ELEVATED ALPHA-1-ACID GLYCOPROTEIN REDUCES THE VOLUME OF DISTRIBUTION AND SYSTEMIC CLEARANCE OF SAQUINAVIR

JOHN W. HOLLADAY, MICHAEL J. DEWEY, BOZENA B. MICHNIAK, HUGH WILTSHIRE, DANIEL L. HALBERG, PAUL WEIGL, ZHENMIN LIANG, KATHRYN HALIFAX, W. EDWARD LINDUP, AND DAVID J. BACK

Departments of Pharmaceutical Sciences (L.W.H.) and Pharmacy Practice (D.L.H.), College of Pharmacy, University of Arkansas for Medical Sciences, Little Rock, Arkansas; Departments of Biological Sciences (M.J.D.) and Pharmaceutical Sciences (B.B.M.), University of South Carolina, Columbia, South Carolina; Roche Discovery, Welwyn, England (H.W.); Roche Laboratories, Nutley, New Jersey (P.W., Z.L.); and Department of Pharmacology and Therapeutics, University of Liverpool, Liverpool, England (K.H., W.E.L., D.J.B.)

(Received March 23, 2000; accepted October 11, 2000)

This paper is available online at http://dmd.aspetjournals.org

ABSTRACT:

The purpose of this study was to characterize the relationship between plasma protein binding and the pharmacokinetic disposition of saquinavir during a normal and elevated alpha-1-acid glycoprotein condition. The extent of plasma binding of [14C]saquinavir to human plasma, human albumin, and human alpha-1-acid glycoprotein was also assessed. Transgenic mice, which overexpress plasma alpha-1-acid glycoprotein, and control mice were given a single intravenous injection of saquinavir (10 mg/kg) and plasma samples were harvested as a function of time. The extent of [14C]saquinavir (0.5–30 µg/ml) plasma protein binding in each group of mice was determined by ultrafiltration. Plasma saquinavir concentrations from in vivo administration were determined by high performance liquid chromatography with tandem mass spectrometry. Saquinavir binding in human plasma and control mouse plasma was similar (approximately 3% unbound). In contrast, the extent of binding was significantly increased in transgenic mice (1.5% unbound). Furthermore, saquinavir was more extensively bound to alpha-1-acid glycoprotein than to albumin (2.1 versus 11.5% unbound). The systemic clearance and volume of distribution of saquinavir were significantly reduced in transgenic mice compared with control mice. The results of this study show that alpha-1-acid glycoprotein is the predominant plasma protein to which saquinavir binds. In addition, elevations in plasma alpha-1-acid glycoprotein considerably alter the pharmacokinetic disposition of saquinavir. This is consistent with the observations that systemic exposure to saquinavir in human immunodeficiency virus patients is greater than that in healthy volunteers and that alpha-1-acid glycoprotein levels increase with the degree of HIV infection.

The variability of pharmacokinetic data for saquinavir is considerable in HIV-positive patients. Systemic exposure of saquinavir, as measured by the area under the plasma concentration versus time curve (AUC), has been reported to vary by approximately 70% in a group of HIV-1-infected patients (Gieschke et al., 1999; Regazzi et al., 1999). The hard gelatin capsule formulation of saquinavir (Fortovase) exhibits low oral bioavailability after a single dose to healthy volunteers (4%) and is estimated to be approximately 10% at steady state in HIV-positive patients (Steimer et al., 1998). A new soft gel formulation of saquinavir (Fortovase) provides a 3-fold increase in the bioavailability of saquinavir compared with the hard gelatin formulation. Several physicochemical and physiological factors reduce the bioavailability, including poor aqueous solubility, metabolism in the gut wall and liver, and the recent finding that saquinavir is a substrate for intestinal P-glycoprotein (Alszen et al., 1998; Kim et al., 1998; Lee et al., 1998).

