OXIDATIVE METABOLISM OF AMPRENAVIR IN THE HUMAN LIVER. EFFECT OF THE CYP3A MATURATION

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ABSTRACT:

Amprenavir is a human immunodeficiency virus-1 (HIV-1) protease inhibitor intended to be used to treat HIV-infected children. Although a pediatric dosage is proposed by the manufacturer, no data are currently available on the pharmacokinetics of amprenavir in neonates and infants. Amprenavir being primarily eliminated after oxidative biotransformation, we explored its in vitro metabolism by cytochrome P450 (P450)-dependent monooxygenases. In our conditions, five metabolites were formed in vitro and subsequently analyzed by liquid chromatography-mass spectrometry; P450-dependent oxidations occurred either on the tetrahydrofuran ring (M3 and M4), the aniline ring (M5), and the aliphatic chain (M2) or resulted from the N-dealkylation and loss of the tetrahydrofuran ring (M1). The two major metabolites, respectively M3 and M2 were formed by human liver microsomes with $K_m$ between 10 and 70 $\mu$M. CYP3A4 and to a lesser extent CYP3A5 were major contributors for the formation of M2, M3, and M5 metabolites, whereas CYP3A7 had no or little activity. This assumption was confirmed by inhibition with ketoconazole and ritonavir (two potent inhibitors of CYP3A) whereas sulfaphenazole (2C9 inhibitor) and quinidine (2D6 inhibitor) were inefficient. The metabolism of amprenavir was negligible in microsomes from either fetuses or neonates and steadily increased after the first weeks of life in relation with the maturation of CYP3A4/5. In conclusion, results demonstrated that the capacity of the human liver to oxidize amprenavir is low during the first weeks after birth and that dosage could be substantially reduced during the early neonatal period.

HIV-1 protease is a proteolytic enzyme involved in the processing of reverse transcriptase and integrase encoded by the genetic material of HIV-1 virus. Inactivation or inhibition of the protease reduce the infectious capacity of virions and are at the basis of treatment of HIV-1 virus. Of interest, a specific pediatric dosage has been recently approved in children aged four years and older in combination with other antiretroviral drugs for HIV infection (Anonymous, 1999).

Previous studies have indirectly suggested that amprenavir should be primarily oxidized by hepatic cytochromes P450 (Decker et al., 1998). In the human liver, the CYP3A subfamily is believed to be responsible for at least 50% of reaction occurring with clinically used drugs. The following four isoforms constituted the 3A subfamily: 3A4, 3A5, 3A7, and CYP3A43. Basically, CYP3A4 is the most abundant isoform present in the adult liver (Schuetz et al., 1994; Shimada et al., 1994) whereas CYP3A5 is expressed in an appreciable amount in only 25% of the adult population (Wrighton et al., 1990). CYP3A7 protein is transiently expressed in the fetal liver whereas 3A43 RNA was detected mostly in prostate and testes (Komori et al., 1990; Domanski et al., 2001). Therefore, the two latter proteins appear to play no significant role in drug metabolism during adulthood.

The situation is different in fetal and neonatal liver; a majority of P450 proteins matures postnataally and explains that monoxygenases develop during the early postnatal period (Cresteil et al., 1985; Treluyer et al., 1991; Vieira et al., 1996; Lacroix et al., 1997; Treluyer et al., 1997; Sonnier and Cresteil, 1998). Thus, CYP3A4 is absent from the fetal liver and rises during the first weeks after birth to reach 30 to 40% of the adult level after 1 month of age. Conversely, the 16α hydroxylation of dehydroepiandrosterone, a specific marker for CYP3A7-dependent activities, is highly active during gestation, maximizing during the first week following birth before progressively declining to reach a very low level in adult liver (Lacroix et al., 1997). Thus one can expect that biotransformation changes occurred during this period may modify the metabolic pathway and pharmacokinetics of amprenavir during the postnatal period. However, no data are currently available on the pharmacokinetics of amprenavir in neonates and infants. The present study was conducted to examine the in vitro metabolism of amprenavir by P450-transfected cells to definitively ascribe the reactions to individual P450 isoforms and compare the biotransformation of amprenavir by human fetal, pediatric, and adult liver microsomes.

