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Original article

Metabolic stereoselectivity of cytochrome P450 3A4 towards deoxypodophyllotoxin: *In silico* predictions and experimental validation

Mattijs K. Julsing [a,1], Nikolay P. Vasilev [b,1], Dina Schneidman-Duhovny [c], Remco Muntendam [a], Herman J. Woerdenbag [a], Wim J. Quax [a], Haim J. Wolfson [c], Iliana Ionkova [b], Oliver Kayser [a,*]

[a] *Department of Pharmaceutical Biology, Graduate School for Drug Exploration (GUIDE), University of Groningen, Antonius Deusinglaan 1, NL-9713 AV Groningen, The Netherlands*
[b] *Department of Pharmacognosy and Botany, Medical University – Sofia, 2 Dunav, Sofia 1000, Bulgaria*
[c] *School of Computer Science, The Raymond and Beverly Sackler Faculty of Exact Sciences, Tel Aviv University, Tel Aviv 69978, Israel*

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Abstract

Deoxypodophyllotoxin is stereoselectively converted into epipodophyllotoxin by recombinant human cytochrome P450 3A4 (CYP3A4). Further kinetic analysis revealed that the Michaelis–Menten K_m and V_{max} for hydroxylation of deoxypodophyllotoxin by CYP3A4 at C7 position were 1.93 μM and 1.48 nmol/min/nmol, respectively. Deoxypodophyllotoxin was subjected to automated docking analysis in order to get better knowledge of the interaction between the CYP3A4 enzyme and the substrate, using the *PatchDock* algorithm with distance constraints. Automated docking showed that the β-hydrogen atom at C7 position is in the most appropriate binding orientation at the site of oxidation. The docking results are consistent with the experimental data for the bioconversion of deoxypodophyllotoxin into epipodophyllotoxin by CYP3A4. In addition, the effects of five lignans, deoxypodophyllotoxin, epipodophyllotoxin, podophyllotoxin, demethylenedeoxypodophyllotoxin, and demethylenepodophyllotoxin, on CYP3A4 were compared in order to investigate the influence of the methylenedioxy group on the biotransformation process, to give insight into the mode of metabolization and to explain inhibitory activity of lignans.

Keywords: Cytochrome P450 3A4 (EC 1.14.14.1); Epipodophyllotoxin; Lignans; Drug metabolism; Automated docking

1. Introduction

Cytochrome P450 3A4 (CYP3A4) is the main human metabolizing enzyme. It accounts for about 30% in the human hepatic metabolism of xenobiotics [1]. It has been shown that CYP3A4 possesses a relatively large substrate-binding cavity, being consistent with its capacity to oxidize bulky substrates such as cyclosporin, statins, taxanes, and macrolide antibiotics [2]. Due to its broad substrate specificity, CYP3A4 may have further interesting pharmaceutical applications. Human cytochrome P450 enzymes can be used to mimic plant cytochrome P450s in a combinatorial biosynthesis strategy.

Podophyllotoxin (Fig. 1) is a lignan with potent antimitotic and antiviral activity. Its semi-synthetic derivatives teniposide and etoposide are widely used as cytostatic drugs. Podophyllotoxin has been isolated from the rhizomes of *Podophyllum peltatum* and *Podophyllum hexandrum* (Berberidaceae). The content podophyllotoxin in these plants is low and the supply of podophyllotoxin from its natural sources is limited due to overcollection [3,4]. For this reason several alternative sources, like chemical synthesis and the use of plant cell cultures [3,5], have been explored in the last decades. Unfortunately, these alternatives were not economically feasible. Whereas, the source of podophyllotoxin in nature is limited, the supply of the structurally closely related deoxypodophyllotoxin (Fig. 1) is sufficient. Deoxypodophyllotoxin can be isolated from rhizomes of *Anthriscus sylvestris* (L.) Hoffm. (Apiaceae) [6,7], an abundantly growing plant that can be easily

* Corresponding author. Tel.: +31 50 363 3299; fax: +31 50 363 3000.
E-mail address: o.kayser@rug.nl (O. Kayser).
[1] Both authors contributed equally to this work and paper.

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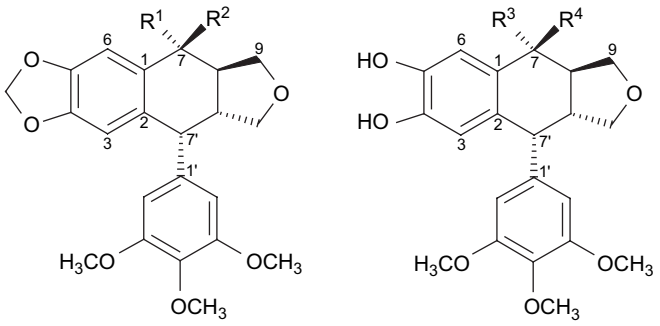

Deoxypodophyllotoxin	R^1 = H; R^2 = H
Podophyllotoxin	R^1 = OH; R^2 = H
Epipodophyllotoxin	R^1 = H; R^2 = OH
Demethylenedeoxypodophyllotoxin	R^3 = H; R^4 = H
Demethylenepodophylloxin	R^3 = OH; R^4=H

Fig. 1. Chemical structures from lignans of interest.

cultivated. As a new biotechnological alternative, we described the successful stereoselective hydroxylation of deoxypodophyllotoxin by recombinant CYP3A4 in *Escherichia coli*. Epipodophyllotoxin (Fig. 1) has been detected as the only metabolite [8]. Therefore, the heterologous expression of CYP3A4 in *E. coli* presents an interesting alternative for a large-scale production of epipodophyllotoxin.