It is known that most HIV protease inhibitors bind primarily to alpha-1-acid glycoprotein (AGP) in the plasma (Kageyama et al., 1994). Additionally, plasma AGP concentrations vary considerably (>50%) in normal and diseased patients, including HIV (Kremer et al., 1988; Øie et al., 1993). This is of particular importance, since plasma AGP levels are highest in the more severe cases of HIV-infected patients as shown by an inverse correlation with CD4 levels (Merry et al., 1996).

Increases in circulating AGP have been reported to alter the pharmacokinetic disposition and pharmacological action of numerous drugs to which it binds. For example, elevated AGP was associated with a higher bioavailability, and lower systemic clearance and volume of distribution of clindamycin in AIDS patients (Gatti et al., 1993). This change in disposition was found to correlate with an increase in plasma binding of clindamycin as a result of an increase in plasma AGP concentration in AIDS patients (Flaherty et al., 1996). In another report, an increase in plasma drug binding in vitro has led to a reduction in the activity of an investigational HIV protease inhibitor (KNI-272) (Kageyama et al., 1994). This reduction in activity was attributable to drug binding to plasma components, including AGP.

These previous in vitro and in vivo observations show the need to...
determine the exclusive influence of elevated plasma AGP on the pharmacokinetic disposition of saquinavir. The information needed in relation to the plasma protein binding of a drug is both quantitative and qualitative. The quantitative information relates to the extent of binding over the therapeutic and toxic plasma concentration range. This will indicate whether saturation of binding is likely and provide a value for the unbound drug concentration that should correlate more closely with the pharmacological activity, particularly if binding is altered by disease. The fraction unbound can also be used in the estimation of pharmacokinetic parameters such as free drug clearance. The qualitative information relates to the protein(s) that is involved in binding.

The aims of this study were to characterize the binding of saquinavir to human plasma, purified human albumin, and AGP. Additionally, the consequences of elevated AGP on the extent of saquinavir plasma protein binding and pharmacokinetic disposition were assessed in a novel strain of transgenic mice.

Materials and Methods

Chemicals and Reagents. Micronized saquinavir and [14C]saquinavir (26.5 µCi/mg) (Wiltshire et al., 1998) were supplied by Roche Discovery, Welwyn, England. Dextrose (5%) injection USP was purchased from VWR Scientific (Suwanee, GA). ScintiVerse II scintillation cocktail, borosilicate scintillation vials, and sterile, 4-ml Vacutainers were purchased from Fisher Scientific (St. Louis, MO). All buffer constituents were purchased from Sigma Chemical Co. Ltd., (London, UK).

[14C]Saquinavir Binding to Human Plasma, Albumin, and AGP. Blood donated by healthy volunteers was collected in heparinized blood tubes at the University of Liverpool, Liverpool, England. Plasma was obtained by centrifugation at 2000g for 15 min. Patient plasma was pooled and used within 2 h. Human albumin fraction V (A1653, 96–99% albumin, remainder mostly globulins) and human AGP (G9885, purity 99%) were obtained from Sigma Chemical Co. Ltd. Solutions of 600 µm albumin (66,500 Da) and 22 µm AGP (41,000 Da) were prepared in a modified Krebs’ mammalian Ringer phosphate buffer: 100 parts 0.154 M sodium chloride, 4 parts 0.154 M potassium chloride, 1 part 0.154 M potassium dihydrogen orthophosphate, 1 part 0.154 M magnesium sulfate, and 21 parts 0.1 M disodium hydrogen orthophosphate. The pH was adjusted to 7.4 with 1 M hydrochloric acid. Protein concentrations before and after dialysis were determined by the method of Lowry et al. (1951) with human serum albumin as standard.

Equilibrium dialysis was used to determine the unbound fraction of [14C]saquinavir in human plasma, albumin, and AGP preparations. Dialysis was performed with a Diacnorm apparatus (Diachema AG, Zurich, Switzerland). The cells consisted of 2 Teflon half-cells each with a capacity of 1 ml and separated by a semipermeable cellulose membrane (molecular mass cut-off 12–14,000 Da; Medicell International Ltd.). The membrane was prepared by scintillation using the Centrifree micropartition system (Amicon Inc., Beverly, MA). This method was different from that used in the assessment of saquinavir binding to human plasma and protein preparations due to preferences of the laboratory animal care and were approved by the institutional animal care and use committee. This transgenic mouse model has been used in previous pharmacokinetic assessments of imipramine and fluoxetine (Yoo et al., 1996; Holladay et al., 1998).

