Metabolism of Phenylahistin Enantiomers by Cytochromes P450: A Possible Explanation for Their Different Cytotoxicity

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ABSTRACT:

Phenylahistin is a fungal diketopiperazine derived from isoprenylated (Phe-ΔHis) cyclodipeptide. The (−)-enantiomer is a cell cycle inhibitor, which can be potentially used as an antitumor agent. By contrast, the (+)-enantiomer exhibits no antimicrotubule activity. To better understand the differences that could arise from a difference in bioavailability, we investigated the interaction and metabolism of both enantiomers with mammalian cytochromes P450 (P450s). We found that both enantiomers were metabolized by various isoforms of mammal P450 with a noticeable activity for the (+)-enantiomer. P450 3A isoforms were mainly responsible for this metabolism, the bioactive (−)-enantiomer being 1.5 to 8 times less metabolized than the (+)-enantiomer. Spectral analysis of the interaction with P450s revealed that (−)-phenylahistin led to a hydrophobic type I signature, whereas the (+)-isomer yielded a Fe-N type II one. Structural analysis of metabolites by liquid chromatography-tandem mass spectrometry allowed us to characterize two major metabolites (P1 and P3) for both enantiomers. In human liver microsomal preparations, P1 was predominant in the (−)-phenylahistin metabolic profile. In contrast, (+)-phenylahistin mainly produced P3 in human microsomes and CYP3A human expressed P450s. (−)-Phenylahistin proved to be less toxic on P450-rich hepatocytes than on P450-deprived KB lines. The slower metabolism of this enantiomer could account for its higher toxicity. This is strengthened by the fact that isolated metabolites of (−)-phenylahistin showed no toxic effects toward KB lines. Finally, differences of metabolism and interaction mode between both phenylahistin enantiomers and CYP3A4 were supported by in silico molecular docking calculations.

Numerous cyclopeptides are known to have biological activities. The smallest cyclopeptides studied for their potential therapeutic effects are diketopiperazines. These compounds are mainly produced in microorganisms such as bacteria and fungi (Prasad, 1995; Demain and Fang, 2000) but are also present in mammals, where they can originate from protein degradation (Scharfmann et al., 1989). The diketopiperazine family exhibits a large variety of biological activities, ranging from cell-cycle inhibition to specific enzyme-activity modulation.

Recently, development of bioassays contributed to the discovery of new cyclodipeptides with potent inhibitory effects on microtubule formation. Tryprostatin (Table 1), a diketopiperazine isolated from Aspergillus fumigatus derived from condensation of proline and iso-prenylated tryptophan (Cui et al., 1996), belongs to this new class. Phenylahistin (PHL), a metabolite isolated from Aspergillus ustus, is another fungal isoprenylated diketopiperazine, composed of phenylalanine and dehydrohistidine. PHL was isolated after screening for new cell cycle inhibitors (Kanoh et al., 1999a,b). The fungus produces PHL as a racemic mixture (Fig. 1), but only the (−)-enantiomer proved to be cytotoxic (Kanoh et al., 1999c). Interestingly, dehydrophenylahistin, the dehydro analog of PHL in which chirality is lost by α-β dehydrogenation of phenylalanine moiety, has also been reported as a novel cell cycle inhibitor 1,000 times more active than (−)-PHL (Kanzaki et al., 2002). Besides, the tertiobutyl analog of PHL, NP2358 (Table 1), exhibits higher cytotoxicity than (−)-PHL against carcinoma cell lines (Nicholson et al., 2006). This product is currently in preclinical development for the treatment of cancer. Because of their cyclic structure, such compounds are not recognized by mammalian peptidases and thus exhibit longer lifetimes in organisms. Recent studies have raised the possibility that the CYP3A subfamily of cytochrome P450 enzymes can metabolize these small cyclopeptides (Delaforge et al., 2001). In a previous work, we ob-

ABBREVIATIONS: c-, cyclo; ANF, α-naphtoflavone; G6P, glucose-6-phosphate; G6PDH, glucose-6-phosphate dehydrogenase; HPLC, high-performance liquid chromatography; K_s, spectrally estimated dissociation constant; LC-MS/MS, liquid chromatography-tandem mass spectrometry; MM-PBSA, molecular mechanics Poisson-Boltzmann surface area; P450, cytochrome P450; (+/-)-PHL, racemic phenylahistin; (+)-PHL, (R)-enantiomer of phenylahistin; (−)-PHL, (S)-enantiomer of phenylahistin; amu, atomic mass unit.
served that roquefortine C, an antibacterial diketopiperazine, which is also isoprenylated (Table 1), efficiently inhibited the metabolic activities of cytochromes P450, especially those of the CYP3A and CYP1A subfamilies (Aninat et al., 2001). A comparison with other cyclodipeptides sharing structural similarities with roquefortine allowed us to highlight the key role of the imidazolyl nitrogen atom of dehydrohistidine in the P450 inhibition process (C. Aninat, L. Perrin, N. Loiseau, Y. Hayashi, R. Ricoux, J. P. Mahy, B. M. Schiavi, M. M. Joullie, and M. Delaforge, manuscript in preparation). Such an inhibitory effect of cytochromes P450 and other hemoproteins is well documented for numerous N-substituted imidazole compounds (Hajek et al., 1982), but there is only little evidence for the inhibitory potency of nonsubstituted nitrogen histidyl derivatives (Brandes et al., 1998).

