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Review

Selective Inhibitors of Vitamin D Metabolism – New Concepts and Perspectives

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Abstract. *Background: Levels of active vitamin D (VD) are controlled by synthesis via CYP27B1 and self-induced metabolism by CYP24A1. Unbalanced high CYP24A1 expression due to induction by diverse endogenous compounds and xenobiotics, and amplification found in various tumours, might lead to local VD deficiency, thereby causing/reinforcing disorders. Materials and Methods: Using primary human keratinocytes, CYP24A1 expression was examined at the mRNA level by dot-blot and Northern blot hybridization, and at the enzyme activity level by analysing HPLC profiles from incubations with ^{3}H-labelled VD metabolites. Results: We have developed a one-step protocol to screen test compounds for potent inhibition of CYP24A1 along with selectivity over CYP27B1 and adequate metabolic stability. These inhibitors amplified hormone levels and, thereby, its function, indicated by increased CYP24A1 expression. Moreover, they stabilized the expression of a CYP24A1 splice variant, possibly serving as a buffer of VD metabolites. In addition, a low abundant, constitutive 24-hydroxylase, active in the low nanomolar range is described. Conclusion: Selective CYP24A1 inhibitors could herald a new era for vitamin D research, as well as for therapeutic application. Inhibitors may be used as single entities or in combination with low doses of potent analogs to prevent and treat various defects of growth and differentiation, and neuro-immuno-endocrine disorders.*

Natural metabolites and synthetic analogues of vitamin D (VD) show a favourable therapeutic potential in a wide variety of indications ranging from defects in bone metabolism to modulation of the immune system, and from neurological disorders to proliferative diseases, in particular cancer (1, 2). However, with few exceptions, broad application of these compounds in the prevention and treatment of diseases is still limited by severe calcemic side-effects, which they evoke at therapeutic doses.

A decade ago, our laboratory started a promising alternative/supplementary strategy that focused on inhibition of CYP24A1, one of the key players in the homeostasis of VD levels and, thereby, VD function. Levels of hormonally-active vitamin D are regulated by two-step synthesis and subsequent multi-step metabolism that eventually leads to loss of VD activity. All the reactions are catalysed by specific hydroxylases, which are members of the cytochrome P450 (CYP) superfamily and expressed in a wide variety of normal cells in the body, as well as in tumour tissues (3-5). Figure 1 displays, in a simplified version, the synthesis of $1\alpha,25(OH)_2D_3$ (or $1\alpha,25(OH)_2D_2$) from sunlight-generated vitamin D_3 (or dietary vitamin D_2) proceeding *via* 25-hydroxylation to $25(OH)D_3$ (or $25(OH)D_2$), consecutive 1α-hydroxylation *via* CYP27B1, and auto-induced metabolism by CYP24A1.

In general, the levels of $1\alpha,25(OH)_2D_3$ are short-lived: acting through its cognate nuclear receptor (VDR),

Abbreviations: VD: vitamin D_3 (D_2); $1\alpha,25(OH)_2D_3$: 1α,25-dihydroxyvitamin D_3; VDR: vitamin D receptor; CYP27A1: vitamin D-25-hydroxylase (EC 1.14.13.15); CYP27B1: 25-hydroxy-vitamin D-1α-hydroxylase (EC 1.14.13.13); CYP24A1: (1α,) 25-dihydroxyvitamin D-24-hydroxylase (EC 1.14.-.-); PXR: pregnane X receptor; HPLC: high performance liquid chromatography; rt: retention time; KGM: keratinocyte growth medium; AUC: area under the curve (concentration-time integral).

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$1\alpha,25(OH)_2D_3$ induces expression of CYP24A1, a multi-catalytic enzyme that exerts repeated oxidative attacks at different positions of the VD C_{20-27} side-chain (Figure 1: grey background). Depending on the site of the primary attack, CYP24A1 oxidation leads to different cascades of metabolites (3): the predominant pathway – termed the C-24 pathway – starts with oxidation at C-24 and leads *via* several intermediates to side-chain cleavage (6). The terminal C-23 acid (calcitroic acid) is only weakly active on the VDR, whereas earlier intermediates still retain $1\alpha,25(OH)_2D_3$-like activity (7, 8). Alternatively, initial oxidation at C-23 (C-23 pathway) results *via* several steps in the formation of a $1\alpha,25(OH)_2D_3$ -23,26-lactone, exhibiting low affinity for the VDR (9). In addition to $1\alpha,25(OH)_2D_3$, CYP24A1 also recognizes the precursor $25(OH)D_3$ as a substrate as well as the 3-epimers generated from both compounds and, in an analogous way, produces from each substrate cascades of metabolites (10-12), the majority of which still have unexplored functions.

Since almost all cells in the body possess the VDR and are therefore targets of VD hormone actions, induction of CYP24A1 can take place all over the body, leading to a transient expression of CYP24 mRNA and enzyme: rapidly induced by up to four orders of magnitude (13), CYP24A1 quickly depletes the active hormone, but also declines in parallel with the hormone(s).

Aiming at increased and stabilized levels of active hormone, we have designed potent azole-type inhibitors of CYP24A1 with IC_{50} values in the low nM range (14, 15). In the past, azole-type inhibitors of various CYPs (CYP51A1, CYP17A1, CYP19A1, *etc.*) had been successfully developed as therapeutic agents in a wide range of indications, and are still gold standards in antifungal therapy and endocrine disorders, including hormone-dependent cancer. However, this class of compounds bears a considerable risk of blocking other members of the CYP family too; concerning specifically CYP24A1, potent inhibitors might also prevent hormone synthesis by the related CYP27B1. Consequently, compounds were screened for, which selectively blocked metabolism by CYP24A1, but not activation *via* CYP27B1. As an appropriate model system primary human keratinocytes were used, which express substantial activities of both enzymes, are easily available human material and one of the major targets considered for therapeutic administration. In earlier reports, we have described how we determined inhibition (IC_{50} values) of CYP24A1 and CYP27B1 from short-term incubations with ^3H-$25(OH)D_3$, using sensitive HPLC techniques to analyse the complex metabolite profiles (14, 15). In a second step, we screened potent selective compounds over extended time-periods for their capacity to amplify the levels and life-time of active VD metabolites (=concentration-time integral (AUC)), and singled out metabolically unstable inhibitors, causing only weak amplification of AUC's. In a final step, promising compounds were examined for their enhancement of $1\alpha,25(OH)_2D_3$ function, demonstrated by amplified expression of the surrogate marker CYP24 mRNA and increased antiproliferative activity. From a total of 418 compounds entering screening, VID400 (see Table III) was selected for development in the primary indication hyperproliferative, inflammatory skin diseases, applicable as a single entity to increase/extend endogenous hormone function or in combination with low doses of potent analogues, thus avoiding calcemic side-effects.

The described screening protocol offered a multitude of valuable information concerning the potency and selectivity of the test compounds, which could be used in pharmacophore modelling (16), but also helped in the understanding of the basic mechanisms regulating the VD metabolic pathways (17). However, the procedures were lengthy, complex and expensive, especially with regard to the recording and quantitative evaluation of HPLC-analyses from thousands of incubations. In order to shorten and facilitate the screening, we have developed a new protocol that, in a one-step process, allows for the identification of potent and selective CYP24A1 inhibitors with sufficiently high metabolic stability. Here, details of this method and its application, description of new aspects of the expression and function of CYP24A1, and discussion of the prospective use of CYP24A1 inhibitors are given.

Materials and Methods

Reagents of the highest purity grade available came from E.Merck (Darmstadt, Germany), Fluka Chemie AG (Buchs, Switzerland), Sigma (St.Louis, MO, USA) and Boehringer (Mannheim, Germany). Azole derivatives were synthesized by H. Egger, according to patented procedures (Schuster, I; Egger, H. Eur. Patent Appl. 0 683 156 A1).

Vitamin D metabolites. ^3H-[26,27]-$25(OH)D_3$ (13 – 15 Ci/mmol), ^3H-[23,24]-$24(R),25(OH)_2D_3$ (95 Ci/mmol) and ^3H-[26,27]-$1\alpha,25(OH)_2D_3$ (131 Ci/mmol) were obtained from Amersham Int. (Buckinghamshire, England), stored in a toluol/ethanol (9:1, v/v; Ar-atmosphere, –20˚C) and checked for purity by sensitive HPLC (described below) before use. Inactive metabolites used as reference compounds for HPLC were obtained from Solvay Duphar BV, (Weesp, The Netherlands); $25(OH)D_3$, $1\alpha,25(OH)_2D_3$ and $24(R),25(OH)_2D_3$). $1\alpha,25(OH)_2$-3epi-D_3 was synthesized at Hoffmann-La Roche (Nutley, NJ, USA). $1\alpha,24(R),25(OH)_3D_3$, $1\alpha,23(S),25(OH)_3D_3$, $1\alpha,25(OH)_2$-24-oxo-D_3, $1\alpha,23(S),25(OH)_3$-24-oxo-D_3, $23(S),25(OH)_2$-24-oxo-D_3, $1\alpha,25(OH)_2$-26,36-lactone and calcitroic acid were produced by G.S. Reddy, using the rat kidney perfusion system, as previously described (6).

Materials for cell culture. KGM (keratinocyte growth medium), EGF, bovine pituitary extract (BPE), 0.5 μg/ml hydrocortisone, and antibiotics (gentamycin and amphothericin B) came from Clonetics, San Diego, CA, USA; fetal calf serum (FCS) from GIBCO; aqua dest (nuclease free), buffer solutions and trypsin (0.125%, pH 7.2) were provided by the medium preparing unit of the Novartis Research Institute, Vienna, Austria.

