Off-target effect of imatinib and nilotinib on human vitamin D_3 metabolism

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Abstract. Prolonged treatment with tyrosine kinase inhibitors (TKI) including imatinib (IMA) or nilotinib (NIL), induces severe disturbances of bone metabolism in patients with chronic myeloid leukaemia. As vitamin D_3 (VD_3) is involved in the complex cycle of bone remodelling, the present study investigated *in vitro*, the influence of IMA and NIL on VD_3 metabolism i) in HaCaT cells and ii) in cultured outer root sheath keratinocytes (ORS-KC) from hair follicles of IMA treated children. Cells were incubated in the presence of IMA or NIL. Concomitantly, specific inhibitors were applied to analyze the inhibition of the VD_3 processing cytochrome P450 isoenzyme family by TKIs. *In vitro,* IMA and NIL significantly impaired the production of calcitriol in HaCaT and cultured ORS-KC cells from hair follicles of IMA treated children. For NIL, this inhibitory effect demonstrated a 4-fold increase. In HaCaT and ORS-KC, application of specific CYP450 inhibitors revealed that CYP27B1 was impaired by IMA and NIL leading to an intracellular accumulation of calcidiol. However, during TKI treatment, KC of IMA treated children revealed no differences in calcidiol and calcitriol levels. In conclusion, IMA and NIL interfere with the vitamin D3 cascade due to their metabolism by CYP27B1.

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Abbreviations: 7-DHC, 7-dehydrocholesterol; CYP2R1, cytochrome P450 family 2, subfamily R, polypeptide 1 (vitamin D 25-hydroxylase); CYP24A1, cytochrome P450, family 22, subfamily a, polypeptide1 (1.25-dihydroxyvitamin D_3 24-hydroxylase); CYP27A1, cytochrome P450, family 27, subfamily A, polypeptide 1 (vitamin D 25-hydroxylase); CYP27B1, cytochrome P450, family 27, subfamily B, polypeptide 1 (1α-Hydroxylase); PXR, pregnan x receptor; VDR, vitamin D receptor; VD_3, vitamin D_3

Key words: CML, growth, imatinib, nilotinib, vitamin D, HaCaT, keratinocytes

Introduction

Chronic myeloid leukemia (CML) is a rare hematologic disease with low incidence but increasing prevalence (1). This progressive, hematopoietic neoplasm is characterized by the presence of the *BCR-ABL1* hybrid gene that is localized on the-so called Philadelphia (Ph+) chromosome [t(9;22) (q34;q11)] (2)-which leads to the constitutively active tyrosine kinase (TK) BCR-ABL1 causing leukemic cell transformation (3-5). As the oncogenic TK BCR-ABL1 is responsible for initiating the disease process (6), selective TK inhibitors (TKI) such as imatinib (IMA; Glivec®/Gleevec®: Novartis, Basel, Switzerland) were developed. Since 2001, (7-12) IMA has become the standard front-line therapy for the treatment of CML in adults (13). For pediatric patients with CML, IMA was approved in Germany in 2003. However, due to the increasing resistance or intolerance of leukemic cells to IMA therapy (14), second-generation TKIs like nilotinib (NIL; Tasigna®; Novartis, Basel, Switzerland) were developed. NIL, an aminopyrimidine-derivative based on imatinib mesylate (15), has a 20- to 50-fold higher inhibitory activity in IMA-sensitive cells and a 3 to 7 times higher inhibitory activity in IMA-resistant cells due to its higher potency and selectivity for the BCR-ABL1 TK (16). Based upon its efficacy, NIL was approved for the treatment of adult patients with CML in chronic and advanced phases after IMA failure or intolerance in 2008 (1).

However, both TKIs show off-target effects on further TKs such as PDGFR and CSF1R (c-FMS), which are involved in the bone remodeling cycle. Especially for IMA it is known that under prolonged treatment, adult CML patients revealed hypophosphatemia and an increased bone mineralization whereas pediatric CML patients develop growth retardation in up to 72.9% of the cases (17-22).