We have previously shown that, among a series of diketopiperazines bearing a histidine residue, PHL was the only one to be significantly metabolized by cytochromes P450 (Aninat et al., 2001). The achievement of total synthesis of (+)-PHL and (−)-PHL (Hayashi et al., 2000) allowed us to study their metabolism in detail, either apart or in racemic mixture. In the present work, we studied the recognition

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**TABLE 1**

<table>
<thead>
<tr>
<th>Structures of the Active Compound</th>
<th>Biological Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryprostatin A: R = OMe</td>
<td>c-(2-isoprenylTrp-Pro) derivatives MDR phenotype reversal: BCRP inhibition; microtubule assembly inhibition&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Tryprostatin B: R = H</td>
<td></td>
</tr>
<tr>
<td>(−)-Phenylahistin</td>
<td>c-(X-isoprenylHis); c-(X-tertiobutylHis); antimicrotubule activity cell cycle inhibitors&lt;sup&gt;d,e,f,g&lt;/sup&gt;</td>
</tr>
<tr>
<td>(−)-Aurantiamine</td>
<td></td>
</tr>
<tr>
<td>NPI-2358</td>
<td>Diketopiperazine derived from c-(isoprenylTrp-Pro) bacteriostatic&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Roquefortine C</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Woehlecke et al., 2003.<br><sup>b</sup> Hajek et al., 1982.<br><sup>c</sup> Hayashi et al., 2000.<br><sup>d</sup> Kanoh et al., 1999a.<br><sup>e</sup> Kanoh et al., 1999b.<br><sup>f</sup> Kanoh et al., 1999c.<br><sup>g</sup> Nicholson et al., 2006.<br><sup>h</sup> Kopp-Holtwiesche and Rehm, 1990.

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![Chemical structure of phenylahistin enantiomers](image)

**FIG. 1.** Chemical structure of phenylahistin enantiomers; (−) and (+) species refer to (S) and (R) enantiomers, respectively.
features of the two PHL enantiomers by human cytochromes P450, compared their respective metabolism, and evaluated their inhibitory mechanisms. We determined the structure of the metabolites and evaluated the possible impact of metabolism of PHL enantiomers on their respective cytotoxicity. Finally, the metabolism results are interpreted in the light of in silico molecular docking experiments of the two PHL enantiomers into CYP3A4 active site.

Materials and Methods

Chemicals. (+)-PHL, (−)-PHL, and (±)-PHL were synthesized as described previously (Kanoh et al., 1999a; Hayashi et al., 2000). NADPH, NADP, glucose 6-phosphate (G6P), glucose 6-phosphate dehydrogenase (G6PDH), furafylline, ketocanazole, α-naphthoflavone (ANF), quinidine, sulfaphenazole, bromocriptine, testosterone, and 6β-hydroxy-testosterone were from Sigma Chemical (Saint-Quentin-Fallavier, France). All other chemicals were of the highest quality commercially available.

Preparation of Microsomes. Human liver samples were kindly provided by the Franche-Comté Hospital surgery service of Besançon University and prepared as previously described (Kremers et al., 1998). Yeast-expressed human P450 microsomes were prepared as already described (Peyronneau et al., 1992) and produced by SPI-bio (Montigny-le-Bretonneux, France). Human-expressed cytochromes Supersomes were from BD Biosciences Gentest Co. (le Pont de Clai, France).

Enzyme Quantification in the Microsomal Fractions. Protein contents in microsomal suspensions were determined using bovine serum albumin as standard (Lowry et al., 1951). P450 concentration was measured as described by Omura and Sato (1964).

Substrate Binding to Microsomal P450s by Difference-Visible Spectroscopy. Binding of substrates to cytochrome P450 was studied by difference-visible spectroscopy as described previously (Delaforge et al., 2001) using a PerkinElmer Life and Analytical Sciences (Waltham, MA) Lambda 18 spectrophotometer, equipped with a turbidity accessory, with 1 μM P450 from human liver microsomes in 0.1 M phosphate buffer (pH 7.4). Yeast microsomes were suspended in 50 mM Tris buffer (pH 7.4) to obtain a 0.2 μM P450 concentration. These buffer conditions were optimized for such yeast-expressed P450s (Peyronneau et al., 1992).

Difference spectra were obtained by addition of increasing amounts of cyclopidetides to microsomal suspensions containing 1 μM P450 from human microsomes or 0.2 μM P450 from yeast-expressed human P450. Apparent spectral dissociation constants, Kᵢ, and ΔAmax, were determined by double inverse representation (1/Δabsorbance function vs PHL concentration). ΔAmax values and Kᵢ values were the means of two to four measurements.

Metabolism. Microsomal incubations. Metabolism of PHL (5–200 μM) was studied at 37°C in 0.1 M phosphate buffer (pH 7.4) using 1 μM P450 from human liver microsomes or 0.04 μM P450s Supersomes CYP1A1, 1A2, 2A6, 2B6, 2C8, 2C9, 2D6, 3A4, and 3A5 using NADPH or an NADPH-generating system (1 mM NADP, 10 mM G6P, and 2 IU G6PDH).

The incubation was stopped at the indicated times by addition of the same volume of acetonitrile. The mixture was then frozen until analysis, before which it was remixed and centrifuged at 10,000 rpm for 10 min. HPLC was performed in a linear gradient mode on a reverse-phase column (Kromasil which it was remixed and centrifuged at 10,000 rpm for 10 min. HPLC was performed in order to verify that more than 90% PHL was metabolized in the presence of NADPH-consuming system and that metabolites were quantitatively eluted from the Sep-Pak column. Equivalent concentrations of (−)-PHL or of its metabolites were added to the culture medium of KB cell lines [CCL 17: American Type Culture Collection (Manassas, VA)], assuming identical molar absorption coefficient for metabolites and PHL. Cell viability was measured after 72 h using the neutral red colorimetric assay and compared with a control culture receiving the same volume of solvent. Docetaxel toxicity was performed in parallel as a positive cytotoxic control. Each determination resulted from the average of three measurements.

Hepatocyte Incubations. Incubations of 1 and 5 μM (±)-PHL were done in 24-well plates (500,000 human hepatocytes/well). Control incubations were performed in the presence of dimethyl sulfoxide. Supernatants were collected at 0, 1, 16, and 24 h. Cellular viability was judged under the microscope by protein measurement of the cellular layer and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium test.