1 Abbreviations used are: HIV, human immunodeficiency virus; Ad 293 cells, human fetal kidney cells transformed by adenovirus; HPLC, high performance liquid chromatography; LC/MS, liquid chromatography-mass spectrometry; THF, tetrahydrofuran; MS/MS, tandem mass spectrometry.

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A final concentration of 20 μM stock solution. When incubated with cells, amprenavir was added at a concentration of 20 μM in 5 ml Dulbecco’s modified Eagle’s medium without fetal calf serum in 75-cm² flask containing Ad 293 cells at near confluence. After 24 h of incubation at 37°C, the culture medium was removed and processed for analysis by HPLC. Mock-transfected Ad 293 cells (transfected with pM72 alone) were used as controls. Three different experiments were performed in duplicate.

Human liver microsomes corresponding to 0.3 nmol P450 were incubated in 0.1 M phosphate buffer, pH 7.45, in the presence of a NADPH-generating system consisting in 10 mM glucose 6-phosphate and 0.5 mM NADP and 20 μM amprenavir in a final volume of 1 ml. For the determination of kinetic parameters, microsomes were incubated for 30 min with concentrations ranging from 1 to 50 μM. The linearity of the reaction was checked in relation with time of incubation and protein content. The reaction, conducted at 37°C, was initiated by the addition of 1 IU of glucose 6-phosphate dehydrogenase and stopped after 30 min by immersing the tube in an ice-cold water bath. For inhibition studies, 30 μM sulfaphenazole, 10 μM quinidine, 10 μM ketoconazole, and 3.7 μM ritonavir were added to the incubation mixture prior to the amprenavir solution. Incubations were performed in duplicate. Controls were incubated under identical conditions but without NADPH-generating system.

**Analytical Procedure.** After centrifugation at 3000 rpm for 5 min, culture medium (1 ml) or the microsomal incubation mixture (0.5 ml) were extracted with 5 ml of a mixture of ethyl acetate-hexane (90:10, v/v). Following agitation for 20 min, all samples were centrifuged at ambient temperature at 4000 rpm for 20 min. The organic phase was transferred into a clean tube and evaporated at 40°C under nitrogen. The residue was reconstituted in 0.2 ml of mobile phase and washed with 3 ml of hexane. Following centrifugation (4500 rpm, 2 min) hexane was eliminated by aspiration, and any excess was evaporated at ambient temperature under nitrogen for 1 min. The clear supernatant was transferred to autosampler vials for HPLC analysis.

The HPLC system consisted of a Beckman model 126 solvent delivery pump, a Beckman model 507 autosampler, coupled to a Beckman model 166 UV spectrophotometric detector (Beckman Coulter, Inc., Fullerton, CA). Data acquisition was performed using Gold chromatography software. Samples (50 μl) were injected at ambient temperature onto a Macherey-Nagel Nucleosil C8
analytical column (3 μm, 125 × 4.6 mm; Macherey-Nagel, Inc., Easton, PA). The mobile phase consisted of 10 mM tetramethyl ammonium perchlorate in 0.1% trifluoroacetic acid-acetonitrile (65:35 v/v). The isocratic elution was performed at 1 ml/min. The absorbance of the eluent was monitored at 205 nm.

Liquid Chromatography-Mass Spectrometry (LC/MS). The HPLC system consisted of a Waters Alliance 2790 pump and autosampler connected to a Waters 996 photodiode array detector (Waters, Milford, MA). The analytical column was a Waters Symmetry C18 (150 × 2.1 mm ID) with a 5-μm particle size. The mobile phase consisted of acetonitrile and 0.1% formic acid (v/v) (A), and 5 mM ammonium acetate with 0.1% formic acid (v/v) (B). The mobile phase was delivered to the column at a flow rate of 0.25 ml/min, as a linear gradient, 0 to 2 min (25% B), 2 to 25 min (25–50% B). The column was maintained at 30°C, and the UV wavelength was monitored at 266 nm.

The mass spectrometer used for the analyses was a MicroMass Q-Tof instrument (Micromass, Manchester, UK) operated in full scan and product ion modes. Electrospray, positive ionization was used with a capillary voltage of 3000 V and a cone voltage of 60 V. The ion source temperature was 100°C, and the desolvation temperature 300°C with nitrogen used as the nebulizer gas. Argon was used as the collision gas for MS/MS experiments with a collision energy of 15 eV. A reference compound, [5-Leucine] enkephalin ([M + H]⁺, m/z 556.2771), 15 μg/ml, was introduced into the ion source with the sample, as a lock mass, at a flow rate of 1 μl/min. Data were acquired and processed using Micromass MassLynx (Version 3.4) software.