Reports of growth retardation due to a long-term application of IMA and related TKIs are increasing (13,17,21,23,24) and are even more prominent in those patients, who started IMA therapy at a prepubertal age. Additionally, pediatric patients display reduced serum levels of 25-hydroxy-vitamin D_3 (25-OH-VD_3; calcidiol) and 1.25-dihydroxyvitamin D_3 (1.25-$(OH)_2$-VD3; calcitriol) (25) under IMA treatment. At least, the effects for NIL are expected to have a similar potential for skeletal effects compared to IMA.

Vitamin D_3 (VD_3) synthesis is initiated by UVB-induced photolysis of 7-dehydrocholesterol (7-DHC) into previtamin D_3 (26) that is then enzymatically hydroxylated to calcidiol by CYP2R1 and/or CYP27A1 (27) in the liver which is further metabolized to hormonally active calcitriol by CYP27B1 (28-30) in the kidney (Fig. 1).

As calcitriol is essential in regulating the blood levels of calcium and phosphorus (32), it plays a key role during bone mineralization (33-35). Clinical studies revealed an impaired growth especially during puberty and prepuberty under IMA treatment (36). Furthermore an association of VD_3 deficiency was shown indicated by low calcidiol/calcitriol blood levels, under IMA treatment as well as impaired longitudinal growth (25).

In a previous study, the effect of IMA on VD_3 synthesis was investigated in HaCaT cells and revealed significantly reduced calcitriol levels up to ~50%, compared to untreated controls (37). However, as the mechanism is poorly understood, the aim of the present study was to investigate the effects of NIL in comparison to IMA and to elucidate the causative mechanisms for this effect by means of the immortalized cell line HaCaT and human keratinocytes expanded in culture from hair follicles collected from pediatric CML patients under IMA treatment.

Materials and methods

Cell culture protocol and cell isolation. The human keratinocyte cell line HaCaT was purchased from Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). Cells were seeded at a density of $1x10^5$ cells/cm^2 and grown in Dulbecco's modified Eagle's medium (DMEM, Gibco, Eggenstein, Germany) supplemented with 10% fetal bovine calf serum (FCS; Gibco, Eggenstein, Germany) at 95% relative humidity, 5% CO_2 and 37˚C for 48 h. Subsequently, the medium was replaced for 18 h by serum-free DMEM to induce synchronization of the cell cycle. Afterwards cells were grown in fetal bovine serum-supplemented DMEM for 8 h until they were almost confluent. To investigate vitamin D_3 metabolism, cells were seeded at a density of $5x10^4$ cells/cm^2 in culture dishes (Ø 30 mm).

ORS-KCs were prepared from human scalp hair follicles of IMA-treated children and their healthy siblings hailing from different regions all over Germany. Because of the disease rareness, 16 IMA-treated children and adolescents between 10 and 22 years (ø16±4 years old; 6 male and 12 female), and 15 healthy subjects between 2 and 33 years old (ø15±11 years old; 7 male and 8 female) take part of this study. An ethic statement of the University Hospital Carl Gustav Carus (EK28212200) and an International Clinical Trials Identifier (NCT00445822) was approved. Hair follicles were plucked by using a pair of tweezers and the bulk of the hair shaft was cropped while the hair follicle was immersed in DMEM buffered with 1 M HEPES (Gibco) and supplemented with 1% PenStrep (Gibco) for 24 h. Afterwards, hair follicles were applied on a feeder layer of 3T3 fibroblasts, previously treated with 0.004 μg/ml mitomycin C (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), and cultivated in a complex medium containing 3 parts DMEM and 1 part HAMS F12 supplemented with 10% FCS, 0.135 mM adenine (Sigma-Aldrich; Merck KGaA), 0.1 nM cholera toxine (Sigma-Aldrich; Merck KGaA), 2 nM triiodothyronine (Sigma-Aldrich; Merck KGaA), 1 pack epithelial cell growth medium supplements (containing epidermal growth factor, hydrocortisone, insulin and transferrine (Promocell, Heidelberg, Germany) 1% PenStrep, 1% sodium pyruvate (100 mM; Gibco) and incubated at 95% relative humidity, 5% CO_2 and 37˚C. Medium was changed 3 times a week. After 2-3 weeks in primary culture, 3T3 cells were removed by trypsination and ORS-KCs were replated at a density of $1x10^5$ cells/cm^2 and grown in DermaLife K complete medium (Cellsystems, Troisdorf, Germany).