Mass Spectrometry Analysis. The LC-MS/MS instrument used was an LCQ DUO Ion Trap coupled with an HPLC system from Thermo-Finnigan. LC was performed on a reverse-phase column 150 x 2.1-mm Kromasil 5C18 column; 20 μl of the 50/50 buffer/CH3CN solution was directly injected into the LC system. Gradient chromatography (run of 33 min) was performed with water/ acetonitrile mixture as mobile phase (eluents A: 90/10 and eluent B: 10/90) at a flow rate of 250 μl/min. The program started with 100% A, then B was increased from 0 to 80% within 25 min and held constant for the next 3 min. The system was then returned to 100% eluent A and allowed to equilibrate for 5 min before next injection. LC-MS/MS measurements were performed using electrospray ionization mode. Electrospray ionization was performed at room temperature in negative mode, the voltage maintained at 4.5 kV and the capillary temperature at 250°C. The collision energy was set at 30%.

Data Fitting. Fitting procedures have been achieved with GraphPad Prism (GraphPad Software Inc., San Diego, CA) and Excel (Microsoft, Redmond, WA).

Computational Details. Ligand data. Quantum calculations were performed using the Gaussian 98 suite of programs (revision A11; Gaussian Inc., Wallingford, CT) (Frisch et al., 2001), at the density functional theory level, using the B3LYP hybrid functional (LYP, Lee et al., 1988; B3, Becke, 1993). All electron 6-311G(d,p) basis sets have been used for C, H, O, and N atoms. Full optimizations have been performed without any geometrical constraints.

Ligand setup. (−) and (±)-PHL have been built up and minimized under Sybyl software (Sybyl Molecular Modeling System, version 7.2; Tripos, Inc., St. Louis, MO). Conformational analyses have been achieved using the genetic algorithm implemented in Sybyl (genetic algorithm conformational search). Atomic charges have been computed on the more stable conformer at the AM1/BCC level according to the AMBER protocol (Jalalian et al., 2000) using Antechamber (Wang et al., 2006). Force field parameters and topologies of these molecules have been built-up using Antechamber in association with general AMBER force field (Wang et al., 2004).

Protein structure preparation for AutoDock procedure. The crystal structure of CYP3A4 corresponding to entry code 1WOE (Williams et al., 2004) recovered from Brookhaven Protein Database was used. Unresolved residues have been rebuilt by homology modeling using Modeller (Marti-Renom et al., 2000) with CYP3A4 structure corresponding to entry code ITQN (Yano et al., 2004) as template. Hydrogen atoms and Gasteiger–Hückel charges have been added using Sybyl. Lys, Arg, Asp, and Glu have been ionized; the protonation state of histidines has been set to i. Charges reported by Oda et al. (2005) have been used for heme. The system has been finally minimized over 500 conjugated gradient steps.

Automated docking procedure. Automated docking was carried out using the docking program AutoDock 3.0 (La Jolla, CA) (Morris et al., 1998).
AutoDockTools was used to set up the rotatable bonds in the ligands, to parameterize the solvent model, and to assign the atomic partial charges of the protein. For the heme, charges were manually assigned with published values as mentioned above (Oda et al., 2005). The initially computed AM1/BCC charges were used for ligands. Docking maps used by AutoDock were defined by considering a grid based on a box, which includes the inner active site of the cytochrome. A grid of 62, 68, and 68 points in the x, y, and z directions was built. A grid spacing of 0.375 Å and a distance-dependent dielectric “constant” were used for the energetic map calculations. The Lamarckian genetic algorithm was used for docking search; the docked compounds were subjected to 100 runs in the AutoDock search. The maximum number of energy evaluation has been raised to 20,10^6, the number of individuals in population has been raised to 300, and the other parameter values were kept at default. The final cluster analysis was performed on the resulting docked structures using a root mean square deviation tolerance of 2.0 Å.

**MM-PBSA binding energies.** Binding energies of docked enantiomers have been estimated according to the MM-PBSA approach. They have been computed directly on the minimized docked structures yielded by AutoDock. All the minimization and computation of MM-PBSA binding energies have been achieved with the AMBER 8.0 suite of programs (Cornell et al., 1995). From the docked structures obtained by AutoDock, hydrogen atoms and atomic charges have been added with Leap according to the AMBER 98 force field. Charges reported by Oda et al. (2005) have been used for heme. The enzyme/ligand complexes were solvated by a TIP3 water box, and the minimal distance between the protein and the border of the box was set to 12.0 Å. Three counter ions (chloride) were added to neutralize the system. Periodic boundary conditions were used in combination with particle mesh Ewald method; for the computation of electrostatic contribution, a cut-off of 10.0 Å was used. Protein and ligand were minimized over 500 cycles while keeping the solvent molecule and counter ions frozen. The ligand was then minimized over 500 cycles, the rest of the system being frozen. The global system was minimized over 500 cycles. Finally, the ligand and its surrounding partners were minimized over 1500 cycles. For this last minimization step, a frozen bulky comprising protein residues and water molecule being at least at 15 Å from the ligand was applied. The default MM-PBSA procedure was then applied for the computation of binding energies.

**Results**

**Spectral Interactions of (−)-PHL, (+)-PHL, and (±)-PHL with P450s.** To determine the nature of PHL/P450 interaction(s), spectral interactions were measured by UV-Vis spectrophotometry. Difference spectra were obtained by addition of increasing amounts of (+)-PHL, (±)-PHL, or (−)-PHL to microsomal suspensions containing 1 μM P450 from human microsomes or 0.2 μM P450 from yeast-expressed human P450. The type II difference spectrum characterized by a maximum at 430 nm and a minimum around 400 nm results from a direct coordination of one imidazolyl nitrogen atom to the heme iron atom, whereas type I difference spectrum characterized by a maximum at 390 nm and a minimum of approximately 420 nm is indicative of a hydrophobic interaction between the substrate and the reactive center of cytochrome P450.