The optimization of the process, which is of basic importance for a possible industrial application, requires more insight into the mechanism of the metabolization process. The structure of CYP3A4 itself has been determined by X-ray crystallography to 2.05 Å resolution [2]. However, it is technically non-feasible to solve the crystal structure of CYP3A4 complexed with any substrate. *In silico* experiments, like molecular modeling should facilitate a better understanding of the substrate selectivity of the CYP3A4 enzyme and can shed a light on the structural aspects of the interactions of this enzyme. Distance constrained docking is possible when the atoms involved in the binding between the enzyme and the ligand are known. For CYP450 enzymes, it is known that the substrates are oxidized using the iron atom of the heme group present in the enzyme. Because it is also known from the HPLC–SPE–NMR data at which position deoxypodophyllotoxin is oxidized by CYP3A4, distance constrained docking was applied in order to determine the orientation of the substrate molecule within the catalytic site.

One of the drawbacks in using CYP3A4 as a converting system is the often occurring inhibition of this enzyme by substrates and/or products [9]. This phenomenon is well known

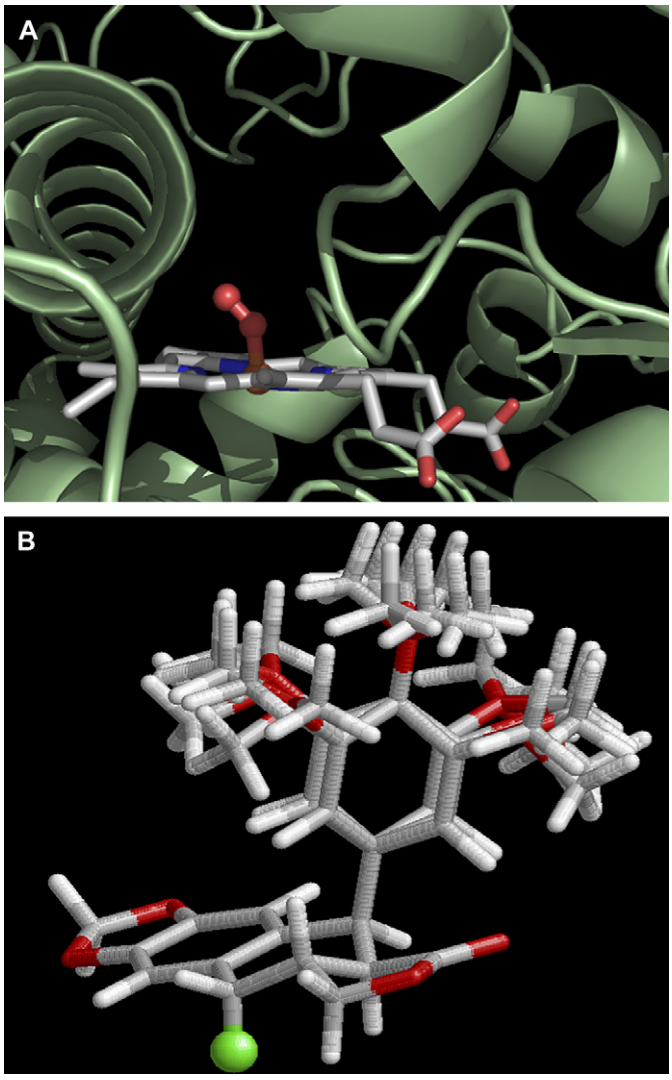

Fig. 2. Input molecules: (A) binding site of CYP3A4 with superimposed O_2 molecule; and (B) deoxypodophyllotoxin molecule conformations, the oxidation site (β-hydrogen atom) is colored green. (For interpretation of color in this figure, the reader is referred to the web version of this article.)

for CYP450 enzymes and influences drug metabolism and endogenous compounds in the human body. The inhibition of CYP3A4 by lignan structures has been described and the methylenedioxyphenyl moiety of some lignans has been considered as the substructure responsible for inhibition [10]. Since deoxypodophyllotoxin and epipodophyllotoxin contain such moiety it is likely that they may cause inhibition of the enzyme as well. Therefore, we determined the kinetic

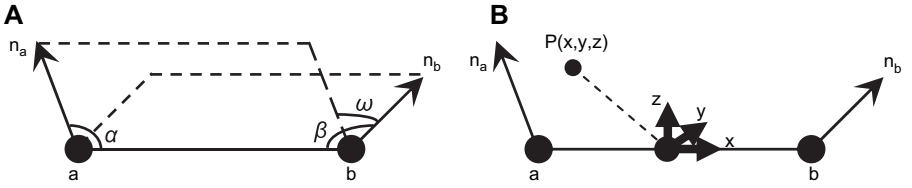

Fig. 3. (A) Reference frame based on two points and their associated normals. The distances (geodesic and Euclidean) and angles are invariant to rotations and translations. (B) Addition of anchor point coordinate to the reference frame for distance constrained docking.

parameters of this reaction and tested the presence of a probable inhibition on the enzyme by deoxypodophyllotoxin or its product epipodophyllotoxin. To correlate the inhibition to the structure of the lignan, the influence of podophyllotoxin and two lignans lacking the methylenedioxy moiety, demethylenepodophyllotoxin and demethylenedeoxypodophyllotoxin (Fig. 1) on the enzymatic reaction were investigated as well. In addition, the same docking procedure as used for substrate binding was applied to construct a model for the inhibition of CYP3A4 by the epipodophyllotoxin molecule with the methylenic carbon atom from the methylenedioxy moiety used as an anchor atom oriented towards the iron atom of the heme group.