Several steps were taken before and during the experiments to minimize the variance between individual mice of each strain. First, the same vendor for C57BL/6 mice was used (Charles River Breeding Laboratories, Wilmington, MA) and mice of similar weight and age were used. In the transgenic strain, mice were obtained by sequential generations of brother-sister mating. Such breeding is common in murine studies and promotes less variance in the physiology in the mice.

[14C]Saquinavir Binding to Transgenic and Control Mouse Plasma. Saquinavir plasma protein binding in mouse plasma was determined by ultrafiltration using the Centrifree micropartition system (Amicon Inc., Beverly, MA). This method was different from that used in the assessment of saquinavir binding to human plasma and protein preparations due to preferences of the individual laboratories. Aliquots of a 10 µg/ml methanolic [14C]saquinavir solution were dried under nitrogen gas and reconstituted with drug-free transgenic or control mouse plasma (500 µl) to obtain saquinavir concentrations of 1, 2, 4, 8, 16, and 32 µg/ml (in triplicate). Plasma samples were incubated at 37°C for 2 h. Immediately before centrifugation, 25 µl of saquinavir-supplemented plasma was harvested to allow the determination of the total drug concentration. Subsequently, each transgenic and control mouse plasma sample was placed in ultrafiltration tubes and centrifuged at 2000g for 30 min. After centrifugation, 25 µl of ultrafiltrate was collected for the determination of the unbound saquinavir concentration. Precentrifugation plasma samples and ultrafiltrate samples (25 µl) were supplemented with 10 ml of ScintiVerse II cocktail and each sample was analyzed by scintillation counting. The fraction unbound was calculated by the method of Lowry et al. (1951) with human serum albumin as standard.


cell = 0.84 ± 0.38
5
P bound = 0.93 ± 0.32
8
P bound = 1.30 ± 0.36
18
P bound = 2.52 ± 0.66
24
P bound = 2.3 ± 0.5
28
P bound = 2.63 ± 0.83

Animals. Adult (20–30 g, 2–4-month-old) control and transgenic mice were used in this study. Male and female mice were used in this study randomly. The transgenic line AGP 9.5-5 contained the rat AGP gene along with sufficient cis-acting regulatory information to direct its liver-specific expression (Dewey et al., 1990). Line AGP 9.5-5 was developed by standard transgenic technology involving microinjection of the rat AGP genomic clone into the pronuclei of C57BL/6Acknowledgments. The authors thank Scientific Corporation for their assistance in conducting the scintillation studies.

TABLE 1

Effect of increasing equilibrium time on the binding of saquinavir (1 µg/ml) to human plasma

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Percentage Unbound</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>0.84 ± 0.38</td>
<td>3</td>
</tr>
<tr>
<td>5</td>
<td>0.93 ± 0.32</td>
<td>3</td>
</tr>
<tr>
<td>8</td>
<td>1.30 ± 0.36</td>
<td>3</td>
</tr>
<tr>
<td>18</td>
<td>2.52 ± 0.66</td>
<td>5</td>
</tr>
<tr>
<td>24</td>
<td>2.3 ± 0.5</td>
<td>2</td>
</tr>
<tr>
<td>28</td>
<td>2.63 ± 0.83</td>
<td>3</td>
</tr>
</tbody>
</table>

Data are expressed as the mean ± S.D.