Addition of (+)- or (±)-PHL to human liver microsomes P450 led to a type II difference spectrum as viewed by difference visible spectroscopy, with K_s values found at approximately 8 μM. However, in the presence of yeast-expressed P450 CYP3A4, the substrate absorbance and the low absorbance of the type II spectrum did not allow precise measurement of the ΔA and K_s values. In contrast, addition of (−)-PHL to human liver microsomes led to a type I spectrum, but its low intensity did not allow precise determination of the K_s value. However, in the presence of CYP 3A4 yeast-expressed human microsomes, (−)-PHL led to measurable type I spectrum with the K_s value at approximately 7 μM.

The (+)-PHL type II spectrum obtained with human liver microsomes was not stable upon iron reduction by dithionite and did not lead to significant decrease of the P450-CO spectrum. This strongly contrasts with the well known interaction between strong imidazole inhibitors, such as miconazole, and P450s, in which the type II spectroscopic signature is maintained under such conditions.

Noticeably, similar K_s values and same interaction data (type I with (−)-PHL, type II with (+)-PHL) were found in either untreated or treated rat liver microsomes (data not shown). The maximal absorbance spectra were obtained for dexamethasone-pretreated rat liver microsomes, in which CYP3A isoforms are mostly induced (Heuman et al., 1982; Wrighton et al., 1985).

**Cytochrome P450-Dependent Metabolism. Biotransformation of PHL in human hepatic P450s.** The HPLC profiles obtained with (+)-, (−)-, or (±)-PHL in human hepatic P450s led to the formation of at least seven detectable metabolites (Fig. 2). For each PHL enantiomer and their racemic mixture, P1 and P3 represented more than 60% of the recovered metabolites (Fig. 2, A–C). In such conditions, production of P3 from (+)-PHL and (±)-PHL was slightly favored compared with the one of P1 (Fig. 2, D and F), whereas a reverse trend was observed for (−)-PHL (Fig. 2E). Kinetic parameters determined from the 0 to 10 min linear part of the incubation profiles are reported in Table 2. For both enantiomers, formation of P1 and P3 displayed similar K_s values. By contrast, V_max values were 3 and 8 times higher for the (+)-PHL than for the (−)-PHL enantiomer, for formation of P1 and P3, respectively. This led to similar V_max/K_s ratios for the P1 formation whatever the enantiomer was, whereas P3 formation was mostly favored for (+)-PHL, as indicated by the highest V_max/K_s ratios.

For determination of the human P450 isoform involved, inhibitory studies were performed with 2 μM ketoconazole, leading to more than 90% inhibition of both P1 and P3 metabolite formation using (+)-, (−)-, or (±)-PHL. Inhibitory effects were observed for both PHL enantiomers upon incubation in the presence of 20 μM bromocriptine (one of the best CYP3A4 substrates; Peyronneau et al., 1994). The IC_{50} of bromocriptine for P3 formation were found equal to 7.8 and 10 μM for (+)-PHL and (−)-PHL, respectively. In addition, 10 μM ANF caused a 10 to 20% increase in P1 and P3 formation and a slight decrease of P5 formation. In contrast, no significant inhibitory effects were observed whatever the PHL enantiomer used upon incubation in the presence of 20 μM sulfaphenazole, furafylline, and quinidine, which are, respectively, CYP2C9, CYP1A2, and CYP2D6 inhibitors. Concomitant incubations of 100 μM testosterone and (+)-PHL led to an increase of 6β-hydroxy-testosterone formation (128 ± 5%), whereas incubation of 100 μM testosterone with (−)-PHL led to a slight decrease of 6β-hydroxy-testosterone formation (93 ± 2%).

**Biotransformation of PHL in human-expressed P450s.** Using human-expressed P450s in Supersomes, CYP1A1, 3A4, and 3A5 (Table 3) were the only isoforms, among the tested ones, to be able to metabolize PHL enantiomers, whereas CYP1A2, 2A6, 2B6, 2C8, 2C9, or 2D6 led to less than 2% of metabolism. Incubations of (−)-, (+)-, or (±)-PHL with CYP3A4 expressed in Supersomes, in the presence of NAPDH, led essentially to the formation of P1 and P3 metabolites. LC-MS/MS analysis showed the presence of a small amount of P4. As in human liver microsomes, (+)-PHL was metabolized by expressed CYP3A4 at least three times faster than (−)-PHL. CYP3A5 was also able to form both P1 and P3 metabolites from each enantiomer. Its activity was found 4 times lower than that of CYP3A4 as regards (+)-PHL metabolism. In contrast, CYP3A5 formed larger amounts of metabolites than CYP3A4 using (−)-PHL. Using CYP1A1 isoenzyme, (−)-PHL was metabolized into (−)-P1 and a more polar metabolite (−)-P5 in nearly equal amounts (Table 3). In contrast, (+)-PHL led to six times more of metabolite called (−)-P5 than of metabolite (+)-P1.
point represents the mean of three experimental values. The solid lines show the best fit using nonlinear tendency curves with Excel software.

**TABLE 2**

Kinetic parameters of P1 and P3 metabolite formation upon incubation of (+)-PHL, (-)-PHL, or racemic PHL with human liver microsomes

<table>
<thead>
<tr>
<th>Substrate</th>
<th>(+)-P1</th>
<th>(+/-)-P3</th>
<th>(+/-)-P5</th>
</tr>
</thead>
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<tr>
<td>(+)-PHL</td>
<td>0.51</td>
<td>0.35</td>
<td>0.38</td>
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<tr>
<td>CYP3A4</td>
<td>1.47</td>
<td>1.47</td>
<td>N.D.</td>
</tr>
<tr>
<td>CYP3A5</td>
<td>1.57</td>
<td>0.38</td>
<td>N.D.</td>
</tr>
<tr>
<td>(-)-PHL</td>
<td>2.37</td>
<td>1.57</td>
<td>N.D.</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>1.57</td>
<td>1.47</td>
<td>1.47</td>
</tr>
<tr>
<td>CYP3A5</td>
<td>1.44</td>
<td>0.36</td>
<td>0.36</td>
</tr>
</tbody>
</table>

N.D., not detected.