2. Results

2.1. Bioconversion of deoxypodophyllotoxin into epipodophyllotoxin

The bioconversion of deoxypodophyllotoxin by CYP3A4, co-expressed in *E. coli* with the human cytochrome p450 NADPH reductase and supplemented with a NADPH generating system, was dependent on the incubation time, CYP3A4 concentration and substrate concentration. Fig. 4A shows linearity for the time of incubation up to 10 min. Extended incubation did not result in a linear increase of product formation anymore. The increase of the amount of enzyme added to the reaction mixture resulted in a linear response up to 69.5 pmol CYP3A4 per ml (Fig. 4B). Based on incubation time and protein concentration in the determined linear range, the increase in epipodophyllotoxin formation out of increasing concentrations deoxypodophyllotoxin followed Michaelis–Menten kinetics (Fig. 4C).

Kinetic analysis of the deoxypodophyllotoxin hydroxylation was further performed in order to calculate the Michaelis–Menten constants. K_m value for this reaction was 1.93 μM and the V_{max} was determined to be 1.48 nmol/min/nmol CYP3A4. Therefore, the efficiency of the catalysis expressed as V_{max}/K_m was 0.77 $nM^{-1}\,min^{-1}$. As a typical substrate compound and well accepted reference compound in studies, testosterone was used in the studies of possible inhibition [11]. Kinetic parameters were determined using the same bioconversion system. For bioconversion of testosterone in the used system K_m was determined as 20.5 μM and the V_{max} was 3.03 nmol/min/nmol CYP3A4, which resulted in a V_{max}/K_m of 0.15 $nM^{-1}\,min^{-1}$.

2.2. Inhibition of cytochrome P450 3A4

Inhibition of CYP3A4 by lignan structures was investigated by examining their influence on the bioconversion of the substrate testosterone into 6β-hydroxytestosterone. The bioconversion was recorded over 1 h. Table 1 shows differences in formation in 6β-hydroxytestosterone after coincubation of several concentrations of the known CYP3A4 inhibitor miconazole [12] and the different lignan structures.

Coincubation with miconazole exerted an inhibiting effect on CYP3A4 activity, showed by the decreased testosterone metabolization after concentration-dependent miconazole exposition. Coincubation with 25 μM miconazole resulted in 4.38% 6β-hydroxytestosterone formed after 1 h incubation in comparison to the control experiment without miconazole. The methylenedioxyphenyl lignans deoxypodophyllotoxin, epipodophyllotoxin, and podophyllotoxin showed all comparable inhibiting effects on the testosterone bioconversion as miconazole. For these three lignans, 6β-hydroxytestosterone contents formed were 4.85%, 11.46%, and 7.04%, respectively, in comparison to the control experiment, without a lignan added. Demethylenedeoxypodophyllotoxin and demethylenepodophyllotoxin are both lacking the methylenedioxy moiety.

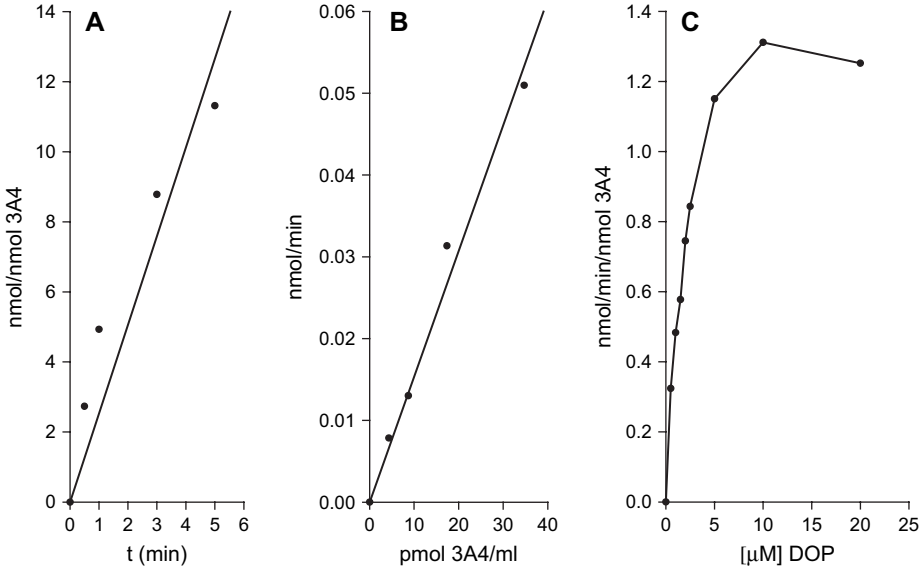

Fig. 4. Dependence of hydroxylation activities catalysed by CYP3A4 on incubation time (A), enzyme contents (B), and deoxypodophyllotoxin concentrations (C). (A) Concentrations of deoxypodophyllotoxin and enzyme were respectively 10 μM and 69.5 pmol/ml. (B) Incubation time was 20 min and concentration of deoxypodophyllotoxin was 10 μM. (C) Incubation time was 5 min and concentration of enzyme was 34.8 pmol/ml.

