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# The binding of HIV-1 protease inhibitors to human serum proteins

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**Abstract**

The non-specific binding of a drug to plasma proteins is an important determinant of its biological efficacy since it modulates the availability of the drug to its intended target. In the case of HIV-1 protease inhibitors, binding to human serum albumin (HSA) and $\alpha_1$-acid glycoprotein (AAG) appears to be an important modulator of drug bioavailability. From a thermodynamic point of view, the issue of drug availability to the desired target can be formulated as a multiple equilibrium problem in which a ligand is able to bind to different proteins or other macromolecules with different binding affinities. Previously, we have measured the binding thermodynamics of HIV-1 protease inhibitors to their target. In this article, the binding energetics of four inhibitors currently in clinical use (saquinavir, indinavir, ritonavir and nelfinavir) and a second-generation inhibitor (KNI-764) to human HSA and AAG has been studied by isothermal titration calorimetry. All inhibitors exhibited a significant affinity for AAG ($K_a \sim 0.5–10 \times 10^5$ $M^{-1}$) and a relatively low affinity for HSA ($K_a \sim 5–15 \times 10^3$ $M^{-1}$). It is shown that under conditions that simulate in vivo concentrations of serum proteins, the inhibitor concentrations required to achieve 95% protease inhibition can be up to 10 times higher than those required in the absence of serum proteins. The effect is compounded in patients infected with drug resistant HIV-1 strains that exhibit a lower affinity for protease inhibitors. In these cases the required inhibitor concentrations can be up to 2000 times higher and beyond the solubility limits of the inhibitors.


*Keywords:* HIV-1 protease; Protease inhibitor; AIDS; Human serum albumin; $\alpha_1$-acid glycoprotein; Calorimetry

## 1. Introduction

An important consideration in drug design is the optimization of the binding affinity against the desired target while simultaneously minimizing the affinity of the drug for unwanted targets. Among the list of unwanted targets are the serum proteins human serum albumin (HSA) and $\alpha_1$-acid glycoprotein (AAG), which are known to bind organic molecules in non-specific ways. These proteins exist in relatively high concentrations (40 mg/ml and 1 mg/ml, respectively) and therefore, have the potential to significantly lower effective drug concentrations to suboptimal levels even if their affinity for drug molecules is not very high. In the case of HIV-1 and other viral infections, the non-specific binding of protease inhibitors to serum proteins is important not only from the point of view of bioavailability, but also from the point of

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view of the development of drug resistance. Antiretroviral therapy at suboptimal plasma levels does not eliminate completely viral replication, thereby contributing to the appearance of drug resistant viral strains [1,2].

Human serum albumin, HSA, is the most abundant protein in plasma (40 mg/ml) and binds preferentially hydrophobic and acidic drugs [3]. Alpha-1-acid glycoprotein (AAG, earlier called orosomucoid) is present at much lower concentrations (normally 1 mg/ml) and preferentially binds hydrophobic and basic drugs [4]. AAG is an acute-phase protein and its concentration can increase up to 4–5 mg/ml during infection including HIV-1 [5]. As with most weak organic bases, HIV-1 protease inhibitors can be expected to bind better to AAG than to HSA.

In order to assess the role of serum proteins on drug bioavailability at a quantitative level, we have investigated the binding thermodynamics of four protease inhibitors in clinical use (saquinavir, indinavir, ritonavir and nelfinavir) and one second-generation inhibitor, currently under development (KNI-764), to AAG and HSA. By combining the affinities for AAG and HSA with that of HIV-1 protease previously determined in this laboratory [6], it is possible to estimate the degree of HIV-1 protease inhibition in the presence of serum proteins. This treatment allows evaluation of the efficacy of each drug against wild type and drug-resistant mutants of the HIV-1 protease.