**TABLE 3**

Metabolism of PHL enantiomers by human-expressed cytochrome P450

<table>
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<tr>
<th>Substrate</th>
<th>P450 Isoform</th>
<th>Metabolite Formation</th>
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<tr>
<td>(+)-PHL</td>
<td>(+)-P1</td>
<td>(+/-)-P3, (+/-)-P5</td>
</tr>
<tr>
<td></td>
<td>CYP3A4</td>
<td>(+/-)-P5</td>
</tr>
<tr>
<td></td>
<td>CYP3A5</td>
<td>(+/-)-P5</td>
</tr>
<tr>
<td>(-)-PHL</td>
<td>(-)-P1</td>
<td>(-)-P3, (-)-P5</td>
</tr>
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<td></td>
<td>CYP3A1</td>
<td>(-)-P5</td>
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<td>(-)-P5</td>
</tr>
<tr>
<td></td>
<td>CYP3A5</td>
<td>(-)-P5</td>
</tr>
</tbody>
</table>

**Fig. 2.** Metabolism profiles of phenylalhistin enantiomers in human liver microsomes. A–C, reverse-phase HPLC profiles of incubations of (+)-PHL (A), (-)-PHL (B), and (-)-PHL (C) using human liver microsomes. Incubations were performed at 37°C for 15 min as described under Materials and Methods. D–F, time dependence formation of metabolites P1 and P3 of (+)-PHL (D), (-)-PHL (E), and (+)-PHL (F). Open squares and filled circles represent P1 and P3 data points, respectively. Each point represents the mean of three experimental values. The solid lines show the best fit using nonlinear tendency curves with Excel software.

**Inhibition of CYP3A4 by (+)-PHL and (-)-PHL.** To understand the origin of nonlinear kinetics behavior for both enantiomers (Fig. 2), inhibitory studies were performed using Supersomes CYP3A4. The IC50 on testosterone 6β-hydroxylase activities of CYP3A4 were measured and found equal to 22 μM for (+)-PHL and 23 μM for (-)-PHL. Inhibition studies revealed that the two enantiomers had at least two Ki values (9 and 17 μM for (+)-PHL and 2 and 15 μM for (-)-PHL). This is particularly noticeable for testosterone concentration under its Km value (80 μM measured under our conditions).

Preincubations of 5 to 15 min of each PHL enantiomer using CYP3A4 in the presence of NADPH-generating system before the testosterone incubation led to important decreases of testosterone 6β-hydroxylation, and this inhibition was found time-dependent (Fig. 3). From the curves displayed on Fig. 3, Krm values of 0.06 and 0.05 min−1 could be derived for (+)-PHL and (-)-PHL, respectively, with K found at approximately 15 μM. This time-dependent behavior of inhibition of testosterone 6β-hydroxylation was also found for both enantiomers using human liver microsomes (data not shown). Using the same protocol, bromocriptine metabolism was only inhibited as a time-dependent manner using 100 μM of each enantiomer. Similarly, ethoxyresorufin deethylation by expressed CYP1A1 was also inhibited.

**Identification of the Main Metabolites.** Identification of PHL metabolites was based on their HPLC retention times and mass spectra using an electrospray source or after collision in the ion trap. The mass spectrum of PHL in negative mode exhibited [M-H]− ion at m/z 349 with a characteristic collision fragment at m/z 258 resulting from cleavage of the benzyl moiety [M-91]− (Fig. 4).

The mass spectra of metabolites P1, P3, and P5 are displayed in Fig. 5. They all exhibited [M-H]− ion at m/z 365, indicating monooxidation. The enantiomer is not specified on Fig. 5 since the MS and MS/MS mass spectra of both (+-) and (+/-)-P1, P3, and P5 metabolites were identical, indicating that their metabolism sites were on the same position. Fragmentation of metabolite P1 (Fig. 5) led to fragments at m/z 347 [M-18]−, 321 [M-44]−, and 229 [M-44-91]−. These results are indicative of oxidation of the isoprenylated moiety. The fragment at m/z 347 arose from dehydration, and the m/z 321 arose from a loss of the C8H8O fragment followed by a loss of benzyl leading to the fragment at m/z 229. These two fragments could result...
from the cleavage of an epoxide on the allylic double bond. The hydroxylation of one of the two isoprenyl methyl groups was excluded by the fact that we did not detect a fragment at m/z 333 corresponding to the loss of CH₂OH moiety.

MS/MS fragmentation of P3 led to a single fragment at m/z 259 (Fig. 5). This loss of 106 atomic mass units (amu) corresponds to hydroxylation of the benzyl moiety either on the aromatic ring or on the benzyl function. The P5 metabolite was relatively more polar than the two other monohydroxyl metabolites and was formed predominantly with P450 1A1 isoform in human-expressed microsomes (Table 3). Its MS/MS fragments were found at 296 [M-69]⁻, 274 [M-91]⁻, and 205 [M-69,-91]⁻. The loss of 69 amu indicated cleavage of the dimethylallyl moiety, whereas the fragment at m/z 205 was indicative of a subsequent loss of the benzyl moiety. This ruled the reaction. Unfortunately, analysis of the MSn fragmentations, did not allow an unambiguous identification of P5.

Detection of at least two dihydroxy metabolites ([M-H]⁻ at m/z 381) was also observed corresponding to metabolite P4 (Fig. 6), which is in reality a mixture of two dihydroxy metabolites. The identity of the rearrangement fragment at m/z 229 and loss of a fragment of 18 then 28 amu ([M-H₂O]⁻ at m/z 363, [M-H₂O-CH₂OH]⁻ at m/z 335) were in favor of an oxidation on the allylic double bond and on the benzyl function. Another metabolite (P4') in Fig. 6) responding to the m/z 383 was also detected. Such a compound can result from hydration of the postulated epoxide metabolite P1. Interestingly, this metabolite is observed in significant amounts only in rat or human liver microsomes, which are known to contain epoxide hydroas, an enzyme able to readily hydrolyze the epoxide function. A schematic metabolic pathway is proposed Fig. 6.