Statistical Analysis. Correlations between the CYP3A content of microsomal preparations, monooxygenase activities, and the formation of amprenavir metabolites were calculated using the Spearman-rank correlation coefficient.

Results

In Vitro Metabolism of Amprenavir by Human Liver Microsomes. In our conditions, five metabolites of amprenavir were generated by human liver microsomes in the presence of NADPH and termed M1 to M5 according to their retention time from HPLC, but the chemical structure details were not defined. In the absence of NADPH no metabolite was formed. In adult liver microsomes, M3 and M2 were the major metabolites formed in vitro followed in decreasing order by M5, M4, and M1 (Table 1). As expected, the formation of individual metabolites as well as the overall metabolism was widely distributed among the adult samples. The kinetic parameters for the formation of the two major metabolites M3 and M2 were determined by incubating adult liver microsomes with increasing concentrations of amprenavir ranging from 1 to 50 μM. A linear double reciprocal relationship was obtained for the formation of these metabolites and allowed the calculation of apparent Kₘ around 19 ± 5 and 32 ± 22 μM for M2 and M3, respectively, with values ranging between 14 to 25 μM and 10 to 72 μM (n = 6). The calculated V_{max} was two times higher for M3 than for M2 (601 ± 309, respectively versus 242 ± 124 pmol/min/mg of protein) and confirmed that the formation of M3 exceeded those of M2 at a concentration of 20 μM amprenavir. Based on these parameters and the circulating concentrations of amprenavir in patients (Sadler et al., 1999) further incubations were performed with 20 μM amprenavir.

When the formation of the major metabolite M3 is tentatively
correlated with the formation of other amprenavir metabolites, a highly positive association can be demonstrated with the formation of M2 and M5 but not with M1 and M4 (Table 2). This suggests that the same P450 isoform could be responsible for the formation of M2, M3 and M5, whereas M1 and M4 could be produced by another P450 protein(s). A CYP3A-dependent monoxygenase (testosterone-6β-hydroxylase) was closely correlated with the formation of M2, M3, and M5 with r values comprising 0.94 to 0.98. Similarly the CYP3A content of microsomes (sum of 3A4 plus 3A5) was positively associated with the production of amprenavir metabolites M2 and M5. The 16α-hydroxylation of dehydroepiandrosterone mostly supported by CYP3A7 was not correlated with the formation of amprenavir metabolites. This strongly suggests that amprenavir metabolites M2, M3, and M5 resulted from the activity of CYP3A4/5 but not CYP3A7. This assumption was confirmed by the inhibition of amprenavir metabolism by adult liver microsomes in presence of P450-specific inhibitors (Table 3). The addition of 10 μM ketoconazole, a specific inhibitor of CYP3A4/5 (Baldwin et al., 1995) to the incubation mixture of adult liver microsomes with 20 μM amprenavir strongly prevented the formation of M2 and M3. This resulted in a 60% inhibition of the global amprenavir metabolism. Ritonavir, another antiprotease inhibitor and substrate of CYP3A (Kumar et al., 1996) produced a more potent inhibition (86–99%) affecting the formation of all metabolites. Quinidine (CYP2D6 inhibitor) had no effect whereas sulfaphenazole (CYP2C9 inhibitor) had only a marginal effect (10–20%) on the formation of M2 (Table 3).

Collectively these data collected from in vitro incubation with liver microsomes suggested a major participation of CYP3A to the biotransformation pathway of amprenavir. This was also confirmed by incubation of amprenavir with recombinant P450 expressed in mammalian cell lines (Table 4). CYP3A4 was the major contributor to amprenavir biotransformation and efficiently formed M3 and M2 as in liver microsomes. Also CYP3A4 was the only isoform capable to generate M5. Cells expressing CYP3A4 were also involved in the production of M1 and M4 but to a much lower extent. Cells expressing CYP3A5 exhibited the same metabolite profile but with a weaker efficiency. In the same conditions CYP2C9 yielded essentially M2 and to a lesser extent M1. CYP3A7 had a low activity as well as CYP1A1, whereas CYP1A2, 2A6, 2C8, 2C18, and 2C19 had virtually no effect on amprenavir metabolism.