Vitamin D_3 assay. For investigation of vitamin D_3 metabolism, HaCaT cells were incubated with 25 μM 7-DHC (dissolved in 100% ethanol; Sigma-Aldrich; Merck KGaA) as substrate and exposed to UVB (300 nm; application rate: 75 mJ/cm^2). Irradiation was carried out by using a tuneable high intensity monochromator (FWHM, 5 nm; Dermolum Um, Müller Optik-Elektronik, Moosinning, Germany). During irradiation, IMA or NIL (provided by Novartis, Basel, Switzerland) were added to the cell culture medium at a concentration of 1 μM (dissolved in 100% DMSO; Sigma-Aldrich; Merck KGaA), respectively. After UVB irradiation and incubation for 24, 48 or 72 h, the medium and detached cells were collected and extracted in a methanol:chloroform (1:1) (Sigma-Aldrich; Merck KGaA) solution. Chloroform phase was used for quantitative determination of calcidiol and calcitriol levels by using commercially available enzyme assays (IDS, Frankfurt, Germany). Results were normalized to $1x10^6$ cells.

To analyze if the VD_3 processing enzymes CYP2R1, CYP27A1 and CYP27B1 are inhibited by IMA or NIL, specific inhibitors of cytochrome P450 enzyme family (VID400, ketoconazol, both Sigma-Aldrich; Merck KGaA) were investigated. Experiments were carried out without irradiation. Cells were incubated for 0, 2, and 4 h with either 5 μM cholecalciferol or 5 μM calcidiol (both Sigma-Aldrich; Merck KGaA and both dissolved in 100% ethanol) as substrate. Before substrate incubation, cells were treated for 1 h either with 200 nM VID400 or 10 μM ketoconazole (both dissolved in 100% ethanol) alone or in combination with 1 μM IMA or NIL, respectively. All experiments were repeated at least 4 times.

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

Results

Inhibitory effect of TKI on calcitriol synthesis in HaCaT and ORS-KCs. To determine the effect of IMA and NIL treatment on VD_3 metabolism, we cultured confluent HaCaT cells for a maximum of 72 h with TKI (clinically effective concentration: 1 μM) and measured calcidiol and calcitriol levels. Using

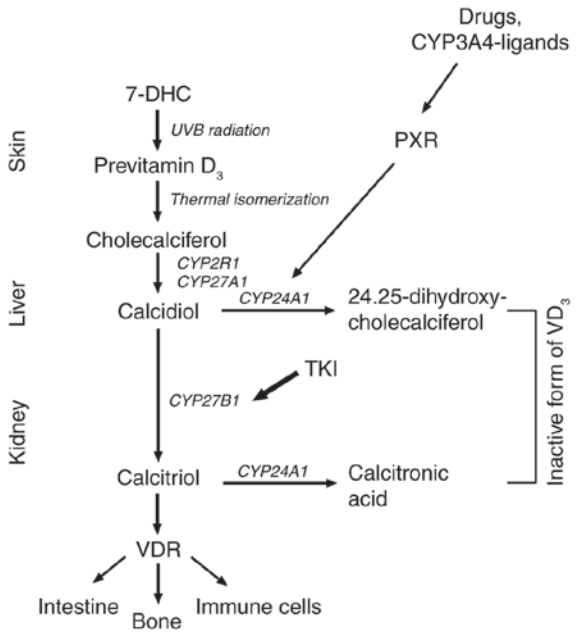

Figure 1. Vitamin D_3 cascade and involved enzymes. Modulation of CYP24A1 by e.g., vitamin D metabolites or other compounds like PXR, can generate a high expression of CYP24A1 and consequentially a vitamin D deficiency as detected in various tumour tissues [modified after Schuster *et al* 2006 (31)]. 7-DHC, 7-dehydrocholesterol; PXR, pregnan x receptor; VDR, vitamin D receptor; TKI, tyrosine kinase inhibitors; VD_3, vitamin D3.