Effects of PHL Enantiomers in Human Hepatocyte Primary Culture. In contrast to various carcinoma cell lines for which cytotoxic effects can be observed in the 0.1 μM range (Kanoh et al., 1999b), no significant toxicity was observed using (+)-PHL in the 1 to 5 μM range on human hepatocytes [using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium test or protein amounts in the cellular layer] (data not shown). In these hepatocyte preparations, metabolite formation was observed in the culture medium at 1-h incubation (approximately 60% of (+)-PHL metabolized). Identification of these metabolites using LC-MS/MS analysis revealed the presence of P1, P3, and P4 metabolites in the culture medium of human hepatocytes. Glucuronide products were not observed upon analysis performed before or after glucuronidase hydrolysis (15 h, 37°C, 5,000 IU glucuronidase, 0.1 M, pH 5.2, acetate buffer).

Additional structural modifications of PHL, incubations of (+)-PHL using dexamethasone-treated rat liver microsomes in the presence or absence of the NADPH-generating system were performed. HPLC analysis showed that more than 90% of the (+)-PHL was biotransformed. The pool of metabolites purified using a Sep-Pak contained less than 5% unmetabolized (+)-PHL. For concentrations of metabolites equivalent to 1.5·10⁻⁶ M and 7.5·10⁻⁷ M of (+)-PHL, no toxicity was observed. By comparison, the observed cytotoxicity of unmetabolized (+)-PHL prepared in similar conditions was 91% and 60%, respectively.

Structural Analysis of PHL: Determination of Conformational Preferences. The molecule of PHL may adopt several structural forms, which could be in equilibrium. In addition to its chiral center, which conducts to two enantiomers, each of them can yield a set of conformers and tautomers. On the one hand, whereas the free rotation of the isoprenyl group has a minor incidence on the shape of the molecule, the rotation around the C=C dihedral angle is estimated to 65°, which is in good agreement with the experimental one (70°) (Kanoh et al., 1999a). Isomer A, characterized by a C=C-C=C-CO₂H dihedral angle of 172°, is found to be slightly more stable than isomer A' by 1.2 kcal · mol⁻¹. In A', the C=C-C=CO₂H dihedral angle is estimated to 65°, which is in good agreement with the experimental one (70°) (Kanoh et al., 1999a).

Considering the precision of the method used for the calculation, a difference of 2 kcal · mol⁻¹ is not meaningful; these two isomers must be considered as isoenergetic.
To provide a clearer description of the energy landscape of the various tautomers, we have also computed their relative energies at the quantum level. Calculations have been carried out starting from the most stable configuration of PHL corresponding to the extended shape (conformer A, Fig. 7), with the histidyl residue bearing the proton on the ε nitrogen atom (isomer A, Fig. 8). A stationary point corresponding to a linear PHL in which the histidyl group is protonated in its δ position (N3) could have been localized on the potential energy surface (isomer B, Fig. 8), with relative energy to A of + 9.9 kcal mol⁻¹. This destabilization is mainly due to steric clash between the imidazolyl N3-H bond and the peptide N2-H bond of the phenylalanine residue. Additionally, this repulsive interaction implies torsion of the C=C-C double bond that contributes to the destabilization of tautomer B. The tautomer C in which the H-bonding nature of N2 and N3 has been inverted with respect to tautomer A has been identified, its relative energy with respect to A being 20.0 kcal mol⁻¹. This unfavorable configuration is due to the internal stability of the amide function with respect to its tautomer form in molecules that cannot aromatize through tautomerism. Finally, a potential prototropic shift can yield to tautomer D, with energy found to be 24.1 kcal mol⁻¹ above A. This high value is mainly due to a strong diminution of the electron delocalization of the dehydrohistidyl motif. As a conclusion, this study shows that conformers A and A’ are isoenergetic, although less stable by roughly 10 kcal mol⁻¹ tautomer B may exist in the active site of the enzyme if it is stabilized through specific interaction with surrounding residues. Based on this study, only conformers A and A’ have been taken into consideration in the modeling of enzyme/ligand interaction.

**Molecular Dockings of PHL Enantiomers into CYP3A4 Active Site.** Docking by automated procedures, such as AutoDock, generally leads to low discrimination between two enantiomers arising from similar binding scores of the calculated poses. Even after extensive sampling, it is common that one of the isomer yields to only few clusters of positioning, whereas its enantiomer yields to numerous clusters, all of them being characterized by similar binding scores. Hence, differentiation of enantiomer binding modes remains tricky. This pitfall could not be avoided in our initial assays of docking of PHL enantiomers by AutoDock 3. To improve the energetic discrimination between clusters, we have fruitfully applied the MM-PBSA binding energy estimation procedure on the automated docking poses.

