 7-dehydrocholesterol (7-DHC), which results in the formation of previtamin D<sub>3</sub>, followed by a thermal isomerization step (21). Thereafter, VD<sub>3</sub> is enzymatically hydroxylated to calcidiol by cytochrome P450 (CYP450) isoenzymes CYP2R1 and/or CYP27A1 (22) in the liver, and further metabolized to hormonally active calcitriol by CYP27B1 (23-25) in the kidney (Fig. 1). In order to investigate the calcitriol pathway and its modulation, the HaCaT human keratinocyte cell line was established by Lehmann (26) as a cellular model, thus demonstrating for the first time that HaCaT cells were capable of hydroxylating calcidiol to calcitriol.

Calcitriol is essential in regulating blood levels of calcium and phosphorus (27) and has a key role during bone mineralization (28-30). Numerous studies have identified an association of vitamin D<sub>3</sub> deficiency (as indicated by low calcidiol/calcitriol blood levels) with impaired growth, particularly during puberty and prepuberty (28,31). However, the detailed mechanisms causing growth delay during imatinib therapy are currently speculative. The aim of the present study was to investigate the effects of the TK inhibitor (TKI) imatinib on vitamin D<sub>3</sub> metabolism in the HaCaT human keratinocyte cell line.

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## Materials and methods

**Cell culture protocol.** The HaCaT human keratinocyte cell line was purchased from Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). The cells were seeded at a density of  $1 \times 10^5$  cells/cm<sup>2</sup> and grown in Dulbecco's modified Eagle's medium (DMEM; Gibco Life Technologies GmbH, Darmstadt, Germany), supplemented with 10% fetal bovine serum (FBS; Gibco Life Technologies GmbH) in a 95% humidified atmosphere containing 5% CO<sub>2</sub>, at 37°C for 48 h. The media was subsequently replaced by serum-free DMEM for 18 h, in order to induce synchronization of the cell cycle. The cells were then grown in FBS-supplemented DMEM for 8 h, until they had reached 80-90% confluency. To investigate the metabolism of vitamin D<sub>3</sub>, the cells were seeded at a density of  $5 \times 10^4$  cells/cm<sup>2</sup> in culture dishes (Ø, 30 mm).

**Vitamin D<sub>3</sub> assay.** To investigate vitamin D<sub>3</sub> metabolism the HaCaT cells ( $5 \times 10^4$  cells/cm<sup>2</sup>) were incubated with 25 µM 7-DHC (dissolved in 100% ethanol; Sigma-Aldrich, Steinheim, Germany) as a substrate, and exposed to UVB (300 nm; application rate, 75 mJ/cm<sup>2</sup>). Irradiation of the cells was performed using a tuneable high intensity monochromator (FWHM, 5 nm; Müller Optik-Elektronik, Moosinning, Germany) over 15 min. At the start of irradiation the cells were incubated with imatinib (supplied by Novartis, Basel, Switzerland), at a concentration of 1 µM [dissolved in 100% dimethylsulfoxide (DMSO)] for 24, 48, or 72 h. Following the incubation, the media and detached keratinocytes were collected and calcitriol was extracted using methanol : chloroform (1:1) (Merck, Darmstadt, Germany). The levels of calcitriol were determined quantitatively from the organic phase using a commercially available enzyme assay (1,25-Dihydroxy Vitamin D EIA; IDS, Frankfurt, Germany). All experiments were performed four times and the results were normalized to  $1 \times 10^6$  cells. Control experiments with ethanol and DMSO were conducted in order to identify any interactions with solvents or other components.

To determine whether the VD<sub>3</sub> processing CYP450 isoenzymes CYP2R1, CYP27A1 and CYP27B1 were inhibited by imatinib, specific inhibitors of the CYP450 isoenzyme family (VID400 and ketoconazole) were applied concomitantly. These experiments were conducted without irradiation. The HaCaT cells were incubated for 0, 2 or 4 h with either 5 µM cholecalciferol or 5 µM calcidiol (both dissolved in 100% ethanol) as a substrate. Prior to substrate incubation, the cells were incubated for 1 h with 200 nM VID400 or 10 µM ketoconazole (both dissolved in 100% ethanol), with or without 1 µM imatinib.