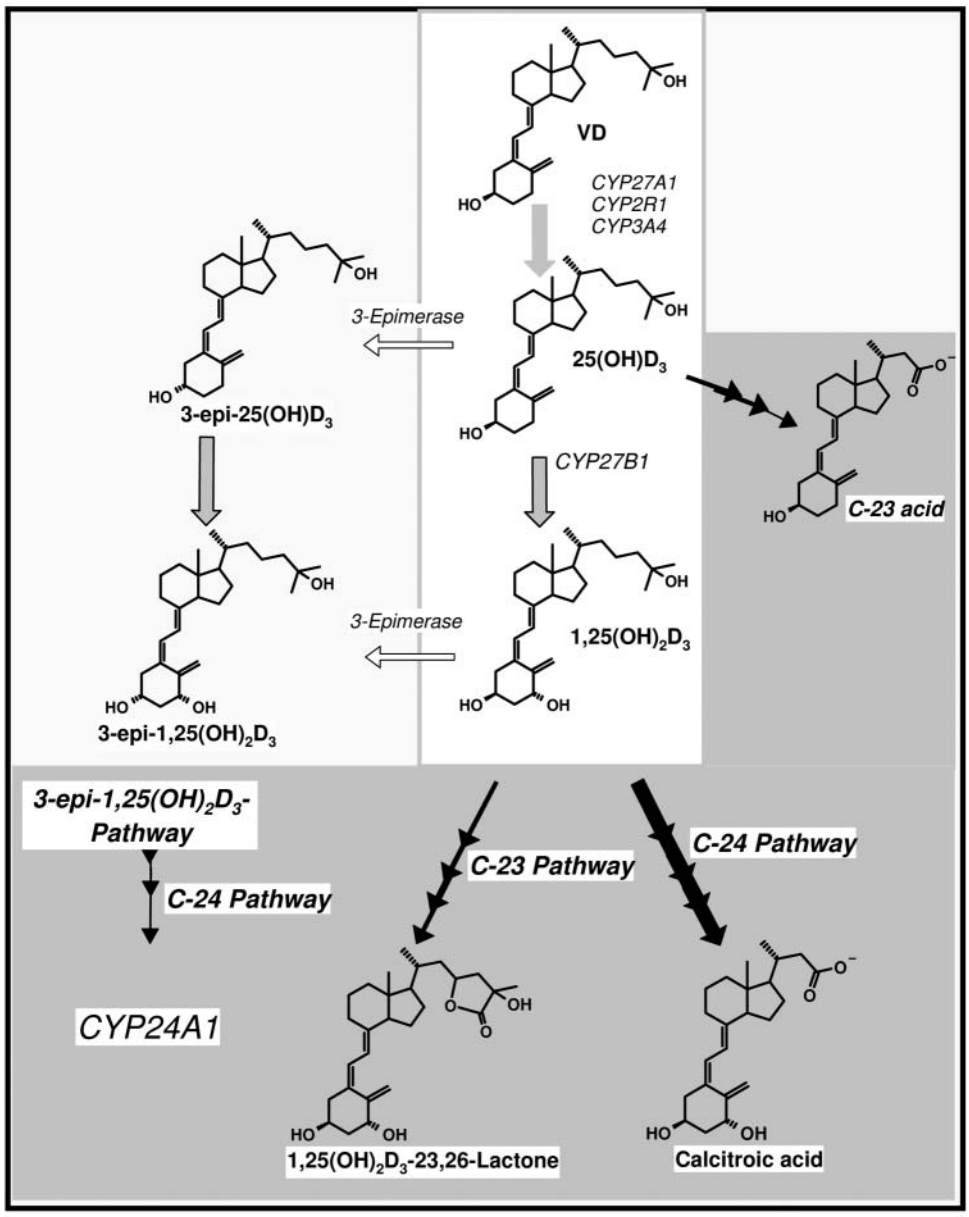


Figure 1. *Synthesis and metabolism of 1α,25(OH)$_2$D$_3$ are catalysed by different CYPs: a simplified view. Synthesis (white background) starts with 25-hydroxylation of VD (VD$_3$ or VD$_2$), which can be catalysed by several different CYPs (CYP27A1, CYP2R1, CYP3A4), followed by 1a-hydroxylation to the active hormone via the specific mitochondrial CYP27B1. 1α,25(OH)$_2$D$_3$ rapidly up-regulates mitochondrial CYP24A1, which repeatedly attacks the C$_{20-27}$ side-chain and, along two pathways, generates terminal products with low or no activity on the VDR. Additionally, CYP24A1 recognizes 25(OH)D$_3$, producing analogous cascades as from 1α,25(OH)$_2$D$_3$. VD metabolites can be converted to their 3α-epimers (light grey background), which are also substrates of CYP24A1. The reaction pathways (multiple arrows) by CYP24A1 are displayed in front of a grey background, the size of the arrows denotes their significance. For details, see text.*

HPLC materials. Solvents gradient grade (n-hexane, isopropanol, LiChrosolv for chromatography), chloroform (p.a.), ethanol (p.a.) and methanol (p.a.) came from Merck (Darmstadt, Germany), Zorbax-Sil columns (250 x 4.6 mm, 5 μM) from Agilent Technologies (Vienna, Austria). Liquid scintillation cocktails for on-line radioactivity detection (Ultima Flow M) and for stationary counting of radioactivity (β-counting, Emulsifier Safe) were purchased from Canberra-Packard (Groningen, The Netherlands).

Cultured human keratinocytes. Human epidermal keratinocytes were isolated from fresh human skin obtained from healthy adult patients, undergoing mammary reduction or cosmetic surgery. Cells isolated from epidermal sheets by trypsin (0.25%) treatment were cultured in serum-free KGM (containing 0.06 mM calcium, 10 nM EGF, BPE, 0.5 μg/ml hydrocortisone and antibiotics), as previously described (18). Culturing and subsequent experiments were done in an incubator at 37˚C in a humidified atmosphere (95% humidity)

with 5% CO_2. At 80-90% confluence, the cells were subcultured and generally used in their second passage, when they had reached 90-100% confluence.

Determination of CYP27B1 and CYP24A1 activities and inhibition by test compounds. Confluent cultures in 6-well plates (around $1.0x10^6$ cells/well), were incubated in 1 ml serum-free KGM with ^{3}H[26,27]-25(OH)D_3 or ^{3}H-[23,24]-24(R),25(OH)$_2D_3$ or ^{3}H-[26,27]-1α,25(OH)$_2D_3$ (purities ≥97%; all substrates plus carrier added dissolved in 5 μl ethanol), optimising a published procedure (19). Experiments in the absence and presence of the test compounds (at a wide concentration range), and with cells pre-incubated with and without VD-metabolites, were terminated by adding 1 ml methanol. Then the cells were scraped off, washed twice with methanol/water, and lipid-soluble compounds extracted from the cells and fluid phase with $CHCl_3$ according to (20). After measuring ^{3}H-activity in the organic and water phases (Tri-Carb liquid scintillation counter (Canberra, Packard)), the $CHCl_3$-phase was evaporated by vacuum centrifugation (Alpha RVC vacuum centrifuge; Christ, Osterode, Germany), redissolved in ethanol and aliquots (mostly containing around 100,000 dpm ^{3}H-activity) subjected to HPLC analysis (described below).

Usually, CYP27B1 activity was determined from the sum of 1α-hydroxylated metabolites formed from ^{3}H-25(OH)D_3 within a 1-h incubation period, according to (14). Keratinocytes depleted of VD metabolites prior to the start of the experiment, converted ^{3}H-25(OH)D_3 to two major products only, ^{3}H-1α,25(OH)D_3 and its sequential product ^{3}H-1α,25(OH)-3epi-D_3, while recovered ^{3}H-activity was around 100%. Inhibition of CYP27B1 activity (IC_{50} value) was determined from the reduced formation of 1a-hydroxylated metabolites in the presence of test compounds (ranging from low nM to 10 μM), using the GRAFIT–IC_{50} software (GRAFIT 4.019; Erithacus Software Ltd).

CYP24A1 activity was determined from the sum of 24-oxidized products formed per h, taking into account the stoichiometry of CYP24A1 attacks leading to each metabolite. For the complexity of metabolite profiles arising from CYP24A1 at a high induction level, we tested under conditions of "initial stage induction". Up-regulated by ^{3}H-1α,25(OH)$_2D_3$ freshly formed from ^{3}H-25(OH)D_3, CYP24A1 activity was monitored during 1 to 3 h (14): after 2-h incubation, only two main products – 1α,24(R), 25(OH)$_3D_3$ and its sequential 24-oxo-product – were detected and, after 3 h, the metabolite profiles were still simple enough to allow for quantitative evaluation. Inhibition of CYP24A1 activity by the test compounds (added in a wide concentration range) was assessed from the reduced conversion of ^{3}H-25(OH)D_3 to 24-hydroxylated metabolites. The sum of 24-hydroxylated metabolites, corrected by the number of attacks leading to each product, was plotted *versus* inhibitor concentration and the IC_{50} value determined using the GRAFIT–IC_{50} software (see above).

HPLC analysis. Straight phase analysis of organic extracts was carried out on a fully automated HPLC-system (HP 1050 series, Canberra Packard; column: Zorbax-Sil, 250x4.6 mm, 5 μm; Hewlett-Packard), using a non-linear gradient (hexane: isopropanol 97:3 - 85:15 v/v, flow rate: 2 ml/min). Metabolite profiles were recorded online by UV (264 nm, Packard) and ^{3}H-radioactivity detection (Canberra, Radiomatic 500TR). In addition, reversed phase analysis was carried out on Ultrasphere ODS (250x4.6 mm, 5 μm, Beckman) with a non-linear gradient (methanol:water 70:30 - 80:20 v/v, flow rate: 1 ml/min). Radioactive peaks in the chromatograms were assigned to chemical structures by matching with cochromatographed authentic standards (see Materials). Peaks were integrated with the software provided with the instrument, the concentration of each peak calculated and given as a percentage of the total radioactivity in the incubation or in moles/10^6 cells.

Gene expression studies. Total RNA was extracted from incubations with VD metabolites in the absence and presence of azole inhibitors, using the TRIZOL reagent (GIBCO-BRL-Life Technologies), according to (21).