**Docking of (−)-PHL.** In the case of the (−)-PHL, the two main clusters were identified (C1-C2, Table 4). In the more energetically favorable one (C1), the isoprenyl group of PHL faces heme but remains rather far from it. The distance between the heme iron atom and the C=C double bond of the isoprenyl moiety equals 5.7 Å (Fig. 9A). In this position, the location of the isoprenyl group prevents any interaction between the nitrogen atoms of the imidazolyl ring and the iron atom. The phenyl tail of PHL points toward the cleft located between the A and F’ helices of CYP3A4 similarly to the binding mode of the distal molecule of ketoconazole in crystal structure of CYP3A4 (entry code 2J0C) (Ekroos and Sjogren, 2006). In the second and energetically less favorable positioning cluster (C2), PHL is closer to the heme, its imidazolyl δ-nitrogen (N3) facing heme iron atom with a distance of 2.38 Å (Fig. 9B). Although the imidazolyl δ-nitrogen (N3) is above the heme iron atom, its lone pair does not point toward the iron atom because of the coplanarity of the di ketoaperazine and imidazolyl rings due to the intramolecular hydrogen bond between them. The two predominant clusters calculated by AutoDock 3 thus correspond to two neighboring configurations, between which the (−)-enantiomer could switch. Hence, while moving between these two configurations, (−)-PHL can only react with heme through its isoprenyl double bond to yield P1. This observation explains why the pose B is not assigned to a type II interaction, but to a type I yielding the P1 metabolite (Table 4). Despite our different assays, no configuration corresponding to the P3 metabolite could have been generated in the docking process for (−)-PHL.
Docking of (+)-PHL. In the case of (+)-PHL, four main clusters of positioning could have been obtained (C3 to C6 in Table 4). In the energetically more favorable one (C3), PHL is located on the top of the active site, below F/H11032-G/H11032 and between A and B/H11032 helices (Fig. 9C). From this positioning, the molecule can flip in the active site to two positioning close to heme. In the more favorable one (C4, Fig. 9D), both the isoprenyl insaturation and the ε-nitrogen (N4) atom of the imidazolyl ring can interact with the heme iron atom [d(N4-Fe) = 4.22 Å, d(C-Fe) = 3.22 Å] and potentially yield P1 or a type II interaction, respectively. The other configuration (C5, Fig. 9E) is almost identical to the more favorable orientation found for the (−)-PHL enantiomer (C1, Fig. 9A). The main differences between these two poses rely on the orientation of the benzyl moiety of PHL and are due to the inversion of the chiral center of the Phe residue. Finally, the fourth docked structure of (+)-PHL (C6, Fig. 9F) puts the ligand phenyl groups in close vicinity to the heme reactive center and accounts for the formation of the P3 metabolite.

Discussion

Previously, we reported that PHL was the only known diketopiperazine having a dehydrohistidine residue recognized and significantly metabolized by rat liver microsomes (Aninat et al., 2001). We also demonstrated that, of the two PHL enantiomers, only the (−)-one exhibited cytotoxic effects (Kanoh et al., 1999c). The question arises as to whether such differences can originate, at least in part, from nonequivalent recognition, or from metabolism by cytochrome P450, leading to different metabolic ratios.

The present results show that both PHL enantiomers are recognized by various P450 isoforms, with the highest spectral signals for CYP3A among the examined P450s. (−)-PHL generates a hydrophobic type I difference spectrum, whereas (+)-PHL gives a Fe-N type II and not a reverse type I as we already reported (Aninat et al., 2001). All results demonstrate unambiguously that CYP3A subfamily is the most involved in the metabolism of PHL, in human liver microsomes where the CYP3A isoforms are the most abundant, or directly using Supersome-expressed P450. Additional evidence is given by the fact that ketoconazole, a potent CYP3A inhibitor (Pelkonen et al., 1998; Zhang et al., 2002), or bromocriptine, a good CYP3A4 substrate, inhibits PHL metabolism. Metabolism of testosterone, an endogenous CYP3A substrate, was inhibited by PHL in a time-dependent manner, suggesting that both compounds compete for the CYP3A active site. In contrast, furafylline, sulfaphenazole, quinidine, and α-naphtho-
flavone, respectively, selective inhibitors of CYP1A2, CYP2C9, CYP2D6, and CYP1A (Guengerich et al., 1986; Bourrie et al., 1996; Guengerich, 1999), had no effect on human liver metabolism of PHL.

The slight increase of P1 and P3 in the presence of ANF is also in favor of CYP3A involvement in the metabolism of PHL since ANF is known to show cooperative heterotropic effects in the CYP3A subfamily (Ueng et al., 1997; Emoto et al., 2001).

Metabolism of PHL by isolated human cytochrome isoforms confirmed these results. The time-dependent and concentration-dependent inhibition of testosterone metabolism by PHL proved that the two PHL enantiomers act simultaneously as direct inhibitors through competition or by formation of Fe-imidazole ligand, and as mechanism-based inhibitors. The latter inhibitory mode explains, in part, the nonlinearity of the metabolite formation upon time. This dual mode of action arising from both a direct Fe-N type II complex and an inhibitory effect of the metabolites has been reported in the case of itraconazole (Isoherranen et al., 2004).

In human liver microsomal or using CYP3A4 preparations, (+)-PHL was metabolized faster than (-)-PHL. The fact that the (+)-enantiomer yields a Fe-N imidazole type II spectrum and is the most rapidly metabolized may be surprising at first. For the (+)-enantiomer, the interaction between the imidazolyl moiety and the heme iron atom may result from a good match between the shape of the enzyme cavity and the one of the molecule and could explain its better affinity for CYP3A4. Additionally, this interaction imposes an orientation of the substrate within the active site of the enzyme that reduces the reaction between the heme reactive species and the isoprenyl group yielding P1. This can account for the inversion of the P1/P3 ratio between the two enantiomers. However, a partial inhibition of CYP3A4 by the Fe-N interaction, as seen spectroscopically, is not consistent with the higher metabolism rate of (+)-PHL as compared with (-)-PHL, which does not show this specific interaction. Several arguments may be advanced: 1) access to the heme active site and the final positioning from which P3 originates are kinetically and/or thermodynamically more favorable for (+)-PHL than for (-)-PHL; 2) (+)-PHL has a positive or higher homotropic effect than (-)-PHL; and 3) an inhibitory complex can be formed following for example protein alkylation by the epoxide (+)-P1 in relation with its higher formation as compared with (-)-P1. Whereas the second assumption cannot be easily addressed experimentally because of the type II spectral signature, one can notice than the first one is supported by the metabolism rates of (+)- and (-)-PHL by expressed CYP3A4. Indeed, we have shown that the rate of production is higher for P3 production than for P1 for (+)-PHL with respect to (-)-PHL. Kinetics data obtained from human liver microsomes showed that $V_{max}/K_m$ values were seven times lower for (-)-P3 and only two times lower for (-)-P1 than for their corresponding (+)-metabolites. Thus, there is more discrimination between P3 and P1 formation depending on the chirality of the compound. This demonstrates that CYP3A4 does not significantly differentiate between enantiomers when they enter the enzyme via their imidazole moiety, whereas a net chiral differentiation occurs when PHL accesses the inner pocket via its phenyl side. In other terms, the higher chiral recognition of PHL by CYP3A4 is associated with the vicinity of its asymmetrical side (Phe moiety, P3

![Fig. 7. Representation of two rotamers of (-)-PHL obtained by rotation around the C-C=C-C=C=C of the PHE residue.](image-url)
metabolism) to the heme reactive center. To confirm this interpretation, we have addressed CYP3A4/PHL binding by molecular docking.