**Statistical analysis.** Statistical analysis at defined time points of incubation was performed using one-way analysis of variance with Bonferoni adjustment to evaluate the effects of IMA-treated samples compared with untreated controls, using the GraphPad Prism 6.0 software (GraphPad Software, Inc., San Diego, CA, USA).  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Inhibitory effects of imatinib on calcitriol synthesis.** Imatinib incubation at the clinically effective concentration of 1 µM,

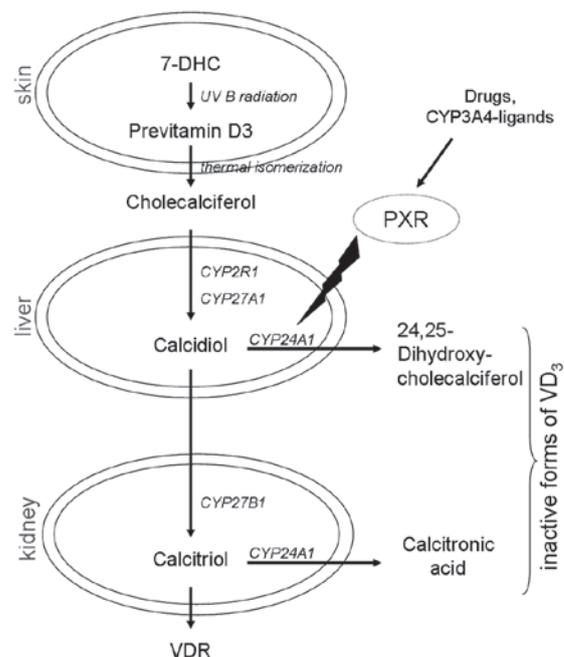


Figure 1. Vitamin D cascade and the enzymes involved. Modulation of CYP24A1 by vitamin D metabolites or other compounds, such as PXR, may generate a high expression and vitamin D deficiency, as detected in various tumor tissues [modified after Schuster *et al* (32)]. VDR, vitamin D receptor; VD<sub>3</sub>, vitamin D<sub>3</sub>; 7-DHC, 7-dehydrocholesterole; PXR, pregnane x receptor; CYP2R1, cytochrome P450 family 2, subfamily R, polypeptide 1 (vitamin D 25-hydroxylase); CYP27A1, cytochrome P450, family 27, subfamily A, polypeptide 1 (vitamin D 25-hydroxylase); CYP27B1, cytochrome P450, family 27, subfamily B, polypeptide 1 (1 $\alpha$ -Hydroxylase); CYP24A1, cytochrome P450, family 22, subfamily a, polypeptide 1 (1,25-dihydroxyvitamin D<sub>3</sub> 24-hydroxylase).

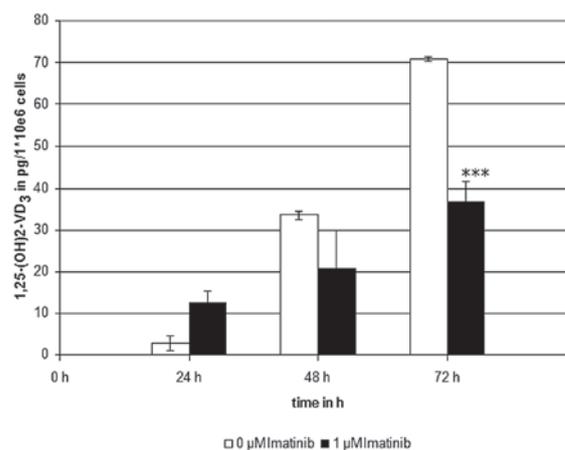


Figure 2. Time-dependent calcitriol (1,25-(OH)<sub>2</sub>-VD<sub>3</sub>) synthesis in the HaCaT human keratinocyte cell line following incubation with 7-dehydrocholesterole (7-DHC) as a substrate and UV irradiation (300 nm). The cells were either untreated (controls; white bars) or treated with imatinib (1 µM, black bars). The data represents the mean  $\pm$  standard deviation of four experiments.