Northern blot analysis of CYP24A1 mRNA followed a standard protocol (22). RNA (10 μg) was denatured at 56˚C, fractionated in a 1.2% agarose gel containing 2.2 M formaldehyde and 0.5 μg/ml ethidium bromide and transferred to a Hybond-N^+ membrane (Hybond-N^+, Amersham) by capillary diffusion. Blots were hybridized with a 60-mer fragment from the 3'-untranslated region of the CYP24A1 gene (Access.number L13286; nucleotide 1981-2040; Pharmacia-Biotech (Pfizer)), labelled with ^{32}P-dATP. After pre-hybridization to block unspecific binding, hybridization was performed at 65˚C overnight in a sodium phosphate buffer (200 mM, pH 7.0) containing 5xSSC (0.75 M sodium chloride, 0.075 M sodium citrate, pH 7.0), 5x Denhardt's solution, 7% SDS, Poly A (1 mg/ml) and salmon sperm DNA (1 mg/ml). Thereafter, the membranes were washed twice with 3x SSC and 1x SSC, and exposed to X-ray films (Kodak X-Omat AR, 1651454) for up to 7 days. Subsequently, the membranes were stripped at 80˚C in 0.1% SDS and re-probed with a 29-mer fragment from the GAPDH gene (Access.number M33197; nucleotide 348 – 376; Pharmacia-Biotech (Pfizer)). Quantification of CYP24A1 mRNA normalized to GAPDH mRNA was done by densitometric scanning of the autoradiograms.

Additionally, dot blot analysis was carried out, since this method required less material and permitted a simultaneous evaluation of a far higher number of samples, under identical conditions. RNA (5 μg / 2 μl denaturing buffer) was directly spotted to Hybond^N membranes in a 1-cm grid template, immobilized at 80˚C/2 h, and hybridised with the ^{32}P-labelled CYP24A1 probe described above. The further processing was the same as in Northern blotting.

Screening for selective inhibition of CYP24A1 by testing induction of CYP24A1 mRNA caused by 1α,25(OH)$_2D_3$, freshly generated from 25(OH)D_3. Besides comparing the inhibition of CYP27B1 and CYP24A1 tested in separate experiments, as described above, amplification of CYP24A1 mRNA levels were examined by test compounds achieved in incubations with 25(OH)D_3. In short, confluent keratinocytes were incubated in 6-well plates with 20 nM 25(OH)D_3 in the absence and presence of 200–500 nM test compounds and also with the test compounds alone, each condition in triplicates, for 24 h. Thereafter, RNA was isolated and subjected to dot-blot analysis, as described above. RNA from incubations with up to 18 test compounds alone and in the presence of 25(OH)D_3 plus the respective controls could be spotted on a single Hybond membrane. Hybridization with the 60-mer fragment of the CYP24A1 gene and reprobing with the GAPDH gene were carried out as described above. Compounds combining strong inhibition of CYP27B1 with relatively weak inhibition of CYP24A1, as well as metabolically labile compounds, had only a minor effect on the CYP24A1 expression and were excluded from further testing.

Calculations. If not otherwise indicated, the data were analysed with the GRAFIT software (GRAFIT 4.019; Erithacus Software Ltd.), which was also used for statistical analyses, curve fitting and drawing of figures.

Results

Constitutive expression of CYP24A1 in human keratinocytes. Besides the well-established induction of CYP24A1 by hormonally-active VD, strong evidence was found for constitutive CYP24A1 at the levels of both mRNA expression and enzyme activity. When cultured in serum-free KGM, devoid of VD metabolites (basal conditions), keratinocytes derived from different donors consistently displayed low, but significant, expression of CYP24A1 mRNA, detectable by real-time RT PCR (Table II; (18)).

Characterization of constitutive 24-hydroxylase activity. Prior to the onset of CYP24A1 induction by active VD, cultures also expressed considerable 24-hydroxylase activity (Figures 2, 3), as demonstrated in a short-term experiment with ^{3}H-1α,25(OH)$_2$D$_3$ as substrate (range 0.5 – 10 nM). After 1-h incubation, substantial formation of 1α24(R),25(OH)$_3$D$_3$ and its sequential oxidation product 1α,25(OH)$_2$D$_3$-24-oxo were noticed, both processes following Michaelis–Menten kinetics and saturable at low nM concentration (Figure 3, Table I). Incubations with ^{3}H-25(OH)D$_3$ (range 1.25 – 20 nM), performed in parallel, showed conversion of freshly formed 1α,25(OH)$_2$D$_3$ (amounting to 25.42 (± 2.21 SD; n=12)% of applied substrate) to 1α,24(R),25(OH)$_3$D$_3$ (peak 6) and its 24-oxo-product (peak 5; Figure 2). Analysing metabolite formation with the GRAFIT 4.0.19 Enzyme kinetics software according to Michaelis-Menten kinetics, gave identical K_m and V_{max} values, irrespective of whether 1α,25(OH)$_2$D$_3$ was exogenously applied or endogenously formed (Figure 3 A, B; Table I).

24-Hydroxylation was also detected in incubations with ^{3}H-24(R),25(OH)$_2$D$_3$ (range 0.5 – 20 nM), from which, after 1α-hydroxylation to 1α24(R),25(OH)$_3$D$_3$ (peak 6), the 24-oxo product (peak 5) was produced (Figures 2, 3 C). This process, characterized by a higher Km value, still became saturated in the low nM range (Table I). No relevant attack on substrates lacking the 1α-hydroxy group was observed.

Several other studies, carried out with keratinocytes from different donors and either ^{3}H-25(OH)D$_3$ or ^{3}H-1α, 25(OH)$_2$D$_3$ as substrates, confirmed the strong interactions with the enzyme (K_m values: range 2.7 – 7 nM) and its low abundance (V_{max} as a measure of enzyme concentration: ≤1.0 pmole / 10^6 cells/h). For these characteristics, it is obvious that constitutive 24-hydroxylase has not been recognized in studies working either at substrate concentrations far above the K_m and/or using insensitive analytical methods.

The existence of a constitutive 24-hydroxylase was substantiated in incubations over extended periods by blocking induction with the transcription inhibitor actinomycin (and, to a lesser extent, with the protein synthesis inhibitor cycloheximide). In the presence of 1 μM actinomycin, 24-hydroxylated products increased steadily with time, pointing also to a high stability of the enzyme (lifetime $t_{1/2}$ >8 h; IS *et al.*, accepted manuscript: Vitamin D: New Research. Nova Science Publishers Inc.).

Inhibition of constitutive 24-hydroxylase activity. Questioning whether selective inhibitors of inducible CYP24A1 blocked the constitutive form likewise, 24-hydroxylase activity was tested as a function of inhibitor concentration in short-term (1 h) experiments prior to the onset of induction, and after the start of induction. As an example, Figure 3D shows incubations with 20.7 nM ^{3}H[26,27]-25(OH)D$_3$ in the presence of the selective inhibitor SDZ-89443 (14), ranging from 33.0 to 2000 nM. After 1-h incubation, some 0.4% of the substrate had been converted – *via* freshly generated 1α,25(OH)$_2$D$_3$ (1.96 pmoles / 10^6 cells/h) – mainly to 1α,24(R),25(OH)$_3$D$_3$; corresponding to a rate 0.08 pmoles / 10^6 cells/h; SDZ-89443 inhibited the conversion with an IC_{50} of 410.8 (± 105.8) nM. After 2 h, around twice as much product was noticed (1.1±0.07%; corresponding to a rate of 0.11 pmoles/ 10^6 cells/h), indicating that induction had not yet started. Inhibition by SDZ-89443 (IC_{50} 369.6±107.3 nM) was close to the 1 h value. After 3 h, a steep burst in product formation (7.0±0.9%; 1.13 pmoles / 10^6 cells/h above basal activity) demonstrated almost 10-fold up-regulated activity. In spite of the excess of induced CYP24A1, inhibition by SDZ-89443 remained almost unchanged (IC_{50} 443.4±230.8 nM, matching an earlier reported value of 350±100 nM (20).

Comparable inhibition data for the basal and inducible forms of 24-hydroxylase have also been obtained with structurally different inhibitors in all studies, in which basal 24-hydroxylase activity was sufficiently high above the threshold to permit inhibition studies (data not shown). For their highly comparable product profiles and equivalent recognition of inhibitors, we assume that the basal and inducible form are due to the same CYP24A1 enzyme.

Inducible CYP24A expression in human keratinocytes. Substantial up-regulation of CYP24A1 mRNA and enzyme activity became detectable with a delay of some 2-3 h after either 1α,25(OH)$_2$D$_3$ had been exogenously added or its endogenous production from the precursor 25(OH)D$_3$ had started (14). Table II describes the kinetics of CYP24A1 induction resulting from treatment of keratinocytes with 20 nM 1α,25(OH)$_2$D$_3$: After different exposure times (and after removing the remaining inducer by washings), CYP24A1 activity was determined in a subsequent 1-h incubation with ^{3}H[26,27]-25(OH)D$_3$ (20 nM) as substrate. Enzyme activity