where fu is fraction unbound; P is protein concentration in solution before dialysis; P’ is protein concentration in solution after dialysis; C’B is radioactivity in buffer compartment after dialysis; C’P is radioactivity in plasma compartment after dialysis; C’bound is concentration of bound drug had no volume shift occurred; C’bound is C’bound (1 + δ); and δ is fractional increase in the volume of the plasma compartment due to volume shift:

\[ \delta = \frac{P - 1}{P} \]
In Vivo Saquinavir Administration to Mice. To characterize the pharmacokinetic disposition of saquinavir, transgenic and control mice (n = 3–4 for each strain at each time point) received a single intravenous injection of unlabeled saquinavir (10 mg/kg, 1.33 mg/ml as free base). The total volume of intravenous drug solution injected was 0.0075 ml/kg of body weight. Each mouse was anesthetized by halothane vapor, and whole-blood samples (0.8 ml) were harvested into Vacutainer, heparinized tubes by cardiac puncture (destructive sampling) at 0, 5, 15, and 30 min and 1, 2, 3, 4, 6, and 8 h post dose. Whole-blood samples were subsequently centrifuged at 3000g for 5 min and plasma was stored in disposable borosilicate culture tubes (100 µl) in duplicate at −20°C until high performance liquid chromatography (HPLC) analysis.

Hepatic Blood Flow Determination. The apparent hepatic blood flow in transgenic and control mice was estimated as the clearance of a bolus injection of indocyanine green (ICG) as described by Wynne et al. (1990). Historically, ICG clearance has been used as a reliable measurement of the apparent hepatic blood flow, since ICG is exclusively eliminated by the liver. Transgenic and control mice (n = 3 for each strain) were given an intravenous bolus dose (1.0 mg/kg) of ICG, and at 2, 5, 10, 15, 30, and 60 min after ICG administration, each mouse was anesthetized with halothane vapors. Whole-blood samples were collected from anesthetized mice by cardiac puncture and the harvested serum was stored at −20°C until HPLC analysis. The possibility of differential binding of ICG to transgenic and control mouse plasma was investigated using the ultrafiltration procedure described for the determination of saquinavir binding in mouse plasma. Briefly, portions of a methanolic solution of ICG were dried and pooled mouse plasma was added to the residue to yield a concentration of 5 µg/ml (n = 3 samples/strain). Plasma ICG samples were centrifuged at 2000g for 30 min. Hematocrits for transgenic and control mice were determined using a Coulter counter and were expressed as percentage values. The hepatic blood flow of each strain of mice was determined by the following formula: ICG_BLOOD = ICG_SERUM × (1 − hematocrit).

Plasma and ultraltrafine ICG quantification was performed by HPLC with ultraviolet detection using a modification of a method described by Hopton et al. (1999). Briefly, the mobile phase of acetonitrile/methanol and 5 mM phosphate buffer (37:3:40) was used to separate ICG and the internal standard, respectively. The range of quantification was 0.5 to 500 ng/ml.

Saquinavir Analysis. Concentrations of saquinavir in transgenic and control mouse plasma were determined by high-performance, on-line high performance liquid chromatography with tandem mass spectrometry using a method based on a previously published protocol (Knebel et al., 1995). Plasma samples (0.1 ml) were supplemented with 0.02 ml of 20 ng/ml D5-labeled saquinavir internal standard (Wilshire et al., 1998). Drug extractions were performed by an automated solid phase extraction procedure using C2 Bond Elut SPE cartridges (Varian, Harbor City, CA) and reconstituted with mobile phase. The reconstituted samples (20 µl) were applied to a Zorbax Rx-C18 (2.1 × 150-mm, 5-µm) column at 30°C with gradient elution by 10 mM ammonium acetate/0.1% acetic acid and acetonitrile. The retention time for saquinavir and its internal standard was 4.3 min. A micrassot II triple quadruple mass spectrometer in positive electrospray mode was used to analyze the samples. The molecular weight/charge ratios of the protonated precursor and product ions (m/z) were 671.3 to 128.0 and 676.2 to 133.0, for saquinavir and the internal standard, respectively. The range of quantification was 0.5 to 500 ng/ml.