significantly reduced the calcitriol levels to ~50%, as compared with the controls, which were not treated with the TKI (Fig. 2). To verify these results, control experiments were conducted in the presence of 7-DHC without irradiation, and in the absence of 7-DHC with irradiation. Furthermore, to screen out any interactions of the solvents used, control experiments with

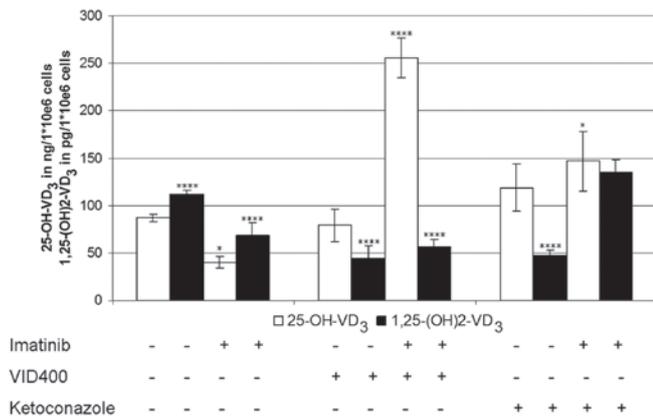


Figure 3. Synthesis of calcidiol (25-OH-VD<sub>3</sub>, white bars) and calcitriol (1,25-(OH)<sub>2</sub>-VD<sub>3</sub>; black bars) in the HaCaT human keratinocyte cell line following incubation with cholecalciferol as a substrate. The cells were either untreated or treated with imatinib (1 μM) and inhibitors VID400 (200 nM) or ketoconazole (10 μM). The data are presented as the mean ± standard deviation of four experiments, in pg/1x10<sup>6</sup> cells for calcitriol and ng/1x10<sup>6</sup> cells for calcidiol.

ethanol and DMSO were conducted. As expected, no generation of calcitriol was detectable in the control experiments.

**Effects of selective inhibitors in combination with imatinib on the vitamin D<sub>3</sub> cascade.** Using cholecalciferol as the vitamin D<sub>3</sub> synthesis-starting substrate, the levels of calcidiol and calcitriol in the cells exposed to imatinib over 4 h were lowered to 50% that of the controls (Fig. 3). Treatment with the CYP450 inhibitors VID400 and/or ketoconazole, in the absence of imatinib, had nearly no effect on calcidiol levels (range, 90–110 ng/1x10<sup>6</sup> cells), whereas calcitriol levels decreased to 60% of the control values. Treatment with imatinib in the presence of VID400, resulted in increased calcidiol levels by 600% but had no effects on calcitriol synthesis. Treatment with ketoconazole and imatinib resulted in increased levels of calcitriol, by 200% (Fig. 3).

Furthermore, the experiments were repeated using calcidiol as the substrate and analyzed in the same way as previously described, resulting in calcitriol levels concordant with those described in Fig. 3, with the exception of ketoconazole. The cells incubated with imatinib in the absence of VID400 or ketoconazole had lower calcitriol levels, as compared with those incubated without imatinib. Identical levels were detected in the presence of VID400 without imatinib, whereas the combination of VID400 and imatinib increased the levels. In the presence of ketoconazole the calcitriol levels, with and without imatinib exposure, were decreased.

## Discussion

During imatinib treatment longitudinal growth retardation has been identified as a frequent side effect in children (13–15,32–43). Jaeger *et al* (20) investigated biochemical skeletal markers in 17 pediatric patients with CML (age, 4–17 years) undergoing imatinib treatment and reported low serum levels of vitamin D<sub>3</sub>, as well as impaired bone metabolism. However, children undergoing treatment for various types of cancer frequently exhibit vitamin D<sub>3</sub> deficiencies (44,45). The

reason for this may be a lack of sun exposure or poor nutrition, but may also be due to drug interactions, or a combination of these factors (44).