**LC/MS Analysis of the Amprenavir Metabolites.** Samples of the incubation mixture were analyzed by LC/MS analysis to help identify the structure of the metabolites, M1 to M5. LC/MS analysis of a standard of amprenavir shows a component with a retention time of 18.3 min with a protonated molecular ion at m/z 506. The product ion mass spectrum of the [M + H]⁺ is shown in Fig. 1, and the proposed assignments for the ions in the mass spectrum are shown in Fig. 2, in agreement with previously reported data (Singh et al., 1996). The major fragments are at m/z 418 (intact molecule-THF ring), 392

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**Fig. 2. Proposed fragments for amprenavir.**

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![Diagram of Amprenavir Metabolites](image)
Incubation mixture, Adult 1

Figure 3. LC/MS chromatograms obtained from the analysis of a typical incubation mixture (Adult 1). Chromatograms for individual metabolites were monitored at m/z = 506 for amprenavir, 392 for M1, 504 for M3 and M4, 522 for M2 and M5. The total ion current was shown on the bottom line of the figure.
in the formation of M2, M3, M4, and M5 is simultaneous in the neonatal liver but differed from the maturation of the biotransformation pathway leading to M1.

**Discussion**

The in vitro metabolism of amprenavir (141W94) has been previously explored using S9 fractions prepared from rat, monkey, and human livers. Using HPLC/MS detection, metabolites were tentatively identified and showed that amprenavir underwent various metabolic routes leading to the formation of multiple derivatives after oxidation and glucuronidation of the parent molecule. Major derivatives resulted from the oxidation at the benzylic position, at the aniline ring or from the opening of the tetrahydrofuran ring (Singh et al., 1996).

In the present study, five metabolites can be reproducibly isolated from incubation of amprenavir with human liver microsomes or P450-expressing cells. Due to the lack of UDP-glucuronic acid in the incubation mixture, no glucuronic acid conjugate can be detected in our incubations. Only oxidation products can be detected using our conditions: the major product M3 resulted from the mono-oxidation of the parent molecule on the THF ring whereas M2 is oxidized on the aliphatic chain. The aniline ring is another potential site of oxidation in metabolite M5, whereas M4 (probably an isomer of M3) is produced in lower amounts. Finally the N-dealkylated metabolite of amprenavir (4228W94) was identified as the minor metabolite M1.

These five metabolites of amprenavir were not formed by native untransfected cells and were also absent from incubation of human liver microsomes in absence of NAPDH implicating P450 as active contributors to their production. When the formation of these metabolites was tentatively associated with each other, a clear-cut dissociation was observed between the formation of metabolites M2, M3, and M5 on one hand and M1 and M4 on the other hand. When correlations were calculated with monoxygenase activities ascribed to CYP3A or with the CYP3A content of microsomes, a positive association was evident for M2, M3, and M5 suggesting the participation of CYP3A isoforms in these reactions, whereas the dealkylation of amprenavir is probably supported by another P450. CYP2C9 appeared to actively participate to the formation of M2, whereas other P450 tested in this study (CYP1A2, 2A6, 2C8, 2C18, and 2C19) did not actively result in oxidation of amprenavir.

**FIG. 4.** LC/MS/MS mass spectrum of metabolites M1, M2, M3, M4, and M5.

**TABLE 5**

Distribution of metabolites generated during in vitro incubation of amprenavir with human microsomes