Table 1
Inhibiting effects of several concentrations of miconazole and lignan compounds on the bioconversion of 25 μM testosterone

Coincubated compound	Relative bioconversion (%)			
	0 μM	1 μM	10 μM	25 μM
Miconazole	100	41.1	10.5	4.38
Deoxypodophyllotoxin	100	61.0	10.4	4.85
Epipodophyllotoxin	100	69.4	7.96	11.46
Podophyllotoxin	100	44.1	7.82	7.04
Demethylenedeoxypodophyllotoxin	100	103.0	69.6	69.7
Demethylenepodophyllotoxin	100	95.0	98.7	97.1

Coincubation of testosterone and 1, 10, and 25 μM demethylenepodophyllotoxin resulted in respectively 95.0%, 98.7%, and 97.1% (Table 1) metabolized testosterone compared to the bioconversion assay of testosterone without a lignan added. Therefore, it can be stated that coincubation of demethylenepodophyllotoxin with testosterone did not influence the formation of 6β-hydroxytestosterone and CYP3A4 was not inhibited by this lignan. Coincubation of testosterone and 1, 10, and 25 μM of demethylenedeoxypodophyllotoxin showed a decreased synthesis of 6β-hydroxytestosterone of respectively 103%, 69.6%, and 69.7% compared to the control assay (Table 1). Nevertheless, the influence on the testosterone conversion was less drastic than the decrease in bioconversion caused by miconazole and the three methylenedioxy lignans.

Since deoxypodophyllotoxin has been identified as a substrate for recombinant CYP3A4 giving epipodophyllotoxin as the metabolite, the possibility of metabolisation of the other, in this study, used lignan structures was investigated as well. Bioconversion assays using these compounds as the single substrate for CYP3A4 were performed. According to the HPLC chromatograms bioconversion assays performed with 10 μM of epipodophyllotoxin, podophyllotoxin, and demethylenepodophyllotoxin as substrates did not show any additional peaks of formed metabolites. Nevertheless, demethylenedeoxypodophyllotoxin ($k' = 6.04$) did show an extra peak with capacity factor $k' = 4.50$, which corresponded with capacity factor of demethylenepodophyllotoxin. HPLC–MS analysis as performed and described before [8], identified this peak as the hydroxylated metabolite.

2.3. Modeling CYP3A4–deoxypodophyllotoxin interaction

The distance constrained docking method was applied to dock the deoxypodophyllotoxin molecule into the CYP3A4 active site with the iron bound oxygen of the O_2 molecule at the heme group and C7 as anchor atoms. The distance constraint between these two atom centers was set to 4 Å. The deoxypodophyllotoxin molecule was subjected to conformational search and each of the 20 conformers was docked separately. The candidate solutions for each conformer of the deoxypodophyllotoxin molecule were clustered using RMSD of 1 Å. The resulting complexes were further refined by the Monte-Carlo based refinement method. The refinement method minimizes the energy of the complex by adjustment of the side-chain rotamers of the receptor and improvement of the ligand position in the active site. As a result, in some of the solutions the anchor atoms were moved apart. We retained only solutions where the distance between the anchor atoms remained below 4 Å. The three top scoring complexes according to the binding energy actually represented very similar solutions (Fig. 5A). This means that three different docking solutions converged to the same minima, adding more confidence to the quality of prediction.

We analyzed the solution (out of three top scoring) with the shortest distance between the anchor atoms (2.64 Å). This solution was ranked second (Fig. 5, blue color). The residues of CYP3A4 in contact with the deoxypodophyllotoxin molecule are Arg105, Arg212, Thr309, Ile369, Ala370, Arg372, Leu373, Glu374, Arg375, and Leu482. A residue was considered as a contact residue, if one of its atoms is within 4 Å from one of the deoxypodophyllotoxin atoms. The model was analyzed using the LPC server [13]. By this method seven