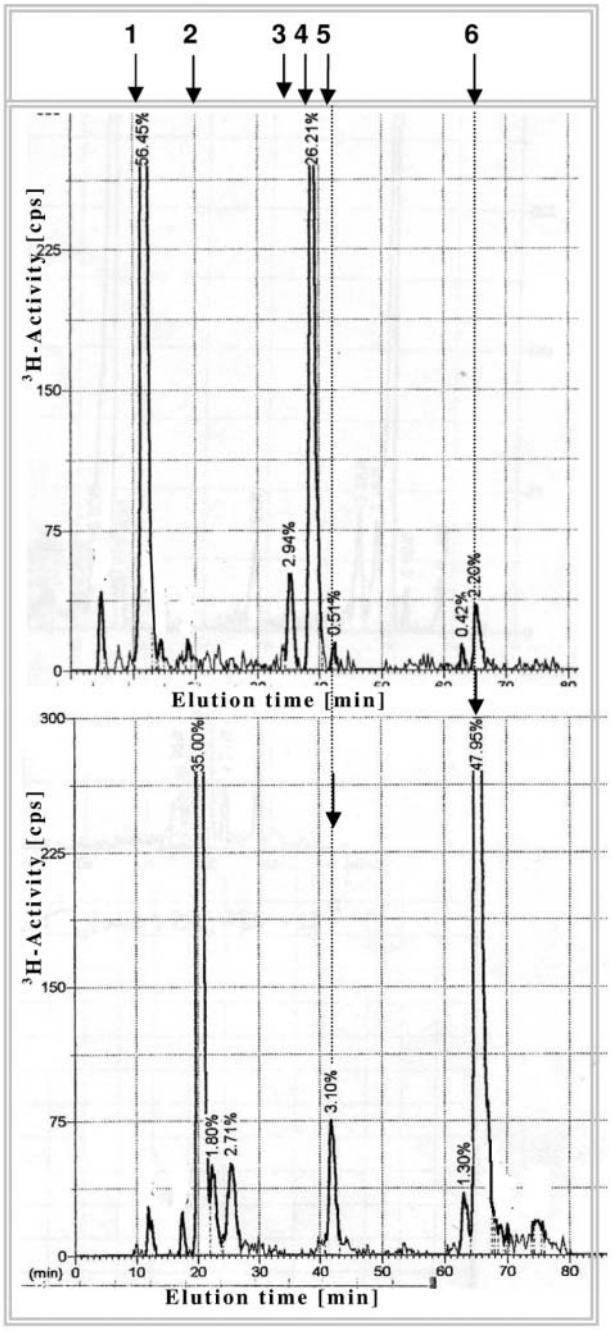


Figure 2. *Constitutive expression of CYP24A1 activity in human keratinocytes: HPLC profiles. Confluent keratinocytes from breast skin were incubated for 1 h with 2.5 nM ^{3}H-25(OH)D_3 (upper profile) or 1.0 nM ^{3}H-24(R),25(OH)$_2D_3$ (lower profile). Thereafter, incubations were extracted with $CHCl_3$ and aliquots containing >97% of applied ^{3}H-activity subjected to HPLC analysis on Zorbax-Sil (see Methods). Peaks were assigned to metabolites by matching with authentic reference compounds. 1=25(OH)D_3; 2=24(R),25(OH)$_2D_3$; 3=1α,25(OH)$_2D_3$-3-epi; 4=1α,25(OH)$_2D_3$; 5=1α, 25(OH)$_2D_3$ –24-oxo; 6=1α,24(R),25(OH)$_3D_3$. Small peaks in front of peaks 2 and 6 were assigned to the respective 3-epimers. Two peaks eluting after peak 2 were artefacts. Metabolites are given as a percentage of the total ^{3}H-activity; the detection limit was ≤0.1%. Profiles show representative HPLC recordings out of twelve chromatograms for each substrate.*

was defined as the sum of metabolites generated by CYP24A1 attacks per h, taking into account the stoichiometry of their formation, as reported (14).

Under the described experimental conditions, induction of CYP24A1 activity was transient; it peaked after 6 to 8 h exposure, exceeding basal activity (in this experiment: v=0.085 pmoles/ 10^6 cells/h) more than 100-fold and declined rapidly thereafter. In comparison, gene expression data obtained from analysing incubations with 25(OH)D_3 by real-time RT PCR (18), showed a similar time course and extent of CYP24 mRNA induction (Table II). The same kinetics were also obtained from Northern blot analyses, however, for the very faint signal of CYP24A1 mRNA from control incubations, a quantification of the extent of induction was problematic (see Figure 6).

The time-course and extent of CYP24A1 expression was crucially dependent on the presence of active VD. However, as a consequence of high CYP24A1 activity, the cells became rapidly depleted of hormone, leading to a cessation of CYP24A1 transcription and a quick disappearance of its mRNA and enzyme activity. A prolonged exposure to active VD, achieved either by high doses of the hormone, which saturated CYP24A1, or by inhibitors of CYP24A1, increased the extent of CYP24A1 induction and its life-time. Figure 4 illustrates the effect of VID400 (200 nM) on metabolite profiles arising from incubation with 20.6 nM ^{3}H[26,27]-25(OH)D_3 for 7 h. At this time, maximum induction of CYP24A1 (Table II) had led to a far-reaching depletion of continuously generated 1α,25(OH)$_2D_3$ in the control sample (around 0.058 nM) and also of earlier 1α,25(OH)$_2D_3$ metabolites, at the expense of polar side-chain cleaved products (not shown). VID400 almost completely suppressed side-chain cleavage and, earlier, biologically-active metabolites reappeared (in particular, 1α,25(OH)$_2D_3$ –3-epi rising to 1.62 nM, 1α,24,25(OH)$_3D_3$ to 0.67 nM, 1α,23,25(OH)$_3D_3$-24-oxo to 0.88 nM and 1α,25(OH)$_2D_3$ to 0.33 nM).

CYP24A1 attacks on compounds lacking the 1α-group played a minor role.

A new one-step protocol to screen for potent and selective CYP24A1 inhibitors with adequate metabolic stability. Based on the transient induction of CYP24A1 mRNA in incubations with 25(OH)D_3, *via* its product 1α,25(OH)$_2D_3$, we developed a rapid and simple procedure to screen for strong, selective CYP24A1 inhibitors:

In general, incubations with 25(OH)D_3 were found mostly depleted of active VD-metabolites after extended periods (24 h) and, at the same time, CYP24A1 mRNA expression had returned to low levels (Table II). Strong inhibitors of CYP24A1 with selectivity over CYP27B1 increased the levels and life-time of active 1α-hydroxylated VD metabolites and, thereby, amplified CYP24A1 expression. However, since maintenance of CYP24A1 expression required the enduring

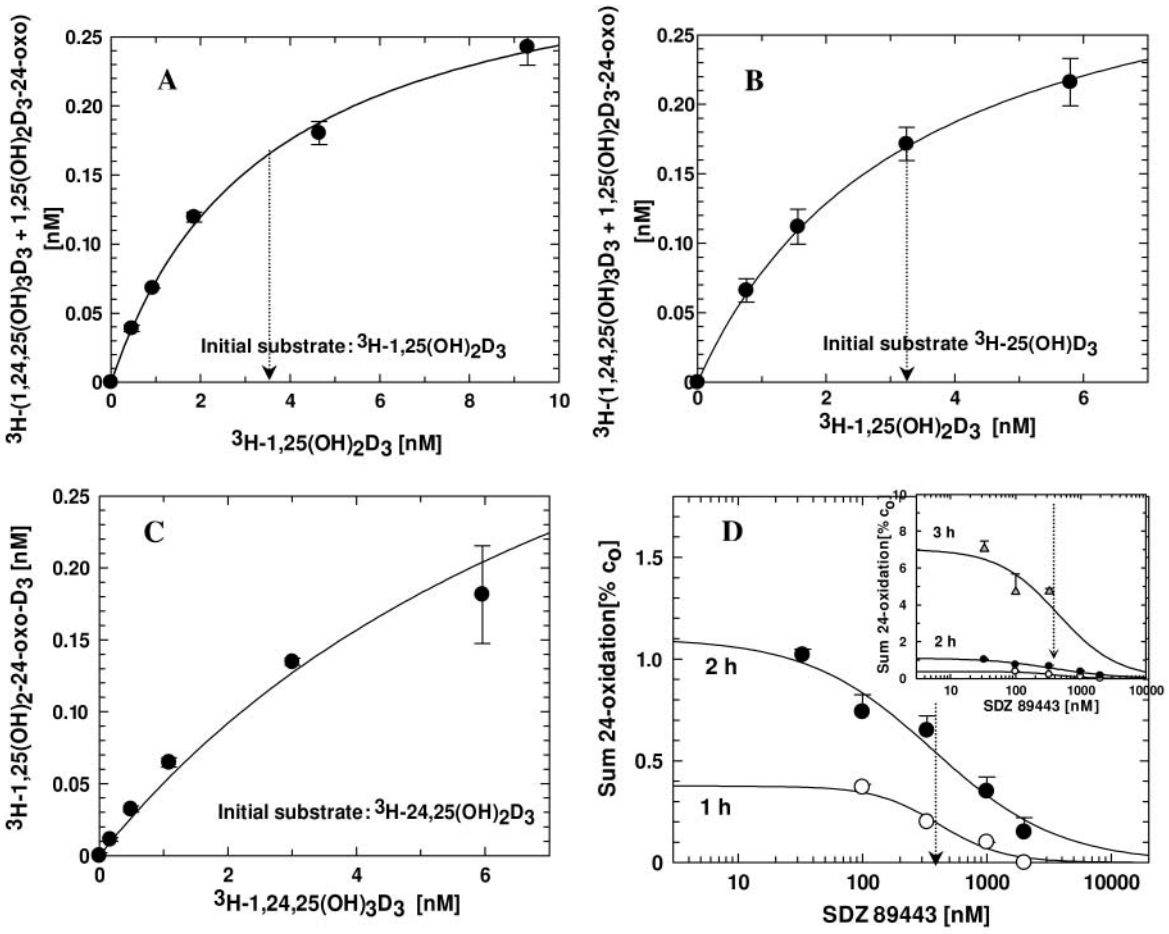


Figure 3. *Constitutive expression of CYP24A1 activity : Kinetic analysis (A-C) and inhibition by SDZ-89443 (D). A-C): Confluent keratinocytes from breast skin were incubated for 1 h with A) ^{3}H-1α,25(OH)$_2$D$_3$ (0; 0.5-10.0 nM), B) ^{3}H-25(OH)D3 (0; 1.25-20 nM), C) ^{3}H-24(R),25(OH)$_2$D$_3$ (0; 0.5-20 nM). In D) incubations were performed with 20.7 nM ^{3}H-25(OH)D$_3$±SDZ-89443 (33-2000 nM) for 1, 2 and 3 h. From all incubations, organic extracts with CHCl$_3$, containing >97% of applied ^{3}H-activity, were analysed by HPLC on Zorbax-Sil, the individual metabolites identified by matching with authentic reference compounds (Figure 2), and quantified as described in Methods. Incubations with ^{3}H-1α,25(OH)$_2$D$_3$ exogenously added (A) or endogenously produced from ^{3}H-25(OH)D$_3$ (B) generated ^{3}H-1α,24(R),25(OH)$_3$D$_3$ and its sequential product ^{3}H-1α,25(OH)$_2$D$_3$-24-oxo. In C) ^{3}H-1α,24(R),25(OH)$_3$D$_3$, endogenously produced from ^{3}H-24(R),25(OH)$_2$D$_3$, was converted to ^{3}H-1α,25(OH)$_2$D$_3$-24-oxo. The concentrations of products [in nM] are plotted versus the applied substrate concentration (A) or versus the endogenously formed substrates from applied precursors (= initial substrate; B,C). D): The concentrations of 24-hydroxylated products are plotted versus inhibitor concentration; the insert illustrates the massive rise of products when induction of CYP24A1 started. Representative data (± error) from one out of two independent experiments, each performed in duplicate, were analysed according to Michaelis-Menten kinetics (A-C), using the Enzyme Kinetics software (K_m and V_{max} values: Table I) or the IC_{50} software (D), both from GRAFIT 4.0.19 (Erithacus). Arrows indicate the concentrations of the K_m (A,B) and the IC_{50} (D).*

presence of inducing agents, only test compounds with sufficient metabolic stability could achieve long-term amplification (Table III).