In humans, vitamin D<sub>3</sub> has a primary role in maintaining extracellular ionized calcium levels and bone mineralization (46). In children, vitamin D<sub>3</sub> is required for growth and also for the prevention of rickets (47). In addition, vitamin D<sub>3</sub> is an important immunomodulator, that has been shown to have antiproliferative effects, potentiate apoptosis and inhibit angiogenesis (45). Pediatric oncology patients have a higher prevalence of vitamin D<sub>3</sub> hypovitaminosis (20,45). The present study aimed to investigate the reasons for vitamin D<sub>3</sub> deficiency and recognize the potentially causative mechanisms for low vitamin D serum levels and growth retardation in prepubertal patients with CML. The results of the present study demonstrate an inhibitory effect of imatinib on the synthesis of calcidiol and calcitriol during vitamin D<sub>3</sub> synthesis in human keratinocytes, leading to decreased levels by 50%. This finding is in concordance with the published clinical data of Jaeger *et al* (20).

To identify the potential target of imatinib within the vitamin D<sub>3</sub> cascade, the synthesis of calcidiol and calcitriol was examined in confluent HaCaT cells treated with two well-known specific CYP450 inhibitors: VID400 and ketoconazole. While ketoconazole is known to be a general inhibitor of P450 enzymes, VID400 specifically blocks CYP24A1, thus allowing the identification of the potential target of imatinib within the vitamin D<sub>3</sub> cascade with enzymes involved, such as CYP24A1, CYP27A1 and CYP27B1 (Fig. 1). Experiments were conducted in combination with and without imatinib using cholecalciferol as a substrate, therefore no irradiation of the cells was required. As previously described, VID400 at a concentration of 200 nM, may dose-dependently inhibit CYP24A1 activity, and partially inhibit CYP27B1 by 30% (48). Ketoconazole, at a concentration of 10 μM, is a general inhibitor of the CYP450 isoenzymes (49), including vitamin D hydroxylating enzymes, such as CYP24A1, CYP27A1 and CYP27B1 (50). The present study demonstrated that exposure to VID400 alone stabilized the levels of endogenously produced calcitriol.

It has been shown that VID400 results in increased expression of Cyp24 (451,52). CYP24 catalyzes the metabolism of calcidiol and calcitriol. The activity is regulated by a negative feedback loop dependent on calcitriol concentration, resulting in decreased calcitriol levels. It has previously been suggested, that in cancer cells, particularly in prostate cancer, a rapid breakdown of calcitriol levels is caused by an overactive CYP24 (53). The combination of the CYP24 inhibitors tested with imatinib, resulted in increased levels of calcidiol levels. These results suggest that besides the inhibition of CYP24 by VID400, the activity of CYP27B1 is impaired by imatinib, resulting in an intracellular accumulation of calcidiol. This is in concordance with a previous *in vivo* study, where it was shown that imatinib is metabolized by various liver CYP450 isoenzymes, mainly CYP3A4 and CYP3A5 (54). CYP3A4 is also known to be a human microsomal vitamin D 25-hydroxylase (55), similar to CYP27B1.

Isolated ketoconazole exposure resulted in increased calcidiol and decreased calcitriol levels, whereas the combination with imatinib increased the levels of calcidiol and calcitriol. Ketoconazole is also known to be a strong inhibitor

of CYP3A4 (56), resulting in poor metabolism of imatinib. Based on a drug interaction study, co-administration of imatinib and inhibitors as well as inducers of CYP3A4 activity (57), requires careful monitoring of the patients to rule out toxic side effects, or decreased TKI effects on the underlying CML.

To catalyze the 25-hydroxylation step in the liver, at least six CYPs are involved *in vivo*, the most prominent ones being CYP27A1 and CYP2R1 (58). CYP27B1 is responsible for the renal 1 $\alpha$ -hydroxylation of vitamin D to hormonally active calcitriol. The vitamin D synthesis cascade is a complex system with numerous enzymes involved at diverse steps; therefore, various enzymes may be affected by imatinib. Imatinib, the inhibitors (VID400 and/or ketoconazole) and the substrates (cholecalciferol and/or calcidiol) may all compete for binding to one or more CYPs in keratinocytes, resulting in interference with vitamin D<sub>3</sub> metabolism. The results of the present study clearly indicate a competitive inhibition of CYP27B1 by imatinib, as concomitant blocking of CYP27B1 with VID400 resulted in elevated levels of calcidiol, but decreased levels of calcitriol. However, the mechanism remains poorly understood, and additional studies are required.

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