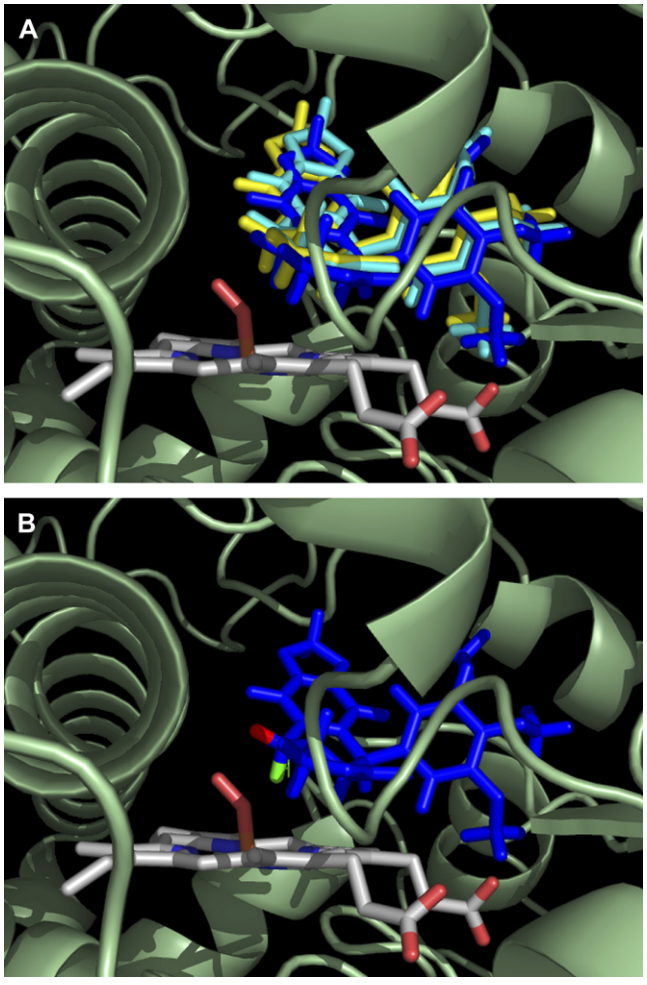

Fig. 5. (A) Top three low-energy docking solutions, showing convergence to the same orientation in the binding site; and (B) stereoselectivity of the solutions towards β-hydrogen atom (green color) at C7 position of deoxypodophyllotoxin compared to α-hydrogen atom (red color). (For interpretation of color in this figure, the reader is referred to the web version of this article.)

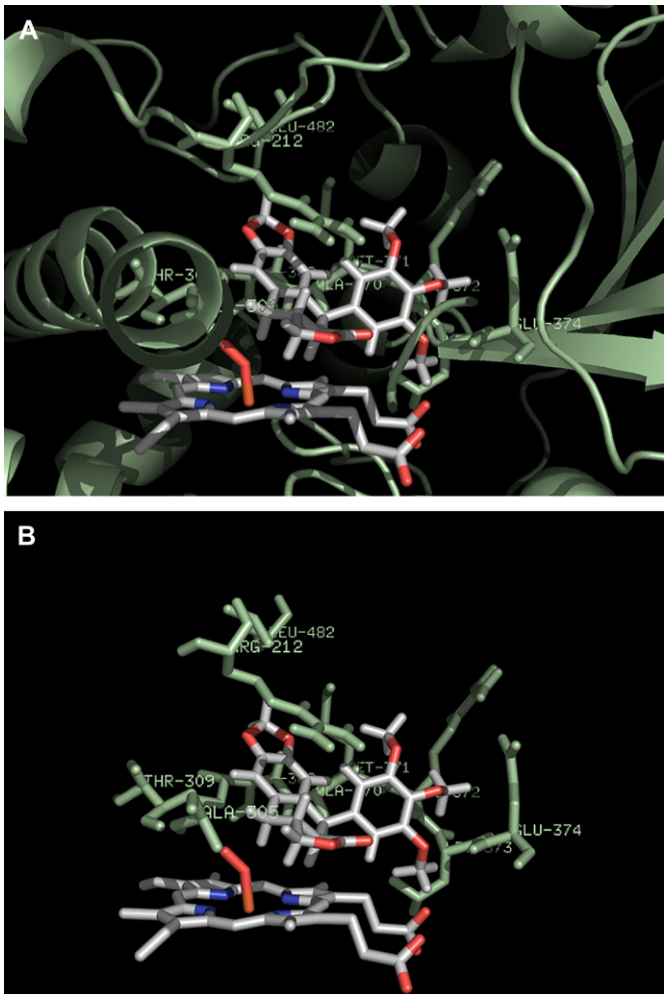

Fig. 6. Residues of CYP3A4 in contact with deoxypodophyllotoxin molecule presented in the complete structure of the active site (A) and with the amino acids involved only (B). Residues Arg105, Arg212, and Glu374 form hydrogen bonds with the substrate according to LPC server [13]. Other interacting residues are Thr309, Ile369, Ala370, Arg372, Leu373, Arg375, and Leu482.

hydrogen bonds were recognized (Fig. 6). However, the distance spanned by two of them was more than 4 Å. Specifically, the oxygen atom from the carbonyl group at C-9′ position of the deoxypodophyllotoxin molecule makes a hydrogen bond with the amide group of Arg212 residue; 3′-methoxy group of the substrate molecule interacts with Arg105; 4′-methoxy group is linked by hydrogen bond to Glu374; and the oxygen from 5′-methoxy group forms hydrogen bonds both with Arg105 and Glu374 residues. In addition, this model agrees with the experimental data about the stereoselectivity of the reaction. The β-hydrogen atom of deoxypodophyllotoxin at C7 position is in the most appropriate orientation at the site of oxidation (Fig. 5B).

2.4. Modeling of possible CYP3A4 inhibition

It has been reported that methylenedioxyphenyl lignans cause mechanism-based inhibition of CYP3A4 [10]. Therefore, we also investigated the case when the methylenic carbon from the methylenedioxy moiety is used as an anchor atom of the epipodophyllotoxin in distance constrained docking. The iron atom of the heme moiety in the active site was used as the anchor atom of the enzyme CYP3A4. The same docking protocol was applied as the one used for the bioconversion model. The lowest energy solution was the one with highest shape complementarity (Fig. 7A). The distance between the anchor atoms was 3.6 Å. The residues of the receptor in contact with the epipodophyllotoxin were Arg105, Phe108, Ser119, Ile120, Phe213, Phe215, Ala305, Ala370, Arg372, and Glu374.