## 2. Experimental

### 2.1. Serum proteins

Human AAG (Cat. No. G9885) and HSA (Cat. No. 3782) were obtained from Sigma. AAG was dissolved in 10 mM sodium acetate, pH 5.0 and dialyzed prior to the experiments. The concentration was calculated from the absorbance at 280 nm using a molar extinction coefficient of $3.9\times10^4$ $M^{-1}$ $cm^{-1}$ [7]. HSA was dissolved in 20 mM sodium phosphate with 150 mM NaCl and monomers were separated from dimers by size exclusion using Sephacryl S-300 HR (Amersham Bioscience). Monomer fractions were identified by native PAGE, pooled, concentrated and finally dialyzed against 10 mM sodium acetate with 150 mM NaCl, pH 5.0. The concentration was calculated from the molar absorbance of $3.52\times10^4$ $M^{-1}$ $cm^{-1}$ at 280 nm [8]. The high ionic strength used for HSA was necessary for the stability of HSA, especially at low pH [9].

### 2.2. Protease purification

Plasmid-encoded HIV-1 wild type protease was expressed as inclusion bodies in *Escherichia coli* 1458 cells [10–12]. The cells were suspended in extraction buffer (20 mM Tris, 1 mM EDTA, 10 mM 2-ME, pH 7.5) and broken with two passes through a French pressure cell ($\geq$16 000 psi). Cell debris and protease-containing inclusion bodies were collected by centrifugation (20 000 $\times g$ for 20 min at 4 °C). Inclusion bodies were washed with three buffers. Each wash consisted of resuspension (glass homogenizer, sonication) and centrifugation (20 000 $\times g$ for 20 min at 4 °C). In each step a different washing buffer was employed: buffer 1 (25 mM Tris, 2.5 mM EDTA, 0.5 M NaCl, 1 mM Gly–Gly, 50 mM 2-ME, pH 7.0), buffer 2 (25 mM Tris, 2.5 mM EDTA, 0.5 M NaCl, 1 mM Gly–Gly, 50 mM 2-ME, 1 M urea, pH 7.0) and buffer 3 (25 mM Tris, 1 mM EDTA, 1 mM Gly–Gly, 50 mM 2-ME, pH 7.0). Protease was solubilized in 25 mM Tris, 1 mM EDTA, 5 mM NaCl, 1 mM Gly–Gly, 50 mM 2-ME, and 9 M urea, pH 9.0, clarified by centrifugation, and applied directly to an anion-exchange Q-Sepharose column (Q-Sepharose HP, Amersham Bioscience) previously equilibrated with the same buffer. The protease was passed through the column and then acidified by adding formic acid to 25 mM immediately upon elution from the column. Protease-containing fractions were pooled, concentrated, and stored at 4 °C at 5–10 mg/ml.

### 2.3. Protease inhibitors

Clinical inhibitors, saquinavir, ritonavir, indinavir and nelfinavir were purified from commercial capsules by HPLC (Waters Inc.) using a semipreparative C-18 reverse-phase column developed with 20–80% acetonitrile containing 0.05% trifluoroacetic acid. Purified inhibitors were lyophilized

and stored at −20 °C as solids or dissolved in DMSO. KNI-764 was kindly provided by Dr Y. Kiso, Department of Medicinal Chemistry, Kyoto Pharmaceutical University, Kyoto, Japan. The latter compound was used without further purification. The inhibitor solution used in the experiments was prepared in 10 mM sodium acetate, pH 5.0, the DMSO concentration being kept constant at 2%.

### 2.4. Isothermal titration calorimetry

Isothermal titration calorimetry was carried out using an MCS titration calorimeter system from MicroCal Inc. The calorimetric cell containing either HSA or AAG was titrated with protease inhibitor dissolved in the same buffer (10 mM sodium acetate, pH 5.0, 2% DMSO). Injections of volumes were 10 μl. In the case of HSA the buffer was supplemented with 150 mM NaCl. All solutions were properly degassed to avoid any formation of bubbles in the calorimeter during stirring. The heat evolved upon each injection of ligand was obtained from the integral of the calorimetric signal. The heat associated with the binding of protease inhibitor to serum protein was obtained by subtracting the heat of dilution from the heat of reaction. All measurements were made at 25 °C except for binding of indinavir to AAG, which was made at 35 °C.