Summarizing the protocol, we examined whether and to which extent the test compounds – usually tested at a single concentration of 200 nM or 500 nM – amplified CYP24A1 mRNA levels arising from incubations with 10 nM 25(OH)D$_3$ for 24 h. Incubations in triplicates were also carried out with the test compounds alone. Thereafter, total RNA was isolated and subjected to dot-blot hybridization on Hybond membranes, using ^{32}P-labelled probes of the CYP24A1 gene and of the standard GAPDH gene for reprobing, as described in Methods. Figure 5 outlines the described principle (upper graph) and gives an example of the results obtained from dot-blot analyses of parallel studies with 12 different compounds, in the presence and absence of 10 nM 25(OH)D$_3$ (lower graph): The faint signals in 1 (Figure 5) denote control samples (treated with ethanol) and are possibly due to the constitutive form of CYP24A1, described in the former section. The spots in 2 stand for incubations with 10 nM 25(OH)D$_3$ in the absence of a test compound, illustrating the reduced expression of CYP24A1 mRNA after 24 h. Spots

Table I. *Kinetic analysis of constitutive 24-hydroxylation.*

Substrate	24-Hydroxylated products	Kinetic parameters		Inhibition by VID400
		K_m [nM]	V_{max} [pmoles/10^6 cells/h]	IC_{50} [nM]
^{3}H-1α,25(OH)$_2$D$_3$ (exogen)	1α,24(R),25(OH)$_3$D$_3$ + 1α,25(OH)$_2$D$_3$-24-oxo	3.51±0.32	0.33±0.01	n.d.
^{3}H-1α,25(OH)$_2$D$_3$ (produced from ^{3}H-25(OH)D$_3$)	1α,24(R),25(OH)$_3$D$_3$ + 1α,25(OH)$_2$D$_3$-24-oxo	3.34±0.51	0.34±0.03	~15.00
^{3}H-1α,24,25(OH)$_3$D$_3$ (produced from ^{3}H-24,25(OH)$_2$D$_3$)	1α,25(OH)$_2$D$_3$-24-oxo	9.48±2.99	0.53±0.10	n.d.

For experimental details, see text. 24-Hydroxylated products are shown in Figure 2. K_m and V_{max} values are means±SD from two independent experiments, each performed in duplicate. The IC_{50} value was roughly assessed from four independent incubations with 10-15 nM ^{3}H-25(OH)D$_3$ in the presence of VID400 at a broad concentration range. n.d., not determined.

in 3 and 4 are due to 200 nM VID400, tested plus 25(OH)D$_3$ (3) and alone (4). The following pairs of spots are each due to a distinct test compound (at 500 nM) plus and without 25(OH)D$_3$, and show different degrees of CYP24A1 stabilization. Besides VID400, a remarkably strong effect was observed with NVP-VAB636 (spots 11 + 12), which had been found to be a superior inhibitor in extended earlier testing (Table III). Interestingly, NVP-VAB636 alone (spots 12), but also VID400 and several other azole-inhibitors, exerted a weak intrinsic effect on CYP24A1 expression, also in the absence of VD metabolites.

Table III lists examples of compounds, which had been examined by the extended protocol already mentioned, and were now tested by the new method. As expected, SDZ-88357, a strong inhibitor of CYP27B1 (14) that stabilized 25(OH)D$_3$ levels, did not amplify CYP24A1 expression, confirming that 1α-hydroxylation was required for CYP24A1 induction. SDZ-286907, a strong and selective CYP24A1 inhibitor when tested in short-term experiments, had shown a weak effect only on the AUC of active VD metabolites when examined over extended periods, possibly due to metabolic instability. Accordingly, dot-blot analysis revealed only a minor increase of CYP24A1 mRNA expression. Kinetic studies with four other structurally different, selective CYP24 inhibitors (VID400, NFP-VAB636, SDZ-89789 and SDZ-284814) had resulted in strongly increased AUC's of important metabolites (14, unpublished data]. A corresponding rise in CYP24A1 mRNA was detected in dot-blot screening.

In summary, in agreement with recent data and the outcome from extended former testing, the new method appears well-suited to single out potent, selective CYP24A1 inhibitors with adequate metabolic stability from large series of test compounds, in a rapid and cost-saving way. Moreover, the extent of CYP24A1 amplification can also be seen as a first valuable functional assay. Concerning a potential concomitant induction of a splice variant of CYP24A1, described in the next section, a careful selection of probes for hybridization will permit determination of both the stabilization of CYP24A1 and of its splice variant.

The described method might be adapted for high-throughput screening.

Table II. *Time course of CYP24A1 induction: expression of enzyme activity and mRNA.*

Preincubation [h]	24-Hydroxylase activity n-fold basal activity	CYP24A1 mRNA* n-fold basal expression
0 (Basal activity)	1 (0.085 (0.01 SD) pmoles/10^6 cells/h)	1 (0.027 (0.011)% GAPDH)
2	31.68 (3.57)	
3	34.79 (2.13)	32.0 (0.5)
4	58.07 (17.80)	
5		65.8 (1.8)
6	85.87 (4.92)	
8	112.56 (12.42)	
20	21.51 (2.28)	1

Confluent keratinocytes in serum-free KGM were pretreated with 20 nM 1α,25(OH)$_2$D$_3$ for the indicated periods, and thereafter incubated with 20 nM ^{3}H-25(OH)D$_3$ for 1 h. $CHCl_3$ extracts of the incubations were analysed by HPLC as described in Methods. The enzyme activity denotes the sum of all 24-oxidized products formed in 1 h, using stoichiometric coefficients for the number of attacks. The data are means (± SD) from two studies, each performed in duplicate.
*For comparison, data (means±SD) on CYP24A1 mRNA expression are listed, obtained by real-time RT-PCR from incubations with 50 pM 25(OH)D$_3$ for the indicated periods (18). Basal expression is given as % of GAPDH mRNA, assuming 100% efficiency of amplification during PCR.

Stabilization of a CYP24A1 splice variant by selective inhibitors of CYP24A1. In different cell types, including keratinocytes, induction of CYP24A1 mRNA by active VD

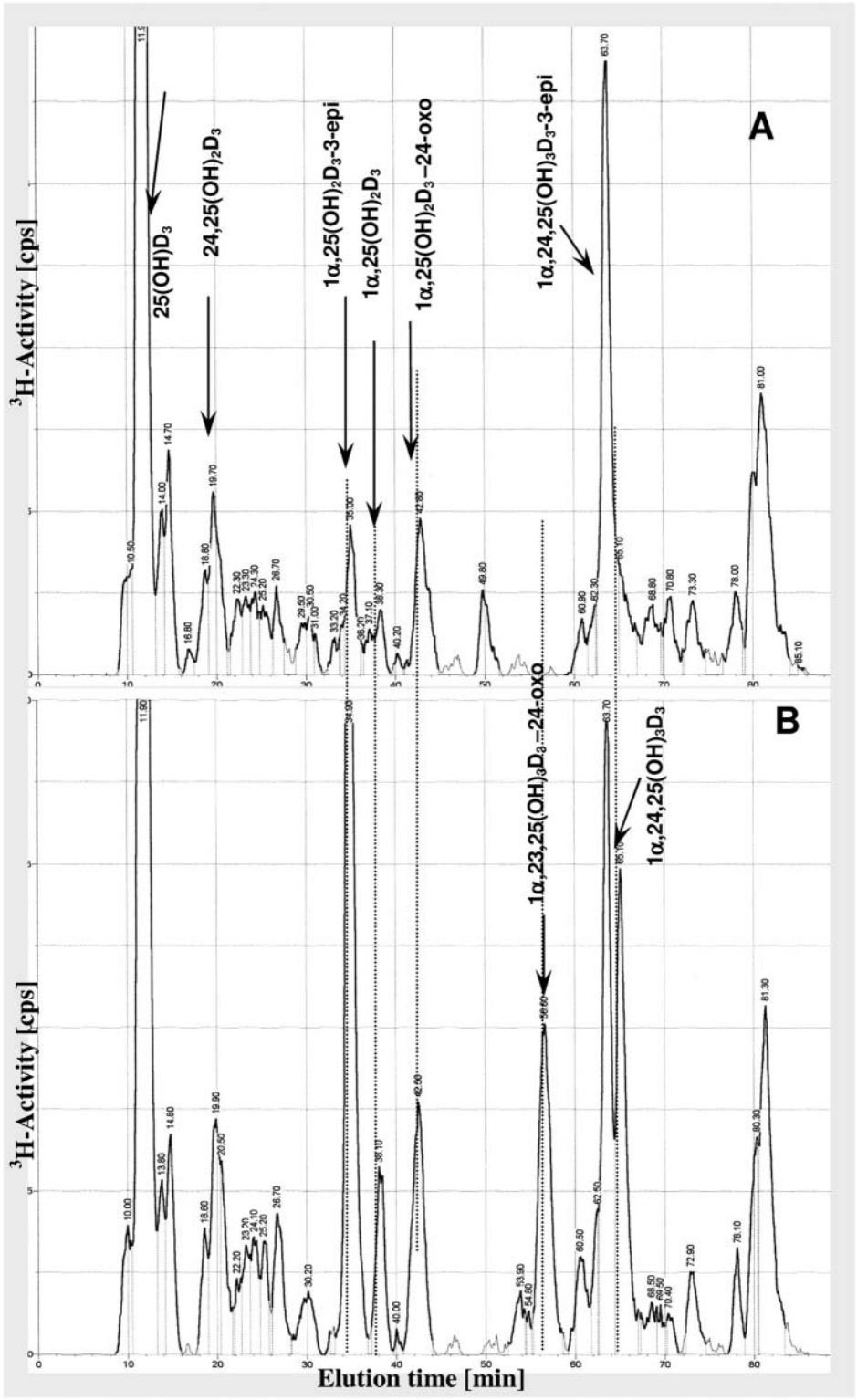
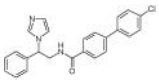
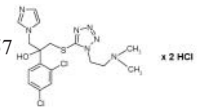
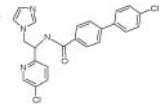
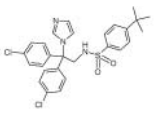
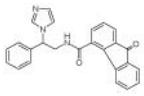
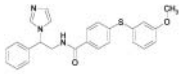
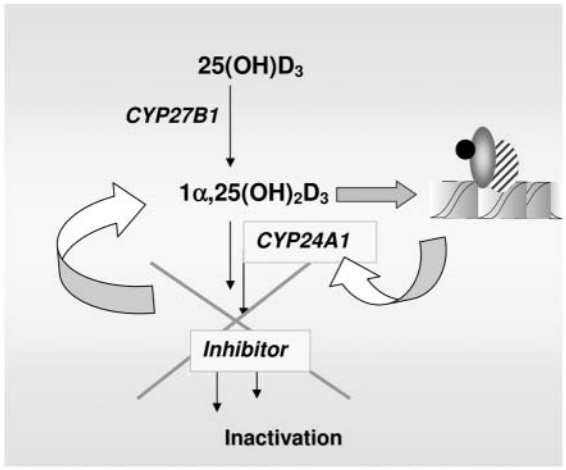