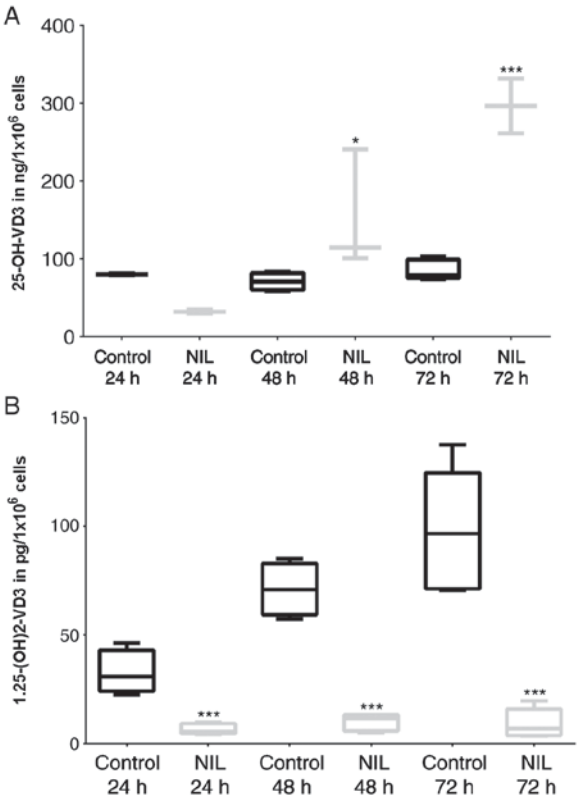

Figure 2. Time dependent synthesis of (A) calcidiol and (B) calcitriol in human keratinocyte cell line HaCaT after incubation with 7-dehydrocholesterol as substrate and UV irradiation without or with NIL. Data were presented as mean ± standard deviation. Statistical significance level compared to untreated control is defined with P-value: $^{*}P \leq 0.05$ and $^{***}P \leq 0.001$. NIL, nilotinib.

7-DHC as substrate, NIL significantly increased calcidiol levels to 300% in comparison to untreated controls (Fig. 2A) and significantly reduced calcitriol levels to 10% (Fig. 2B). These data were verified by repeating the experiments without irradiation and using cholecalciferol as synthesis starting substrate.

The same effect was found by repeating the described experiments using ORS-KCs from IMA-treated children with CML and their healthy siblings as controls. The experiments with ORS-KCs were performed with IMA and NIL, respectively. Data of IMA-treated children and healthy subjects were summarized and shown as one bar, respectively (Fig. 3). However, compared to KCs of healthy subjects, KCs of children with CML revealed no differences in their capability to synthesize calcitriol under identical physiological conditions.

Effects of TKI in presence of specific inhibitors on the vitamin D_3 cascade. For identification of the potential target of TKI within the VD_3 cascade, we examined confluent HaCaT cells under exposure to selective cytochrome P450 inhibitors such as VID400 and ketoconazole. While ketoconazole is known to be a general inhibitor of P450 enzymes, VID400 only blocks CYP24A1 at a specific concentration. Experiments were carried out in combination with and without TKI by using cholecalciferol as synthesis-starting substrate, so that no irradiation of cells was necessary.