### 2.5. Assays of protease inhibition in the absence and presence of serum protein

The effect of inhibitor on the catalytic activity of HIV-1 protease with and without AAG present was monitored spectrophotometrically at 25 °C by following the hydrolysis of the chromogenic substrate Lys-Ala-Arg-Val-Nle-nPhe-Glu-Ala-Nle-$NH_2$ (California Peptide Research Inc.). Protease was added to a 120 μl cuvette containing substrate at 25 °C. Final concentrations in the standard assay were 50 nM active protease, 50 μM substrate, 10 mM sodium acetate, and 1 M NaCl (pH 5.0). Hydrolysis rates were obtained from the initial portion of the data, where less than 20% of the substrate is hydrolyzed. The inhibition constant, $K_i$ was derived from the different rates of hydrolysis obtained by varying the concentration of drug in presence of 0.3–1.3 mg/ml AAG. The absorbance was monitored at 300 nm using a Cary 100 spectrophotometer (Varian).

## 3. Results and discussion

### 3.1. Isothermal titration calorimetry

Isothermal titration calorimetry was utilized to determine the binding affinity and binding enthalpy of different protease inhibitors to AAG and HSA. Fig. 1a and b shows the microcalorimetric titrations of AAG with saquinavir and ritonavir. As summarized in Table 1 the binding enthalpy to AAG is exothermic at 25 °C for all inhibitors except for indinavir, which has an enthalpy close to zero. When measured at 35 °C, the binding of indinavir to AAG was associated with a small enthalpy change (−1.8 kcal/mol) and a low binding affinity ($K_a = 0.37 \times 10^5$ $M^{-1}$). Ritonavir had the highest affinity towards AAG ($K_a = 1.3 \times 10^6$ $M^{-1}$), followed by nelfinavir and KNI-764 ($7 \times 10^5$ $M^{-1}$). The binding affinity of saquinavir was somewhat lower ($K_a = 2.4 \times 10^5$ $M^{-1}$) but characterized by a large negative enthalpy change. It should be noticed that the stoichiometry deviates significantly from 1 for saquinavir and nelfinavir, both having a value close to 0.2. A low number of binding sites has been previously observed for binding of other drugs to AAG and has been attributed to lack of affinity to some of the three main genetic variants known to be present in serum as well as in commercial preparations of AAG [13–15].

As expected for basic hydrophobic molecules, all inhibitors studied in this report had lower affinity for HSA than for AAG. Binding of indinavir to HSA could be considered too small to have any impact on the affinity for HIV-1 protease and is not included in the calculations below. The affinity of saquinavir for HSA was, however, somewhat larger ($K_a = 14.9 \times 10^3$ $M^{-1}$) and characterized by a binding enthalpy of −20 kcal/mol. Nelfinavir, ritonavir and KNI-764 had affinity constants on the order of $5.0 \times 10^3$ $M^{-1}$.