Figure 4. *Metabolism of ^{3}H[26,27]-25(OH)D$_3$ in human keratinocytes: a snapshot taken after 7-h incubation in the absence (A) and presence of VID400 (B). Confluent keratinocytes from lid skin were incubated for 7 h with 20.6 nM ^{3}H-25(OH)D$_3$ without (A) and with 200 nM VID400. Thereafter, incubations were extracted with CHCl$_3$ (56% of applied ^{3}H-activity extracted in controls and 82% in VID400-treated samples) and aliquots subjected to HPLC analysis on Zorbax-Sil, as described in Methods. The peaks were assigned to metabolites by matching with authentic reference compounds. The inhomogeneous peak at the front contains, e.g., 1α,23,24(R),25(OH)$_3$D$_3$ and polar conjugates.*

Table III. *One-step screening for strong, selective inhibitors of CYP24A1 with sufficient metabolic stability.*

Compound (concn.)	Chemical structure	CYP24A1 mRNA (score)		IC_{50} [nM]	
		Cpd	Cpd+25D	CYP27B1	CYP24A1
VID400 (R-form) (200 nM)		1.0 (0.0)	8.00 (0)	616,17 (113.2)	15,21 (3.52)
SDZ-88357 (1 μM)		0.5 (0.0)	2.5 (0.0)	60.2 (6.5)	>5000
SDZ-89787 (500 nM)		0.5 (0.0)	8.50 (0.0)	1070,0 (133.6)	284.9 (128.4)
SDZ-284814 (500 nM)		0.50 (0.0)	8.00 (0.0)	6090.0* (540.1)	56.5 (13.3)
SDZ-286907 (R-form) (200 nM)		0.50 (0.0)	4.17 (0.29)	1696,9 (421.9)	34.9 (6.9)
NVP-VAB636 (R-form) (500 nM)		1.33 (0.29)	9.33 (0.58)	266.1 (32.5)	5.08 (0.31)
Blank (5 μl ethanol)		0.5 (0.0)	–	–	–
$25(OH)D_3$ (10 nM)		–	2.0 (0.0)	–	–

Confluent keratinocytes in serum-free KGM (triplicates) were incubated for 24 h with test compounds (Cpd)±10 nM $25(OH)D_3$ (25D). Thereafter, RNA was isolated and subjected to dot-blot hybridization on Hybond membranes, as described in Methods. The intensity of the spots was scored on a scale from 0 to 10. For comparison, inhibition constants (IC_{50} values) for CYP27B1 and CYP24A1 are given, which had been determined from HPLC analyses of metabolic profiles obtained from incubating ^{3}H-$25(OH)D_3$ with the test compounds in a broad range of concentrations.
*Because of solubility problems, the IC_{50} for CYP27B1 might be too high.



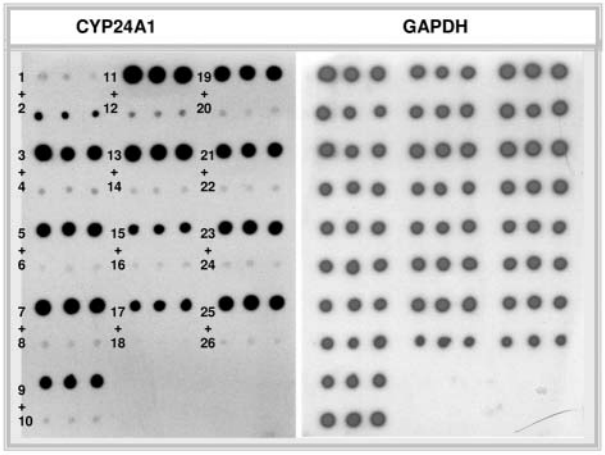


Figure 5. *A rapid one-step protocol to screen for strong selective CYP24A1 inhibitors: principle of testing (upper graph) and a representative example of dot-blot hybridization (lower graph). After extended (24 h) incubation, only compounds which selectively blocked hormone metabolism and were sufficiently stable, led to increased hormone levels and, accordingly, amplified hormone function, causing a massive rise of CYP24A1 mRNA expression. The lower panels display the expression of CYP24A1 mRNA (left) after 24-h incubation and, the same panel after reprobing, GAPDH mRNA (right). Three spots shown at each condition were derived from three incubations. The numbers stand for the following conditions: 1=blanks (5 μl ethanol as solvent); 2=10 nM $25(OH)D_3$ (25D); 3 + 4=200 nM VID400 plus 25D (3) + 200 nM VID400 (4). The following 11 compounds (tested at 500 nM) are listed in the same way: 500 nM compound plus 25D (n) + 500 nM compound alone (n+1). The strongest stabilization of CYP24A1 mRNA was noticed with NVP-VAB636 (spots 11), which itself was a weak inducer of CYP24A1 (spots 12). Spots 23 + 24 are derived from SDZ-284814 ± 25D; spots 25 + 26 from SDZ-89787 ± 25D; the three compounds are listed in Table III.*

can go along with the induction of a splice variant. This phenomenon had been recognised for a long time (*e.g.* (7, 23)), but was only recently investigated in detail (24). The splice variant was identified as a truncated version coding for a protein shortened by some 150 amino acids, which missed exon 1 and 2, including the N-terminal mitochondrial targeting sequence. As the variant was catalytically inactive, however retaining (most of) the substrate recognition sites, it was suggested that it might compete with the wild-type for substrate binding (24).

When we followed the time-course of CYP24A1 induction in keratinocytes from various donors by Northern blot analysis, our probe (from the 3'-untranslated region of the CYP24A1

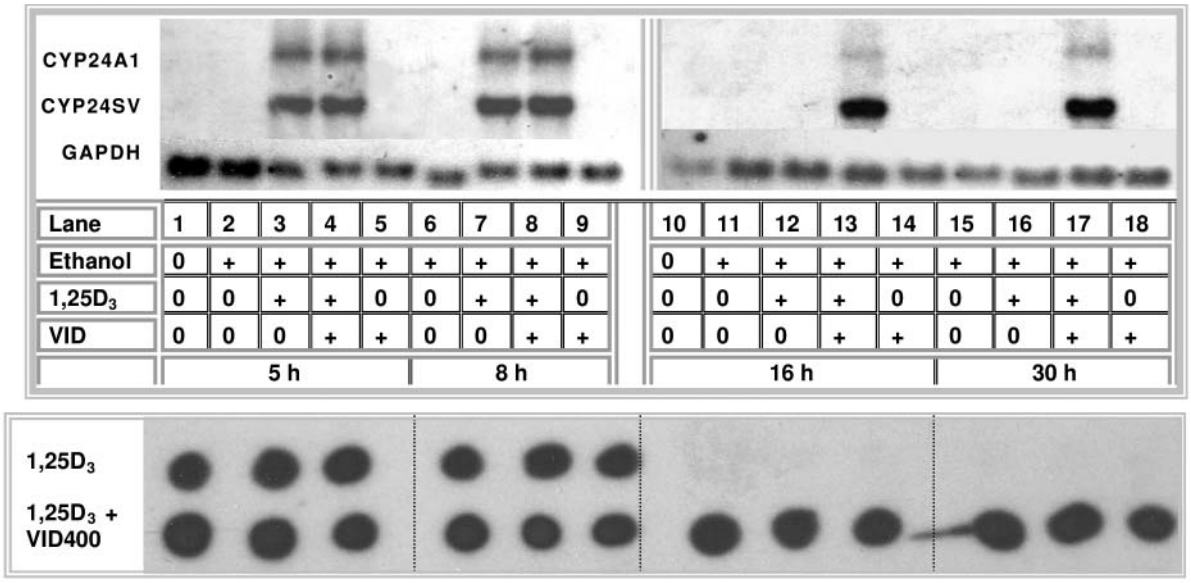


Figure 6. *Transient induction of CYP24A1mRNA and CYP24A1sv-mRNA by 1α,25(OH)$_2$D$_3$ and amplification by VID400. Confluent keratinocytes were incubated with 10 nM 1α,25(OH)$_2$D$_3$±200 nM VID400 for the indicated periods. Thereafter, RNA was extracted and subjected to Northern blot analysis (upper panel), using a probe from the 3'-untranslated region of the CYP24A1 gene (nucleotide 1981-2040), and GAPDH as reference. In addition to the 3.4 kb band of CYP24A1, a second band around 2.7 kb was detected, which was strongly amplified by VID400. The dot blot analysis, shows triplicates of each condition, at the same time points as used for the Northern blot and hybridized with the same probe (middle panel). Since it could not distinguish between both CYP24A1-forms, it showed maintained high expression levels in presence of VID400, during the entire observation period. Low panel: densitometric evaluation of the upper Northern blot; c-CYP24 and c-CYP24sv stand for incubations with 1α,25(OH)$_2$D$_3$ in absence of VID400.*

gene (nucleotide 1981-2040)), consistently recognized also a band corresponding to the reduced form and more abundant than the wild-type, in agreement with (24). Figure 6 illustrates the transient up-regulation of CYP24A1 and the splice variant (CYP24A1sv) by 10 nM 1α,25(OH)$_2$D$_3$; both forms peaked between 5 and 8 h and disappeared rapidly thereafter. When hormone depletion was inhibited by 200 nM VID400, the expression of the CYP24A1 gene became prolonged in the expected way. Surprisingly, VID400 caused a much higher stabilization of CYP24A1sv mRNA: its levels declined only slightly, exceeding expression of the wild-type by four- to five-fold. The high overall expression and its stability over extended periods were confirmed by dot-blot analysis, which, however, did not distinguish between the two forms.