The atomic contacts of the model were analyzed with the LPC server. Two hydrogen bonding contacts were detected between CYP3A4 and epipodophyllotoxin. In both hydrogen bonds the hydroxyl group at C7 was involved. This group interacted with the amide group and hydroxyl group of Ser119 residue. The interaction of epipodophyllotoxin with CYP3A4 involved more aromatic–aromatic contacts compared to

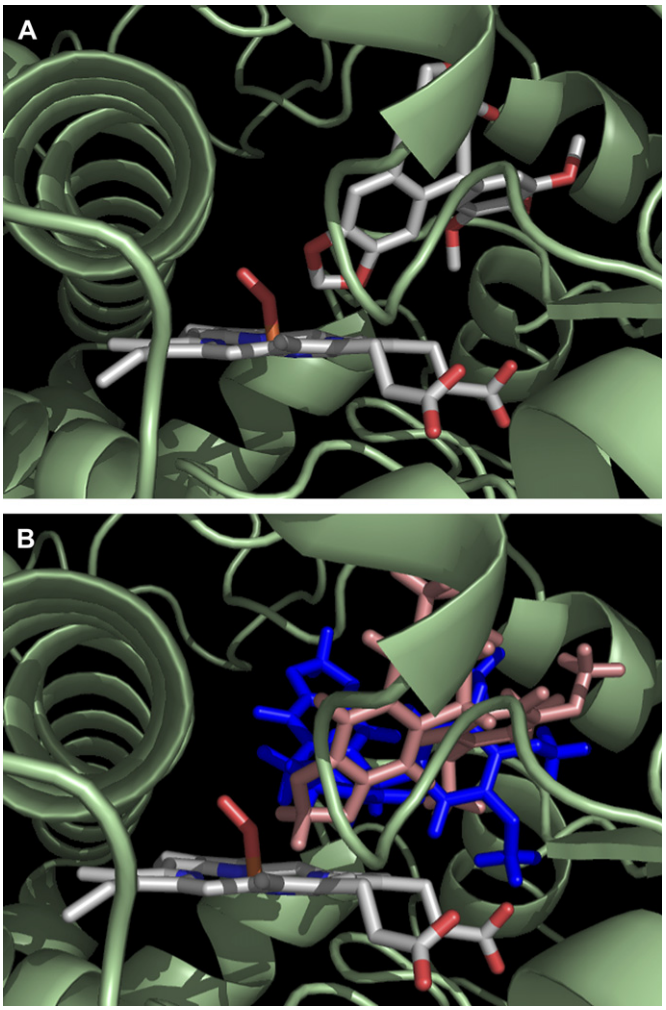

Fig. 7. (A) Epipodophyllotoxin in complex with CYP3A4, inhibition by methylenedioxy moiety; and (B) comparison to deoxypodophyllotoxin complex model (blue), suggesting that the product of the reaction slides inside the cavity from substrate position (blue color) to inhibitor position (pink color). (For interpretation of color in this figure, the reader is referred to the web version of this article.)

deoxypodophyllotoxin, according to LPC (nine contacts for epipodophyllotoxin compared to two for deoxypodophyllotoxin).

3. Discussion

Deoxypodophyllotoxin is an important precursor of podophyllotoxin in the plant biosynthesis [3]. In contrary to *Podophyllum* the metabolism of deoxypodophyllotoxin by human CYP3A4 results in the stereoisomer epipodophyllotoxin as the only metabolite. The stereoselective bioconversion of deoxypodophyllotoxin at the C7 position by CYP3A4 has been identified by HPLC–SPE–NMR [8]. The distance constrained docking technique described in this study was able to explain the stereoselectivity, suggesting preference of the β-hydrogen of the C7 atom towards the oxygen bound to the heme group in the active site of the enzyme (Fig. 5B).

From the amino acid residues hypothesized by the model as being involved in inhibition, only four residues did overlap with the deoxypodophyllotoxin model binding site, suggesting that after the oxidation reaction the product slides inside the active site cavity to the inhibition position (Fig. 7B). These docking results are consistent with the examples of mechanism-based inhibition caused by methylenedioxyphenyl compounds as described in the literature above.

The kinetic parameters determined for deoxypodophyllotoxin and testosterone made it possible to decide the conditions used for the inhibition studies. The concentration of testosterone was set at 25 μM, which is approximately around the K_m value of 20.5 μM. Inhibition by the lignan structures arises from the ability of the methylenic carbon in methylenedioxyphenyl compounds to undergo oxidation to a carbene, which then interacts with the heme iron of CYP3A4 to form a stable heme-adduct complex [14,15]. The experimental data presented in this work confirmed the specific role of the methylenedioxy moiety causing inhibition of CYP3A4. Both epipodophyllotoxin and podophyllotoxin showed a strong inhibitory effect on the bioconversion of the substrate testosterone. The same accounts for deoxypodophyllotoxin. Because deoxypodophyllotoxin is a substrate for CYP3A4 as well, this inhibition could also be caused by a competition for the enzyme or inhibition by the product formed. These findings are in agreement with the determined kinetic parameters. Although the velocity of the deoxypodophyllotoxin conversion seemed to be lower than the testosterone conversion, the K_m value for deoxypodophyllotoxin was 10-fold lower than the K_m value of testosterone. From the calculated efficiency it can be concluded that deoxypodophyllotoxin can compete easily with testosterone for the active site.

Demethylenepodophyllotoxin lacks the methylenedioxy moiety. This compound did not cause inhibition, which confirms the hypothesis that the inhibition is caused by the methylenedioxy moiety of the lignan. The results suggested a weak inhibition effect caused by demethylenedeoxypodophyllotoxin. This is probably the influence of competition for the active site of the enzyme, rather than inhibition. The identification of demethylenedeoxypodophyllotoxin as a substrate for CYP3A4 supports this observation.