<table>
<thead>
<tr>
<th>Microsomes</th>
<th>M1</th>
<th>M2</th>
<th>M3</th>
<th>M4</th>
<th>M5</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fetus</td>
<td>0.3</td>
<td>0.3</td>
<td>0.75</td>
<td>&lt;0.15</td>
<td>&lt;0.15</td>
<td>1.35</td>
</tr>
<tr>
<td>Neonate 1 day</td>
<td>&lt;0.15</td>
<td>0.65</td>
<td>2.6</td>
<td>0.5</td>
<td>&lt;0.15</td>
<td>3.75</td>
</tr>
<tr>
<td>Neonate 2 days</td>
<td>1.6</td>
<td>4.75</td>
<td>3</td>
<td>1.4</td>
<td>1.25</td>
<td>12.0</td>
</tr>
<tr>
<td>Infant 1 month</td>
<td>0.6</td>
<td>7.6</td>
<td>8.2</td>
<td>3.0</td>
<td>3.3</td>
<td>22.7</td>
</tr>
<tr>
<td>Infant 3 months</td>
<td>1.8</td>
<td>23.2</td>
<td>21.7</td>
<td>9.4</td>
<td>13</td>
<td>69.2</td>
</tr>
<tr>
<td>Adult 1</td>
<td>8</td>
<td>58.7</td>
<td>44.2</td>
<td>22.5</td>
<td>23.9</td>
<td>157.2</td>
</tr>
</tbody>
</table>
These conclusions were also confirmed by inhibition of amprenavir metabolism in liver microsomes; ketoconazole a well characterized inhibitor of CYP3A consistently reduced the formation of the two major metabolites M2 and M3, whereas sulphanemazole (CYP2C9 inhibitor) or quinidine (CYP2D6 inhibitor) had either a marginal or no effect. The participation of CYP2C9 to amprenavir metabolism was restricted to the formation of M2 and the inhibition of 2C9 by sulphanemazole observed in the present study did not exceed 10 to 20% of the formation of M2 by liver microsomes. Taking into account that the hepatic concentration of CYP2C9 is lower than the concentration of CYP3A4 and 3A5 and its lower efficiency to form M2, we can expect only a moderate inhibitory effect of sulphanemazole on amprenavir biotransformation by liver microsomes. To test interactions between amprenavir and protease inhibitors, we assessed the metabolism of amprenavir in presence of 3.7 μM ritonavir; this concentration of ritonavir completely inhibited the biotransformation of amprenavir by human liver microsomes as expected from previously published data (Decker et al., 1998).

Recombinant CYP3A4 and to a lesser extent CYP3A5 actively catalyzed these reactions and preferentially produced M2 and M3 in the same ratio as in liver microsomes. This is of considerable interest since hepatic CYP3A5 is expressed in an appreciable amount in only 25% of the population and then could actively participate to the overall metabolism of amprenavir. Furthermore, CYP3A5 is also expressed in a negligible amount in the intestine and could increase the elimination of amprenavir (Kivisto et al., 1996).

CYP3A7, the third member of the CYP3A subfamily had virtually no activity. In human neonates, Lacroix et al. (1997) have shown that CYP3A4 protein was close to detection limits during the early neonatal period whereas CYP3A7, which is present during the fetal and early neonatal period, did not catalyze the same activities as CYP3A4. Recently a similar pattern of expression has been observed for CYP3A5 and 3A4. The major raise of both proteins occurred postnatally, and CYP3A5 was polymorphically expressed in about 25% of the population whatever the group of age considered, including 33 fetuses, 69 neonates, and 18 adults (submitted to publication). Hepatic CYP1A2, CYP2E1, CYP2D6, and CYP2C9 were also shown to rise during the postnatal period in children (9–13). Thus it could be postulated that amprenavir metabolism should follow the ontogenic profile of CYP3A4/5 and not be metabolized in young neonates. These results were confirmed by incubation of amprenavir with hepatic microsomes from neonates and newborns. Microsomes of fetuses and neonates less than 1 week are deprived of CYP3A4 and had no or low activity toward amprenavir, likely due to CYP3A7. Thereafter the CYP3A4 content of neonatal liver rises, and the biotransformation of amprenavir becomes effective in infant livers. Thus from an enzymatic point of view the in vivo hepatic clearance of amprenavir should be very low in early neonates and increase during the first week of life.

The in vivo pharmacokinetics of amprenavir is currently unknown in neonates and infants. Our data are consistent with a low amprenavir clearance in neonates, and a high plasma concentration if the dose delivered is not carefully adjusted to this population. However, our data suggest that in 3-month-old infants the capacity of the human liver to oxidize amprenavir is close to the adult level and could be a valuable basis to adapt dosage to the maturation of drug-metabolizing enzymes. These in vitro data are not a substitute to pharmacokinetic studies but can help to focus the in vivo studies on the critical cut-off age of development.

**References**