The results were comparable to those described before. Cells treated with NIL alone revealed an increase of calcidiol level to 250% whereas calcitriol levels were lowered down to 50% in comparison to those without TKI (Fig. 4). Treating cells with NIL and VID400 revealed calcitriol levels at the same level as cells treated with NIL alone, while calcidiol levels were significantly increased to 400% (Fig. 4). Treatment with NIL and ketoconazole had no remarkable effect in HaCaT cell line. Experiments with ORS-KCs were repeated with TKI and VID400 treatment only. Similar to the results for the HaCaT cell line, ORS-KCs from IMA-treated children with CML and their healthy siblings showed under TKI and VID400 treatment reduced calcitriol levels whereby this effect was more pronounced with NIL as IMA (Fig. 5). No difference was detectable between the VD_3 synthesis of ORS-KCs from IMA-treated children with CML and their healthy siblings. Repeating the experiments with calcidiol as substrate showed the same effect confirming the observed data.

Discussion

VD_3 plays a primary role in the human body by maintaining the extracellular calcium level, acts as an important immune modulator, potentiates apoptosis or inhibits angiogenesis (38). Especially in children, VD_3 is necessary during bone mineralization and in this context for growth but also for prevention of rickets (39).

The presented study describes an off-target effect of the TKIs IMA and NIL on human VD_3 metabolism, which might play a central role in the complexity of longitudinal growth retardation during CML therapy with TKI treatment. Under

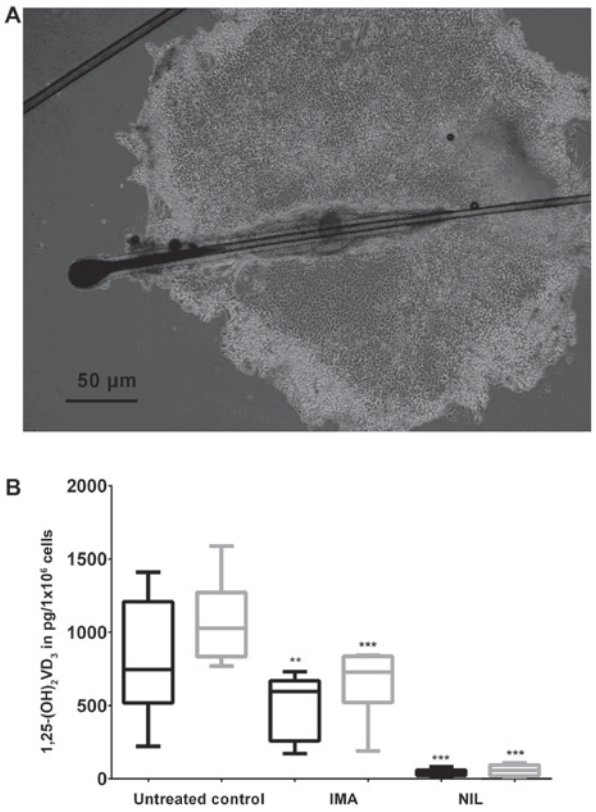

Figure 3. Synthesis of calcitriol in (A) cultured ORS-KCs from healthy subjects (black bars) and TKI treated subjects (grey bars) after incubation with cholecalciferol and during exposure with (B) IMA or NIL. Statistical significance level compared to untreated control is defined with P-value: $^{**}P\leq0.01$ and $^{***}P\leq0.001$. TKI, tyrosine kinase inhibitors; IMA, imatinib; NIL, nilotinib.