The results obtained by the computational modeling in this study showed the potency of distance constrained docking especially for the stereoselectivity of CYP3A4 towards the substrate deoxypodophyllotoxin. As a result the predicted model of the interaction of deoxypodophyllotoxin with CYP3A4 was in good agreement with experimental data obtained by bioconversion of deoxypodophyllotoxin into epipodophyllotoxin by CYP3A4. The presented docking method appears to be a useful tool for the cases in which biological information can be translated to distance constraint. This is the case in many enzymatic reactions, including CYP450 oxidation. The distance constraint does not necessarily have to be tight, i.e. the anchor atoms do not have to be in contact in the resulting complex. Distance constraints can be obtained by various experimental techniques, in particular NMR and FRET. Explicitly employing the distance constraint into the search stage of the docking method yields very accurate models, significantly reducing the number of potential false positive hypotheses and decreasing the running times of the method. As a result the predicted models of the interaction of deoxypodophyllotoxin and its product epipodophyllotoxin with CYP3A4 are in good agreement with experimental data obtained by bioconversion of deoxypodophyllotoxin to epipodophyllotoxin by CYP3A4. Notably, the suggestion rose by the model for the inhibition caused by the interaction between the methylenedioxy moiety and the CYP3A4 was in agreement with the experimental data as well. The methylene moiety causes inhibition of the CYP3A4 activity. However, further experiments are needed to verify the hypothesis that deoxypodophyllotoxin causes mechanism-based inhibition of CYP3A4 enzyme. These additional experiments will show whether the distance constrained docking method may also serve as a model for prediction of possible enzyme inhibition. This would be of influence for the application of the described bioconversion system using recombinant CYP3A4 as an alternative source for the production of epipodophyllotoxin as a precursor for the semi-synthesis of cytostatic drugs. Techniques like dialysis, 2-phase bioprocessing, and immobilizing the cytochrome P450 enzyme can be used to overcome the problems discussed.

4. Materials and methods

4.1. Chemicals

Deoxypodophyllotoxin was isolated from *A. sylvestris* rhizomes, as published elsewhere [6,7]. Identity and purity of deoxypodophyllotoxin were determined by HPLC and ^{1}H and ^{13}C NMR. Podophyllotoxin, miconazole, testosterone, and 6β-hydroxytestosterone were purchased from Sigma (St. Louis, USA). Epipodophyllotoxin was made from deoxypodophyllotoxin by bioconversion using CYP3A4 and subsequently isolated by semi-preparative HPLC. The amount of isolated epipodophyllotoxin was calculated as the concentration of the podophyllotoxin using a standard curve of several concentrations. Demethylenepodophyllotoxin and demethylenedeoxypodophyllotoxin were

a kind gift of Dr. M.A. Castro (Departamento de Quimica Farmaceutica, Universidad de Salamanca, Spain). Medium components and glucose were purchased from Duchefa (Haarlem, The Netherlands), and all other chemicals from Merck (Darmstadt, Germany).

4.2. Production of heterologous CYP3A4

The human gene encoding the CYP3A4 was cloned together with a NADPH-P450 reductase gene into a bicistronic pCW vector [16]. This vector allowed independent expression of the monooxygenase and the reductase genes. A plasmid without the monooxygenase encoding gene was used for control experiments and was referred to as the control plasmid in this study. The described constructs were a kind gift from Prof. F.P. Guengerich (Vanderbilt University School of Medicine, Nashville, USA). Expression was performed in *E. coli* DH5α (Gibco BRL, Gaithersburg, USA) as described before [8]. Measurement of the amount of CYP3A4 was performed by CO saturation difference analysis [8,17].

4.3. Bioconversion assay

The bioconversion assays were performed in potassium phosphate buffer (0.1 M, pH 7.4) containing glucose (12.5 mM). Cells with expressed CYP3A4 were thawed on ice and the specified amount was added to the buffer. The substrate solutions were all prepared in DMSO and diluted to yield a final volume of 10 μl added to the reaction mixture. NADP (1 mM), glucose-6-phosphate (5 mM) and glucose-6-phosphate dehydrogenase (1 U ml^{-1}) were added subsequently to the reaction mixture. Finally, $MgCl_2$ (30 mM) was added, followed by incubation for the specified time at 37 °C and shaking at 250 rpm. The conversions were performed in a total volume of 1.0 ml in Pyrex glass tubes.

4.4. Inhibition assay

Inhibition assays were performed with 25 μM testosterone as a natural substrate, coincubated with various concentrations (0, 1, 10, and 25 μM) of the known inhibitor miconazole [12] and the different lignan compounds, deoxypodophyllotoxin, podophyllotoxin, epipodophyllotoxin, demethylenepodophyllotoxin, and demethylenedeoxypodophyllotoxin, using 69.5 pmol CYP3A4 as described above. The bioconversion reactions were followed for 60 min incubation (37 °C, 250 rpm). Testosterone and the coincubated compound were added to the reaction mixture in a total volume of 10 μl DMSO for all bioconversion assays. The effect of coincubation on the bioconversion was calculated by the amount of 6β-hydroxytestosterone formed in comparison to assays without coincubated compound. The concentration of 6β-hydroxytestosterone was determined from a standard curve of several concentrations of a reference compound. The bioconversion of only testosterone was set as 100% and the other assays were compared to this amount.

4.5. Sample preparation

After incubation the reaction was stopped and extracted twice with 2 ml ethyl acetate by vigorous shaking. The layers were separated by centrifugation. The combined ethyl acetate layers were dried over anhydrous sodium sulphate and evaporated to dryness in a stream of air. Subsequently, the residue was dissolved in 150 μl of methanol and analyzed by HPLC.