Comparable effects on stabilization of CYP24A1 and its splice form have been observed with other strong selective inhibitors, pointing to a longer life-time of the splice variant and possibly also extended expression of its gene product.

Discussion

Practically all cells in the body contain the VDR and are targets of the pleiotropic functions of biologically-active VD. In the past few years, improved microarray techniques have given some insight into the multitude of genes, which can be expressed in direct or indirect response to active VD. In sum, several hundred genes were found affected by VD in normal tissues and cells derived from tumours (*e.g.,* from squamous carcinoma and prostate cancer), with marked differences among various cell types (25-27). Concerning skin, a recent report listed 98 mostly new genes, which were found crucially involved in the "VD-regulated keratinocyte terminal differentiation network" (27). Taken together, the broad array of genes suggested to be regulated by VD covers almost all major aspects of cell function. Concerning activities like growth control and differentiation an extended exposure to the hormone is required, during which the cell experiences the actions of a complex mixture of transiently-modulated genes. It appears likely that these VD functions result from coordinated actions of gene ensembles rather than from regulation by a single or a few key genes.

High CYP24A1 expression: causes and consequences. Irrespective of the mechanisms by which active VD exerts its effects, the extent and duration of these functions are mainly tuned by CYP24A1, which is rapidly up-regulated by active VD in all cells containing the VDR. *In vivo* studies in the mouse confirmed the fast and broad response to active VD: 4 h after administering $1\alpha,25(OH)_2D_3$ in a pharmacological dose, induction of CYP24A1 mRNA was noticed, especially in kidney and intestine, and at a lower level in skin, thymus, bone, and a variety of other tissues (28).

Induction of CYP24A1 reaches highest amplification among all VD-regulated genes, rising up to ten thousand-fold levels in fibroblasts (13) and increasing more than one hundred-fold over basal levels, *e.g.,* in keratinocytes (Table II). The concomitant burst of 24-hydroxylase converts active hormone rapidly *via* several intermediates to terminal products, with low or no activity on the VDR (Figure 1).

For its rapid and high expression, CYP24A1 is widely used as a sensitive indicator of the presence and hormonal function of active VD. However, recent data provide strong evidence that increased CYP24A1 expression is also possible in the absence of active VD. Induction of CYP24A1 can take place in response to various structurally different compounds from endogenous and exogenous sources (*e.g.,* lithocholic acid, calcitonin, retinoic acids; PXR-ligands), which act *via* the VDR but also *via* other receptors (29-32). VD deficiency resulting in impaired bone mineralization has been associated with prolonged treatment with antiepileptic drugs like carbamezipine or phenobarbital or therapy with rifampicin (33). These drugs, like a host of other large, lipophilic compounds, are ligands to the orphan receptor PXR, which induces expression of CYPs crucially involved in drug metabolism (34). Sharing 37% homology with the VDR, the PXR in association with the RXR has been found capable of substituting for the VDR-RXR complex at the VD response element of the CYP24A1 gene and inducing its expression (31, 33). Among our selective CYP24A1 inhibitors, several compounds also showed a slight induction of CYP24A1, possibly also through PXR-signalling (Figure 5).

In addition, amplification of the CYP24A1 gene has been detected by array-based comparative genomic hybridization in a range of cancer tissues (*e.g.,* from breast, prostate, oesophagus and gastrointestinal tract), pointing to CYP24A1 as a candidate oncogene (35, 36). As a consequence of amplification, a too rapid depletion of VD (locally activated hormone or therapeutically applied analogue) in these tissues will counteract desirable antitumour effects.

Figure 7 summarizes the major causes of unbalanced high CYP24A1 expression, which might lead to (local) vitamin D deficiency and, thereby, contribute to or even cause disorders.

As highly sensitive detection methods like real-time RT-PCR are increasingly applied, information accumulates on basal CYP24A1 expression in the absence of pharmacological doses of VD (analogues) or other inducers. Besides the kidney, varying levels of CYP24A1 expression have been demonstrated in diverse human organs, *e.g.,* in untreated normal skin, and normal tissues from breast, cervix and ovary (37, 38), suggesting that the "vitamin D endocrine system" is permanently effective in a large part of the body. Surprisingly, the expression levels in the liver were close to the detection limit, in spite of the wide variety of potential inducers that had passed through the organ before (M.Schüssler, unpublished data). Low CYP24A1 mRNA concentrations in human skin (38) possibly reflect the constitutive form, which we described in cultured keratinocytes (Figures 2, 3). Speculating on the role of constitutive CYP24A1 enzyme, its low K_m for $1\alpha,25(OH)_2D_3$ (Table I) points to an important function at the physiological hormone level; an attractive possibility might be seen in the production of 24-oxidized metabolites which, as "oxysecosterols", could be involved in (intra)cellular signalling.

Inhibition of CYP24A1: therapeutic implications. For the key role of 24-hydroxylase in controlling the levels and function of active VD (analogues), its unbalanced high and/or long-lasting expression can result in severe vitamin D deficiency and the various manifestations arising from it. Inhibitors of CYP24A1 offer a new strategy to increase and extend levels of active hormone and, thereby, its function.

CYP24A1 expression is found throughout many normal tissues of the human body, which may therefore be targets of inhibitors. Most of these tissues also comprise CYP27B1, implying the capacity of hormone synthesis in the same tissue (18, 37, 38) and an autocrine/paracrine regulation of VD

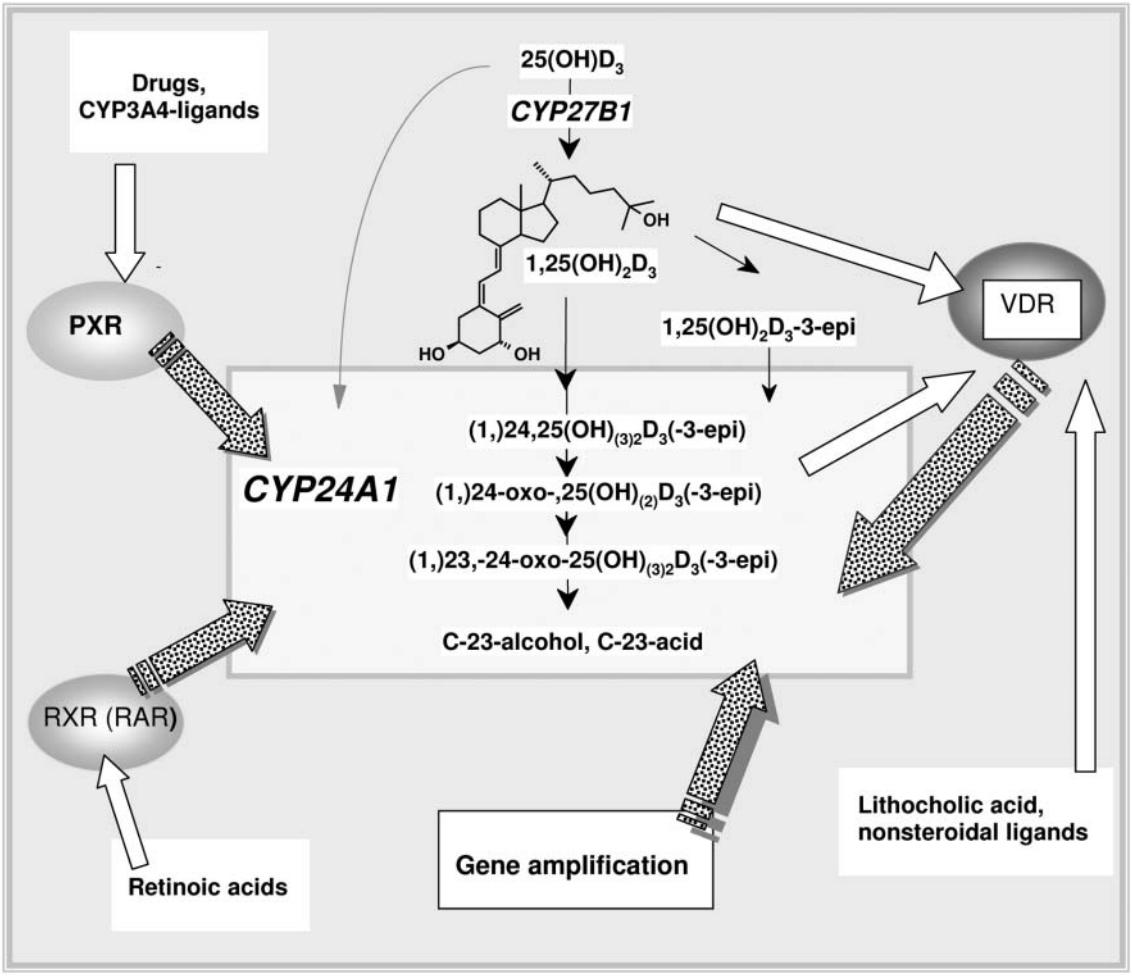


Figure 7. *Major causes of high CYP24A1 expression. Besides VD metabolites, other ligands of the VDR can induce CYP24A1, as well as a wide variety of endogenous and exogenous compounds that are ligands to the nuclear receptors RXR(RAR) and PXR. In addition, high expression of CYP24A1 due to gene amplification has been detected in various tumour tissues. High CYP24A1 expression, that is unbalanced by equivalent synthesis of or supply with 1α,25(OH)$_2$D$_3$ (analogues) can generate (local) vitamin D deficiency.*

signaling. Applied as single entity, CYP24A1 inhibitors with selectivity over CYP27B will, therefore amplify local hormone levels and function. Indications may include all diseases, in which a beneficial effect of active VD is expected (1, 2), but also prevention of defects, *e.g.,* in persons at risk from developing distinct malignancies. It is obvious that a preventive treatment requires especially high safety standards of the compounds.