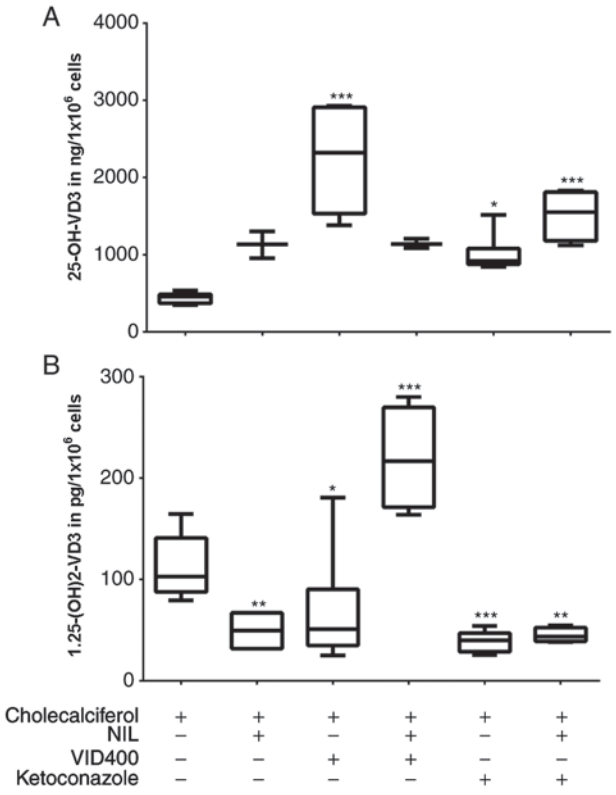

Figure 4. Synthesis of (A) calcidiol and (B) calcitriol in keratinocyte cell line HaCaT after incubation with cholecalciferol during exposure with VID400 or ketoconazole. Data were presented as mean ± standard deviation. Statistical significance level compared to untreated control is defined with P-value: $^{*}P\leq0.05$, $^{**}P\leq0.01$, and $^{***}P\leq0.001$. NIL, nilotinib.

prolonged IMA therapy, growth retardation is increasingly reported as a main side effect in children (18-20,40-50). Additionally, VD_3 deficiency is often described in children who have been treated for different kinds of cancer (38,51) may due to lack of sun exposure and/or poor nutrition and/or drug interactions (51). Concerning pediatric CML patients, Jaeger *et al* (25) investigated for the first time serum bone markers in 17 pediatric patients with CML (age: 4-17 years) under ongoing IMA therapy and reported VD_3 insufficiency or deficiency in addition to impaired bone metabolism (25). As it is now speculative if VD_3 insufficiency or deficiency is caused by the disease itself, the impaired bone metabolism or due to a direct effect of TKI on VD_3 metabolism, we investigated the inhibitory effect of IMA and NIL on VD_3 metabolism in human keratinocyte cell line HaCaT and ORS-KCs of IMA-treated children.

In the skin synthesized VD_3 undergoes 25-hydroxylation in the liver followed by 1α-hydroxylation in the kidney to build the biologically active hormone. For catalysing the 25-hydroxylation step in the liver, at least six cytochrome P450 enzymes (CYPs) are involved whereby CYP27A1 and CYP2R1 (52) are the most viable ones. In the kidney, CYP27B1 is responsible for 1α-hydroxylation of VD_3 to hormonally active calcitriol (Fig. 1). These enzymes are also found in various extra renal tissues including epidermal keratinocytes. Keratinocytes are able to synthesize and catabolize calcitriol as well as harbouring the vitamin

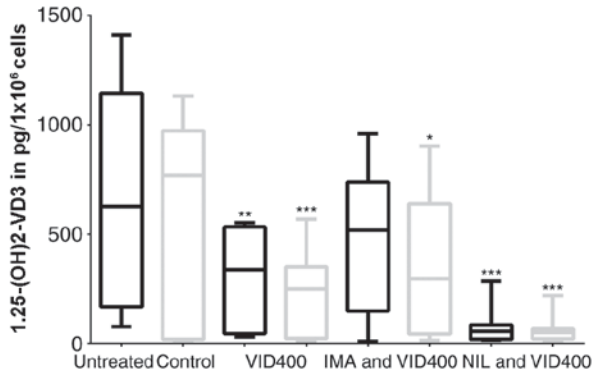

Figure 5. Synthesis of calcitriol in cultured ORS-KCs from healthy subjects (black bars) and IMA-treated subjects (grey bars) after incubation with cholecalciferol during exposure with VID400 in combination with IMA or NIL. Data were presented as mean ± standard deviation. Statistical significance level compared to untreated control is defined with P-value: $^{*}P\leq0.05$, $^{**}P\leq0.01$, and $^{***}P\leq0.001$. ORS-KC, outer root sheath keratinocytes; IMA, imatinib; NIL, nilotinib.