Data Analysis. Plasma saquinavir concentration versus time data in transgenic and control mice were analyzed noncompartmentally using the least-squares nonlinear computer program WinNonlin (Scientific Consulting, Cary, NC). Pharmacokinetic parameters were calculated using standard, noncompartmental equations. The AUC determined using log-trapezoidal method (Bailar, 1988; Nedelman and Gibiansky, 1996; Gagnon and Peterson, 1998). In addition to methodological control with respect to mice, an analysis of covariance was used to test the difference in the mean pharmacokinetic parameters between transgenic and control mice, since the data from these mice were obtained by destructive sampling (i.e., population data). Analysis of covariance allowed the determination of whether the differences in the data are a result of AGP-related events or a function of the inherent variance in mice. An unpaired t test was used to test the difference in the mean unbound fraction of [14C]saquinavir in transgenic and control mice. Pharmacokinetic data are reported as the mean ± standard deviation. Statistical significance was set at p < 0.05.

Results

Initial studies focused on the time required for saquinavir to reach binding equilibrium in the dialysis experiments with human plasma and albumin. On the basis of these results (Table 1), 28 h was chosen as the optimum equilibration time of saquinavir with human plasma and purified proteins. The extents of [14C]saquinavir binding in the range of 0.1 to 30 µg/ml to human plasma, human albumin, and human AGP are shown in Table 2. The percentage of unbound saquinavir was approximately 3% in human plasma over this concentration range. In addition, binding to human AGP was markedly greater than to human albumin (98 compared with 88%). At the highest concentrations of saquinavir (20 and 30 µg/ml) the binding to AGP decreased from 98.5 to 97%, but such changes did not occur in whole plasma. Volume shifts due to osmosis were seen in the plasma binding studies, smaller volume shifts occurred in the binding studies involving albumin, but no volume shift was observed with AGP. Consistent with the values obtained from human plasma, the extent of in vitro murine plasma saquinavir binding was approximately 97% over the concentration range studied (Table 2). In addition, saquinavir binding was significantly increased in transgenic mouse plasma as opposed to control mouse plasma (i.e., 1.5 versus 3.0% unbound) (Table 2). This increase in binding was consistent with the elevation of plasma AGP in transgenic mice. The mean recovery of [14C]saquinavir from the ultrafiltration devices ranged from 72% for the lowest concentration (1 µg/ml) to 81% for the highest concentration tested (30 µg/ml).

The mean plasma clearance of ICG was significantly reduced in transgenic mice compared with control mice (32.1 versus 46.7 ml/min/kg). The extent of ICG plasma binding in the transgenic (81%) and control (79%) mouse sera was similar. Thus, the hepatic blood flow was significantly reduced in transgenic mice (17.0 ± 0.91 versus 23.4 ± 3.3 ml/min/kg). The mean plasma saquinavir concentration versus time profiles in transgenic and control mice after 10-mg/kg intravenous bolus doses are shown in Fig. 1 and the pharmacokinetic parameters are reported in Table 3. Significantly slower clearance in the transgenic mice caused a doubling in systemic exposure. In addition, as a consequence of increased protein binding, the steady-state volume of distribution was reduced by 87% in transgenic mice and the central compartment volume of distribution in transgenic mice was only 5.2% of the control value (Table 3).

Discussion

Considerable variability in the serum level of AGP exists in the general population. The basal AGP level in persons of the same race,
For transgenic mice, observed values are displayed as black boxes and predicted values as a continuous line. For control mice, observed values are displayed as open circles and predicted values as a dashed line.