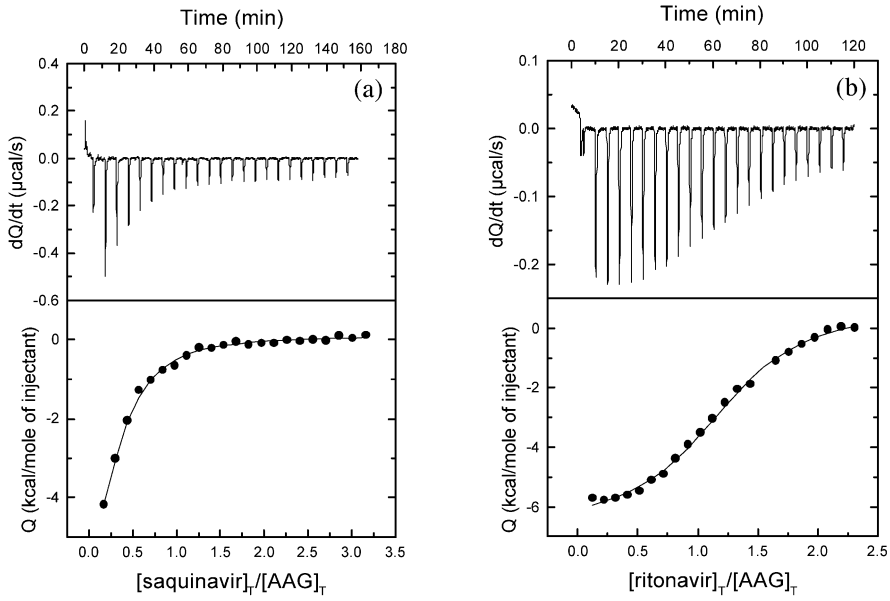


Fig. 1. Calorimetric titrations of AAG with saquinavir (a) and ritonavir (b) at 25 °C in 10 mM acetate buffer, pH 5.0, 2% DMSO. The concentrations were 19.6 μM AAG and 345 μM saquinavir (a), and 7.7 μM AAG and 99 μM ritonavir (b). Injection volumes were 10 μl in both cases. The experiments were performed using a MCS titration calorimeter system (MicroCal Inc.).

### *3.2. HIV-1 protease inhibition in the absence and presence of serum proteins*

The HIV-1 protease inhibition constant, $K_i$, for ritonavir and saquinavir, the inhibitors with the highest and lowest affinity for AAG, were determined in the presence of varying concentrations of AAG. As expected, ritonavir, having a higher affinity for AAG and a stoichiometry of 1, was more affected by the presence of AAG than saquinavir. The apparent $K_i$ values at an AAG concentration of 1 mg/ml were 300 and 60 nM for ritonavir and saquinavir, respectively. Their respective $K_i$ values in absence of AAG are 0.05 and 0.5

Table 1
Results from titration experiments with AAG

| Inhibitor | $T$ (°C) | $K_a \times 10^5$ ($M^{-1}$) | $\Delta H$ (kcal/mol) | $n$ |
|---|---|---|---|---|
| Indinavir | 35 | 0.37±0.06 | −1.8±0.1 | 1 |
| Saquinavir | 25 | 2.4±0.4 | −14.5±3.2 | 0.2 |
| KNI-764 | 25 | 6.7±0.7 | −6.9±0.2 | 1 |
| Nelfinavir | 25 | 7.1±1.3 | −5.3±0.5 | 0.2 |
| Ritonavir | 25 | 13±1.7 | −5.9±0.2 | 1 |

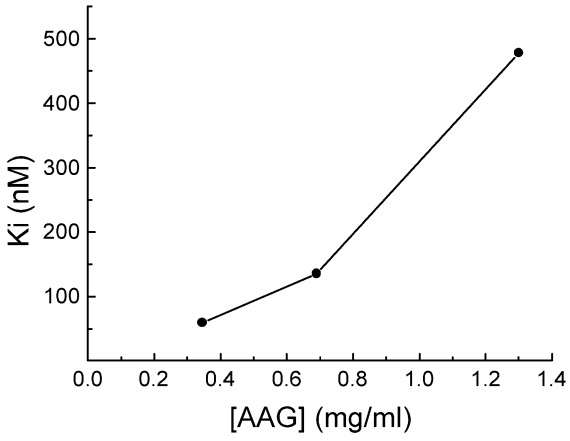


Fig. 2. The dependence of the inhibition constant, $K_i$, of ritonavir for the HIV-1 protease on the concentration of AAG. 50 nM HIV-1 protease and 50 μM chromogenic substrate were used in all experiments. The data points in the figure were derived from the fit of hydrolysis rates at varying concentrations of ritonavir at 0.3, 0.7 and 1.3 mg/ml AAG.

nM [16] indicating that non-specific binding to AAG lowers their effective inhibitory potency by a factor of 6000 and 120, respectively. The dependence of $K_i$ on the concentration of AAG for ritonavir is shown in Fig. 2.