CYP24A1 inhibitors may also be applied in combination therapy. Used together with a potent, however metabolically labile, VD analogue, CYP 24A1 inhibitors will reduce the dose of the analogue required for the desired action and, thereby, the risk of calcemic side-effects. Used together with drugs exhibiting a strong CYP24A1-inducing effect, like antiepileptics, antivirals *etc.*, CYP 24A1 inhibitors may protect from the VD deficiency that these drugs may elicit (33).

As a particular important indication for CYP24A1 inhibitors, we suggest the treatment of malignancies. Compared to normal tissues, various tumours show much higher expression of 24-hydroxylase, but also much higher CYP27B1 (35-38). Suppression of 24-hydroxylase may therefore enable the tumour cell to provide substantial amounts of active VD, which could switch on its anticancer program. Efficient strategies may also result from combinations of CYP24A1 inhibitors with potent analogues, as well as with various antitumour protocols like, *e.g.,* antiestrogen or anti-androgen treatment in hormone dependent cancer, thereby lowering the risk of resistance to therapy.

Concerning the wide field of potential therapeutic applications, CYP24A1 inhibitors might be screened for additional criteria: *e.g.,* appropriate tissue distribution that might enable targeting to specific sites in the body, low potential of drug-drug interactions that could predispose for

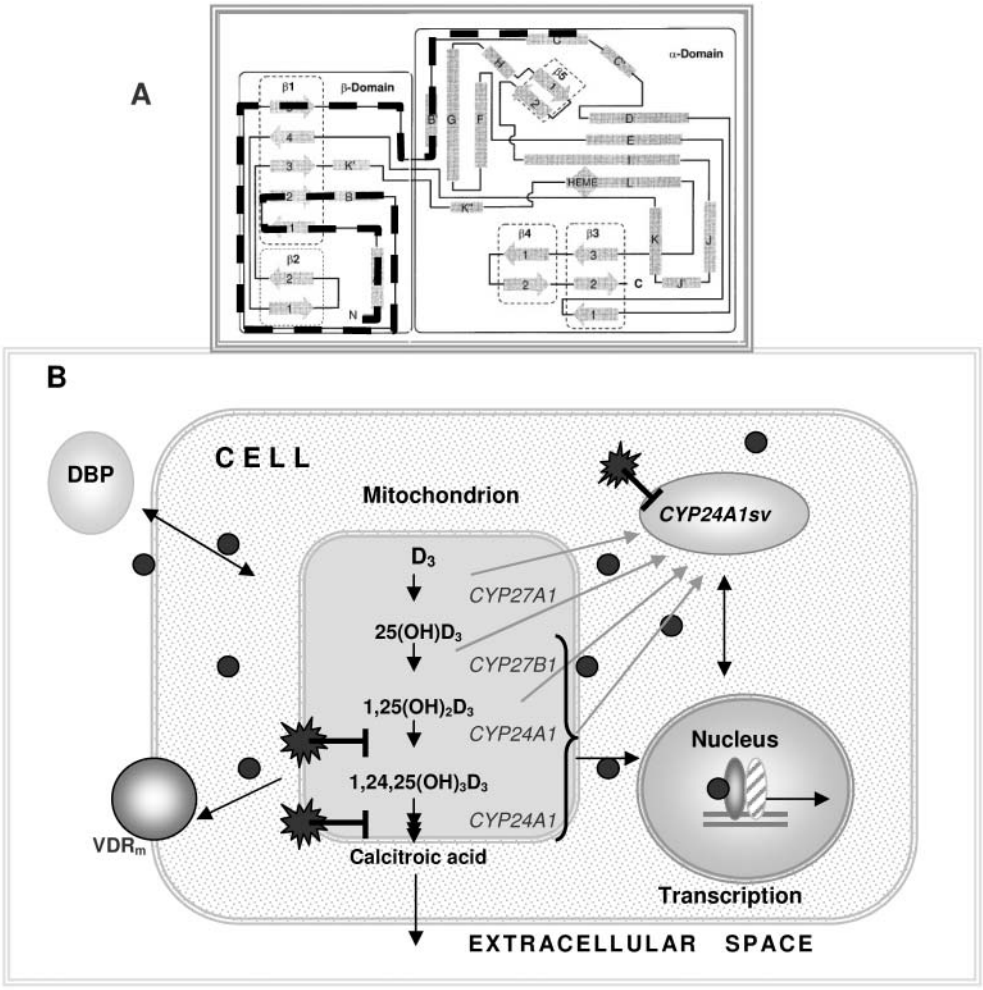


Figure 8. *Hypothetical interference of CYP24A1sv with active VD signalling – a simplified view. A: The splice variant CYP24A1sv has lost some 150 amino acids from the N-terminus (black dotted line), which constitute a major part of the β-sheet rich domain of CYP24A1, and additionally include a part of the a-helix rich domain. The heme binding site and a major part of the putative substrate recognition sites are still available for ligand binding. The depicted secondary structural elements have been modified after (39), describing CYP102. B: CYP24A1sv may compete with the wild-type for substrate (black circles) binding and, thereby modulate hormone function, but also serve as buffer for active VD metabolites. Inhibitors of CYP24A1 (stars) stabilize expression of the splice variant (Figure 7) and may also bind to the protein and make ligands available to other sites.*

combination therapy, a suitable metabolic stability, which would be required to define the duration of amplified hormone action. Basic to all improvements is a rapid and simple test for potency and selectivity over CYP27B1. The new screening method, described in this paper, is uncomplicated and inexpensive, and can be carried out by a small team in a standard biological laboratory. Applying the method in an unmodified form will allow for testing several hundred compounds within one month. Since the model uses intact cells, only compounds with appropriate uptake characteristics can pass this screen. Promising compounds might be then examined for criteria specific for the foreseen indication. Alternatively, the method could be adapted to a gene expression-based high-throughput screening.

Inhibition of CYP24A1: future perspectives. Besides their primary potential as novel therapeutics and also as agents applicable in prevention of disease, selective CYP24A1 inhibitors are valuable new tools for research. In particular, CYP24A1 inhibitors may help to reduce the complexity of the "vitamin D endocrine system" by an improved understanding of its components and their role therein. Activities observed in experiments with $1\alpha,25(OH)_2D_3$ over extended time-periods are commonly ascribed to this compound, irrespective of its conversion to a wide spectrum of transient metabolites, most of them with still unexplored function. Stabilizing the lifetime of distinct VD metabolites by CYP24A1 inhibitors may help to identify whether and which intrinsic activities these products possess. It appears likely that some metabolites

acting as "oxysecosterols" might reveal interesting functions through receptors different from the VDR.

A challenging new issue may arise from the expression of the CYP24A1 splice variant and its stabilization by CYP24A1 inhibitors (Figure 6). CYP24A1sv has been shown to code for a truncated, catalytically inactive protein that still retains (a major part of) its substrate binding domain and, therefore, competes with the wild-type for substrates (24). This was concluded from a reduced synthesis of $1\alpha,25(OH)_2D_3$ in cells overexpressing CYP24A1sv, and increased $1\alpha,25(OH)_2D_3$ synthesis in the presence of an antisense CYP24A1sv (24).

As competition for substrates between CYP24A1 and its splice variant was shown to affect hormone synthesis (24), it may also slow down 24-oxidation. CYP24A1sv might act as a buffer, sequestering substrates when they are abundant and releasing them when their cellular levels decline. Consequently, inhibitors of CYP24A1 could increase the levels and life-time of VD metabolites by a dual mechanism: by directly blocking their metabolism, and by stabilizing CYP24A1sv that might protect them from too rapid inactivation. Figure 8 illustrates suggested effects of CYP24A1sv expression on VD-dependent processes. Besides interfering with VD synthesis and metabolism, CYP24A1sv may also tune signalling through the VDR and possibly prolong hormone action. CYP24A1 inhibitors may extend hormone function by directly amplifying hormone levels and by stabilizing the protein, which stores them.

An important difference between the wild-type and the variant protein could be a different substrate specificity, resulting from the loss of sites possibly involved in substrate access and recognition: in spite of their low sequence homology and enormous variety of functions, all P450 structures analysed so far show the same overall structural fold, consisting of a β-sheet rich domain, a larger α-helix-rich domain and a conserved heme-binding core. Figure 8 displays a sketch, in which the missing 150 amino acids (dotted line) of the splice variant are tentatively overlaid over the secondary structure of a representative CYP (CYP102; (39)). The truncation comprises a large fraction of the β-sheet rich domain, including parts involved in a putative substrate access channel (at the BC-loop/B'helix (40)) and possibly one of the substrate recognition sites (at the B' helix (41)). We speculate that these changes can cause a different specificity of both forms for substrates, but also for inhibitors, and that it might be possible to find inhibitors, which selectively block metabolism by the wild-type, but leave the variant available for interactions with VD metabolites. The up-regulated persistent splice variant might then serve as a hormone carrier and enable sustained action.

Conclusion

Inhibitors of CYP24A1 offer a very wide field of application, ranging from basic research on the complex endocrine network of vitamin D, the function of its components and its cross-talk with other signalling systems, to numerous options for a successful use in prevention and treatment of diseases. Like VD analogues in the nineties of the past century, inhibitors of VD hydroxylases could herald a new era for vitamin D research as well as for therapeutic application.

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