4.6. HPLC

HPLC analysis was performed using a Shimadzu-VP system (Shimadzu, 's-Hertogenbosch, The Netherlands) consisting of a LC-10AT*vp* pump, a Kontron 360 auto sampler, a SPD-M10A*vp* DAD detector, a FCV-10AL*vp* low pressure gradient mixer, a SCL-10A*vp* system controller, a FIAtron systems CH-30 column heater (USA), and CLASS-VP software, version 6.12SP4. The column we used was a Luna C18(2) (250 × 4.6 mm, 5 μm), together with a Phenomenex guard cartridge C18 (ODS, 4 × 3 mm) (Phenomenex, USA). The injection volume was 20 μl and the method of analysis was used as described before [8]. The capacity factor (k') was calculated using the formula $k' = (R_t - R_{t0})/R_{t0}$.

4.7. Structures preparation

The crystal structure of CYP3A4 with highest resolution (2.05 Å) was used (PDB code 1tqn) [2]. In order to perform correct reaction modeling, O_2 was added bound to the heme group iron. The addition of the oxygen molecule was done by superposition of the heme group with the heme group from CYP450cam where the oxygen is bound (PDB code, 1dz8) [18]. The obtained structure, i.e. CYP450 with O_2 molecule, was used as the receptor in the docking procedure (Fig. 2A). According to Schlichting et al. [18], the oxygen atom that is bound directly to the iron is the one that is transferred to the substrate in the oxidation reaction. Therefore it was used as an anchor atom for distance constrained docking.

The structures of the ligands, deoxypodophyllotoxin and epipodophyllotoxin, were generated using ChemDraw. The structures were energy minimized and subjected to conformational search using OMEGA [19,20]. There were 20 conformers generated for each ligand after clustering with RMSD of 0.3 Å. Since the ligands are relatively rigid, the difference between the conformers was very small and concentrated on the pending trimethoxy aryl ring (E ring) and methoxy groups orientations (Fig. 2B).

4.8. Distance constrained docking

In many enzymatic reactions we know exactly which substrate atom should be in contact with a specific enzyme atom so that the catalytic reaction can happen. This contact can be translated into a distance constraint and used in computational 3D modeling of the reaction to restrict the search space only to those complexes that satisfy the given constraint.

Motivated by this observation we have developed the distance constrained docking algorithm. The method receives as an input two molecules (generally, nicknamed) *receptor* and *ligand*, and one distance constraint between one *anchor* atom in the receptor and one *anchor* atom in the ligand. The output of the algorithm is a list of complexes exhibiting significant shape complementarity between the receptor and the ligand and satisfying the distance constraints.

The method is an extension of the PatchDock algorithm [21,22]. This method has three major stages: (i) surface representation by a set of sparse features; (ii) generation of candidate transformations (rotation and translation) by matching of receptor and ligand features; and (iii) filtering and scoring. In order to generate only those complexes that satisfy the distance constraint, stage (ii) was modified.

The matching stage generates transformations by matching two *bases*: one from the receptor and one from the ligand. Each base is a pair of surface points and their associated normals (Fig. 3A). For each base we compute a signature, which is invariant to rotations and translations. Originally, in the PatchDock method, the signature consists of five parameters: Euclidean and geodesic distances between the two points: d_E and d_G, the angles α, β formed between the line segment ab and each of the normals and the torsion angle ω formed between the planes (Fig. 3A). Only bases with similar signatures can be matched.

In the distance constrained docking method we extend our signature in the following way. For each base, we place a reference frame on it, such that its center overlaps with the mid point, the x-axis is in the direction of the line segment ab, the y-axis is the cross-product of the vectors $\boldsymbol{ab}$ and $\boldsymbol{n_a}$ and, thus, is perpendicular to the plane formed by a, b and n_a, and the z-axis unit vector is the cross-product of the x and y axes unit vectors. We compute the coordinate of the anchor atom in this reference frame (Fig. 3B). Therefore, the signature is extended to eight parameters: (d_E, d_G, α, β, ω, x, y, and z), where (x, y, z) is the coordinate of the anchor atom in the reference frame defined by the base.

If two bases, one from receptor and the other from ligand produce a transformation that satisfies the given distance constraint, then the distance between the coordinates of the anchor atoms in the reference frames defined by the bases should be below the given threshold.

The matching of the surface points was performed using Geometric Hashing [23]. In the first preprocessing stage all the ligand bases were created and stored in a hash table according to their signature. In the second recognition stage, we access the table with each receptor base signature and create all potential transformations. Note that inclusion of anchor point coordinates in the signatures of receptor and ligand bases ensures that only base pairs that satisfy the distance constraint are being matched.

The method preserves the patch based matching of PatchDock, however using the given constraint it significantly reduces the search space and therefore the computational time required by the method, allowing for more accurate sampling with denser surface representation.

4.9. Refinement

The refinement method [24] starts with rigid docking candidate complexes and optimizes them by allowing side-chain flexibility and small rigid-body movements of the ligand molecule. Side-chains flexibility is limited to the interface high-energy residues only and is solved by the linear programming technique. The relative partners position optimization is performed by local minimization of the binding energy function in the 6-dimensional translation and rotation space of rigid movements. In the final stage of the method the refined candidates are ranked by binding energy, which includes softened Van der Waals, hydrogen bonding, knowledge-based solvation terms and others.

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