For both enantiomers, the positioning corresponding to (−)-P1 and (+)-P1, (+)-P3 and (+)-PHL Fe-imidazole type II interaction could be characterized in silico (Table 4). In all cases, the binding energies computed at the MM-PBSA level were found lower for the (+)-enantiomer. This is in agreement with the experimental Kᵈ. In the case of (+)-PHL, the best MM-PBSA scoring (−88 kcal · mol⁻¹) is associated to the location of the substrate in a high affinity secondary binding site, then the positioning corresponding to P1 and Fe-imidazole interaction appear to be less favorable with similar binding scores (−62 and −65 kcal · mol⁻¹, respectively). Finally, poses corresponding to P3 are the less favorable ones. The presence of a high affinity secondary binding site as well as three other binding modes for (+)-PHL does not necessarily contradict the experimental spectroscopic type II signature. Indeed, it has been recently shown that some type II ligands can prove to be substrates of P450 in the case of nitrogenous heterocyclic drugs (Locuson et al., 2007). Hence, in silico results are in agreement with type II spectral signature of (+)-PHL as well as with the favored formation of (+)-P3 in CYP3A4, as no positioning with good MM-PBSA energy favoring P3 formation was found for (−)-PHL. In the case of (−)-PHL, the most favorable positioning corresponded to P1 followed by the pose B (Fe-imidazole proximity, not spectroscopically observed). Despite our effort, no structure corresponding to P3 could have been obtained via molecular docking. However, the positions found in silico are in agreement with the finding of a type I interaction and with a favored formation of P1 with respect to P3. Finally, the binding energy computed for the prepositioning of P1 of (+)-PHL (Fig. 9C) is energetically more favorable than the one of P1 for (−)-PHL (Fig. 9A), which accounts for the higher production rate of metabolites obtained for (+)-PHL.

Hence, the type II binding signature is not in contradiction with a high metabolism activity. This is due to the fact that the molecule is linear and can enter the active site by both ends, one leading to weak Fe-imidazolyl interaction or P1 metabolite, the other to the formation of metabolite P3.

No toxicity was observed during ex vivo studies on primary cultures of rat or human hepatocytes, these cells being able to metabolize PHL. Consequently, one of the origins of the lower toxicity could result in lower reactivity of the formed metabolites on the cellular macromolecules. As a confirmation, we carried out a cytotoxicity study on KB lines with (−)-PHL and a mixture of its metabolites. The results gave evidence that the biotransformation of (−)-PHL led to an efficient detoxification. Besides, studies of biological activity of synthetic derivatives of PHL indicated that the presence of an alky group with proper length or a quaternary carbon at position 5 of the imidazole ring, and the spatial arrangement of the benzyl group of the phenylalanine residue, are very important for inhibition of microtubule formation activity (Kanoh et al., 1999a). Our HPLC-MS/MS analyses demonstrate that hydroxylation of the two major metabolites P1 and P3 occurs at these two positions of the molecule, thereby leading to a loss of toxicity of the metabolites formed.

As a conclusion, CYP3A and CYP1A1 are the main isoforms involved in biotransformation of PHL and are able to discriminate between PHL enantiomers. Such discrimination has been assessed through metabolite ratios and metabolism rates. Our docking approach associated with MM-PBSA scoring agreed with the structure of metabolite produced and spectral interaction observed. It also accounted for differences in metabolite ratios and supported a mechanism of recognition. We have shown that metabolism leads to nontoxic metabolites and the differences between the two enantiomers arise from two complementary phenomena: 1) an intrinsic toxicity of the parent enantiomer [−]-PHL ≫ (+)-PHL and 2) a slower metabolism of (−)-PHL versus (+)-PHL. As a consequence, (−)-PHL remains for

![Fig. 8. Tautomerism equilibrium of (−)-PHL.](image-url)
longer times in cells and exerts its inhibitory effects on microtubule formation. In a structure activity relationships study about NPI-2358 (Table 1), a t-butyl-dehydro-PHL analog that is now in clinical phase I trial (Nicholson et al., 2006), chemical modifications by the OH group at various positions of the phenyl ring drastically decreased the cytotoxic activity on HT-29 cell line (Y. Hayashi, unpublished results). Hence, it seems reasonable that the metabolites (OH derivatives) of phenylahistin generated by P450 exhibit no cytotoxicity as shown in the present data, if we consider the high similarity of chemical structure between phenylahistin and this analog.

Fig. 9. Three-dimensional representation of docked PHL enantiomers into the active site of CYP3A4 after minimization [A and B, (-)-PHL; C–F, (+)-PHL]. The side chains of the closest residues (<3.5 Å) are represented in sticks. Phenylalanine side chains belonging to the CYP3A4 phenylalanine cluster are displayed in orange, and basic side chains (Arg) are in green. Ligands are represented in thicker sticks with standard atom color code (oxygen in red, sulfur in yellow, nitrogen in blue). Carbon atoms closest to heme are highlighted in red, and their distance to heme iron atom illustrated in red. For the sake of clarity, the orientation of the representation has been slightly changed between panels.
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