**Table 3**

Pharmacokinetic parameters of saquinavir in transgenic and control mice (n = 3–4) after a single 10-mg/kg i.v. injection

<table>
<thead>
<tr>
<th>Pharmacokinetic Parameter</th>
<th>Transgenic Mice</th>
<th>Control Mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC (μg · min/ml)</td>
<td>438.1 ± 46.7*</td>
<td>124.4 ± 24.0</td>
</tr>
<tr>
<td>V₁ (l/kg)</td>
<td>2.6 ± 0.7</td>
<td>8.2 ± 0.8</td>
</tr>
<tr>
<td>V₉₃ (l/kg)</td>
<td>0.7 ± 0.1*</td>
<td>4.6 ± 0.5</td>
</tr>
<tr>
<td>T₁/₂Z (min)</td>
<td>80.0 ± 19.7</td>
<td>71.4 ± 17.9</td>
</tr>
<tr>
<td>fu (%)</td>
<td>1.5 ± 0.4*</td>
<td>3.0 ± 1.4</td>
</tr>
<tr>
<td>CLₚ (ml/min/kg)</td>
<td>22.7 ± 2.4*</td>
<td>80.1 ± 17.8</td>
</tr>
<tr>
<td>CLₚₙ (l/min/kg, 0.2a)</td>
<td>1.5 ± 0.2*</td>
<td>2.7 ± 0.6</td>
</tr>
</tbody>
</table>

*Mean transgenic mouse value is significantly different from the control mouse value (p < 0.05).

Gender, and age may vary 40% or more (Blain et al., 1985; Kremer et al., 1988; Duchêne et al., 1998). For example, a recent study showed that the mean basal serum AGP concentration in Caucasians was 20% lower than that observed in comparable African-American subjects. In addition, a 45% variance in the level of AGP was found within each group (McCollum et al., 1998). The results of this study show that saquinavir is bound extensively in the plasma (>95%) and primarily to AGP. Additionally, saquinavir binding to AGP increases in proportion to increases in plasma AGP levels. This is of importance in the treatment of HIV illness due to the fact that serum AGP levels are proportionally elevated as a function of the severity of the disease (Merry et al., 1996) as well as the observations of intersubject variability in AGP levels in AIDS patients (Øie et al., 1993). This variation in basal and elevated serum AGP level has direct and multiple consequences on drug disposition and action that may produce considerable interpatient variability in drug response.

Elevations in serum AGP led to an expected increase in saquinavir plasma binding and a resultant decrease in the volume of distribution. In addition, the clearance of saquinavir in the transgenic mice was significantly reduced. The fundamental mechanisms that define these differences may not be strictly due to alteration of the unbound plasma concentration of saquinavir. Our data suggest that the volume of distribution at steady state of the unbound portion of drug is substantially lower in AGP mice as opposed to control mice. Although our study was not designed to specifically address this issue, this discrepancy in the unbound portion of drug can be explained by the possible existence of more AGP in the microvascular endothelium of AGP mice as opposed to control mice, and thus, a restriction of drug passage into the tissues of control mice. The issue of endothelial AGP overexpression in our transgenic mice will be explored in the future.
Therefore, the contribution of albumin to saquinavir protein binding may become more important at higher saquinavir plasma concentrations.

Previous studies have shown a proportional decrease in the in vitro efficacy of experimental HIV protease inhibitors (e.g., A80987, A77003, CGP61755) and incremental increases in AGP. However, this reduction in activity apparently reaches the maximum when blood AGP levels reach 2 mg/ml (Billelo et al., 1995, 1996; Lazdins et al., 1997). As seen in the transgenic mouse model, elevations in plasma AGP led to reductions in the volume of distribution as a result of increased plasma saquinavir binding. Despite the higher total saquinavir exposure during an elevated AGP condition, saquinavir efficacy is predicted to be less, primarily due to the increase in plasma binding. Therefore, during elevated AGP conditions, the efficacy of HIV-protease inhibitor therapy may not directly correlate to total plasma HIV-protease inhibitor exposure.

In summary, the extent of [14C]saquinavir binding to purified human AGP and albumin confirmed that the major drug binding protein of saquinavir is AGP, and this binding displayed a slight trend toward saturation. Nevertheless, [14C]saquinavir did substantially bind to human albumin. Taken together, these results indicate that during elevated AGP conditions, as in patients with low CD4 counts, the extent of saquinavir binding may increase, and thus lead to a significantly higher systemic exposure and significantly lower systemic clearance and volume of distribution of saquinavir.

References

References