D receptor (VDR) (53). As described for the TKI IMA before (37), IMA inhibits CYP27B1 leading to a decrease of calcitriol in combination with an increase of calcidiol in HaCaT and ORS-KC cells. However, here we could show that NIL, according to its 20-fold stronger inhibition properties to BCR-ABL1 (16), demonstrated more pronounced inhibition of calcitriol synthesis up to 95% in comparison to untreated controls. While IMA needs to be metabolized by CYP3A4

and CYP3A5 to an active metabolite (54-56), NIL itself is an orally active drug (15). This probably leads to an even more rapid effect in comparison to IMA and agrees with our results.

Interestingly, independent of starting substrate, TKI treatment, or application of CYP450 inhibitors differences between OTC-KCs of IMA-treated patients and healthy siblings and their ability to synthesize calcidiol or calcitriol were not detected. This could be explained by the extensive cultivation period of the primary culture where the majority of OTC-KCs from IMA-treated children seem to be TKI naïve and thus a possible effect of long-term application of TKI on the cells would be lost. Gender and age of the IMA-treated children, adolescents and healthy subjects had no influence on the outcomes. Therefore, concerning their physiological VD3 metabolism, OTC-KCs of IMA-treated children are comparable to cells of healthy siblings.

For inhibition of specific enzymes involved in the VD_3 cascade (CYP24A1, CYP27A1, CYP27B1), we used VID400 and ketoconazole. VID400 acts dose-dependently with complete inhibition of CYP24A1 activity and partial inhibition of 30% of CYP27B1 (57).

Here we could demonstrate that VID400 treatment alone stabilized the levels of endogenously produced calcitriol in HaCaT. In general, it is described that under VID400 treatment the expression of the CYP24A1 enzyme is strongly amplified and prolonged (58,59). CYP24A1 catalyses the metabolization of calcidiol and calcitriol (Fig. 1) and is thereby regulated by a negative feedback loop of calcitriol concentration. For cancer cells, especially for prostate cancer cells, it has been suggested, that a rapid breakdown of the calcitriol levels are caused by an overactive CYP24A1 (60).

VID400 in combination with TKI increased calcidiol levels whereby the effect was more pronounced for NIL treatment in comparison to IMA. However, this result indicates that beside an inhibition of CYP24A1 by VID400, CYP27B1 might be affected by IMA (37) and NIL resulting in an accumulation of calcidiol. This may be due to the binding affinity of IMA and NIL to microsomal 25-hydroxylases. IMA and NIL are both metabolized by cytochrome P450 isoenzymes like CYP3A4 and CYP3A5 in the liver (54,61). Like CYP3A4, CYP27B1 in VD3 cascade is known to be a human microsomal vitamin D 25-hydroxylase as well (62).

The antifungal agent ketoconazole is a known general CYP inhibitor (63) including vitamin D hydroxylating enzymes such as CYP24A1, CYP27A1 and CYP27B1 (64). Here we could demonstrate that a treatment with ketoconazole led to increased calcidiol and decreased calcitriol levels. The same effect was shown with an application of ketoconazole and NIL.

We conclude that NIL interferes with the binding of ketoconazole and might compete for binding sites on one or more CYPs. In regard to the described interaction with CYP27B1 (37) this is also displaying the reason for the interference of TKI with the vitamin D_3 metabolism.

To summarize, our results indicate a competitive inhibition of CYP27B1 by IMA and NIL, but being more pronounced by NIL. Because CYPs in general act dose-dependently to redress a balance of metabolites, increasing calcidiol levels resulted in decreasing calcitriol levels. Keeping in mind the stronger properties of NIL in comparison to IMA possibly such distinctive effects in another context e.g., calcitriol synthesis are supposable.

In addition to the inhibition of CYP27B1 and as described for different drugs, an additional impairment of CYP24A1 is imaginable, leading to elevated calcidiol levels. However, the detailed mechanism remains weakly understood and additional investigations are needed. Knowing that pediatric oncology patients would have a- at least transiently-higher prevalence of VD_3 hypovitaminosis (25,38), further investigations are needed to identify the reasons for VD_3 deficiency in children with CML exhibiting growth delay.

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