### 3.3. Calculation of the fraction of HIV-1 protease bound to drug

The degree of HIV-1 protease inhibition is equal to the fraction of protease bound to any given inhibitor and can be calculated for different serum conditions using the affinity data determined in this article. The affinity constants of indinavir, saquinavir, ritonavir, nelfinavir and KNI-764 to the wild type HIV-1 protease are $4.5\times10^8$ $M^{-1}$, $4.5\times10^8$ $M^{-1}$, $7.9\times10^9$ $M^{-1}$, $2.3\times10^8$ $M^{-1}$ and $3.1\times10^{10}$ $M^{-1}$, respectively [10,6]. For each inhibitor, the fraction of protease, $F_{PR}$ bound to drug is given by:

$$F_{PR}=\frac{K_{PR}\times[X]}{1+K_{PR}\times[X]} \quad (1)$$

where $K_{PR}$ is an association constant of the inhibitor to the protease and $[X]$ its free concentration. The free concentration of inhibitor can be calculated if its total concentration, $[X]_{tot}$, is known as well as the affinities and concentrations of all the proteins that are able to bind the inhibitor:

$$[X]_{tot}=[X]+\sum_i [P_i]\frac{K_{P_i}[X]}{1+K_{P_i}[X]} \quad (2)$$

where $[P_i]$ is the concentration of protein $i$, and $K_{P_i}$ the binding affinity of the drug for that protein. For the case under consideration in which a drug binds to its target in the presence of the two serum proteins, HSA and AAG, Eq. (2) reduces to:

$$[X]_{tot}=[X]+\frac{[HSA]\times K_{HSA}\times[X]}{1+K_{HSA}\times[X]}+\frac{[AAG]\times n_{AAG}\times K_{AAG}\times[X]}{1+K_{AAG}\times[X]}+\frac{[PR]\times K_{PR}\times[X]}{1+K_{PR}\times[X]} \quad (3)$$

where [HSA] is 40 mg/ml (600 μM) and [AAG] = 1 mg/ml (23 μM) or 4 mg/ml (92 μM) depending on whether basal or acute infection concentration levels are considered. $K_{HSA}$, $K_{AAG}$ and $K_{PR}$ are the affinity constants for HSA, AAG and HIV-1 protease, respectively. The protease concentration, [PR], was assumed to be 0.03 μM.[1] Affinity constants for AAG ($K_{AAG}$) are listed in Table 1. $n_{AAG}$ is the binding stoichiometry to AAG. The affinity of saquinavir for HSA ($K_{HSA}$) was $14.9\times10^3$ $M^{-1}$. A binding affinity to HSA of $5.0\times10^3$ $M^{-1}$ was used for ritonavir, nelfinavir and KNI-764. The binding of indinavir to HSA was negligible.

Fig. 3 a–e show $F_{PR}$ as a function of total drug concentration in the absence and presence of serum proteins. In order to illustrate the increased binding to serum components expected in an HIV-1 infected patient results for both serum having 1 and 4 mg/ml AAG are included in the graphs. These results clearly indicate that the presence of serum proteins significantly reduces drug availability and consequently the degree of HIV-1 protease inhibition for any given inhibitor concentration.

The effect of serum proteins on different protease inhibitors can be assessed by comparing the inhibitor concentrations required to achieve 95% protease saturation, $C_{95}$. Table 2 shows the $C_{95}$ values in the absence and in the presence of 40 mg/ml HSA and 4 mg/ml AAG. In order to illustrate the effect of serum proteins, the ratio $C_{95}^{serum}$ to $C_{95}$ was calculated for each inhibitor. This analysis indicates that $C_{95}^{serum}/C_{95}$ can be as high as 11.8 indicating that in the presence of serum proteins a 10-fold increase in inhibitor concentration is required to attain the same level of inhibition achieved in the absence of serum proteins. Indinavir is less affected by serum proteins than other inhibitors in clinical use suggesting that a drug molecule can be simultaneously optimized for high affinity against a given target and low affinity towards unwanted targets. The second-

[1] The action of protease inhibitors can be assumed to take place intracellularly [17]. If a cell with a volume of $1\times10^{-12}$ l [17,18] is considered to have 200 virions [19] and each virion produces 100 protease molecules [20] the concentration of protease molecules per cell is on the order of 0.03 μM.

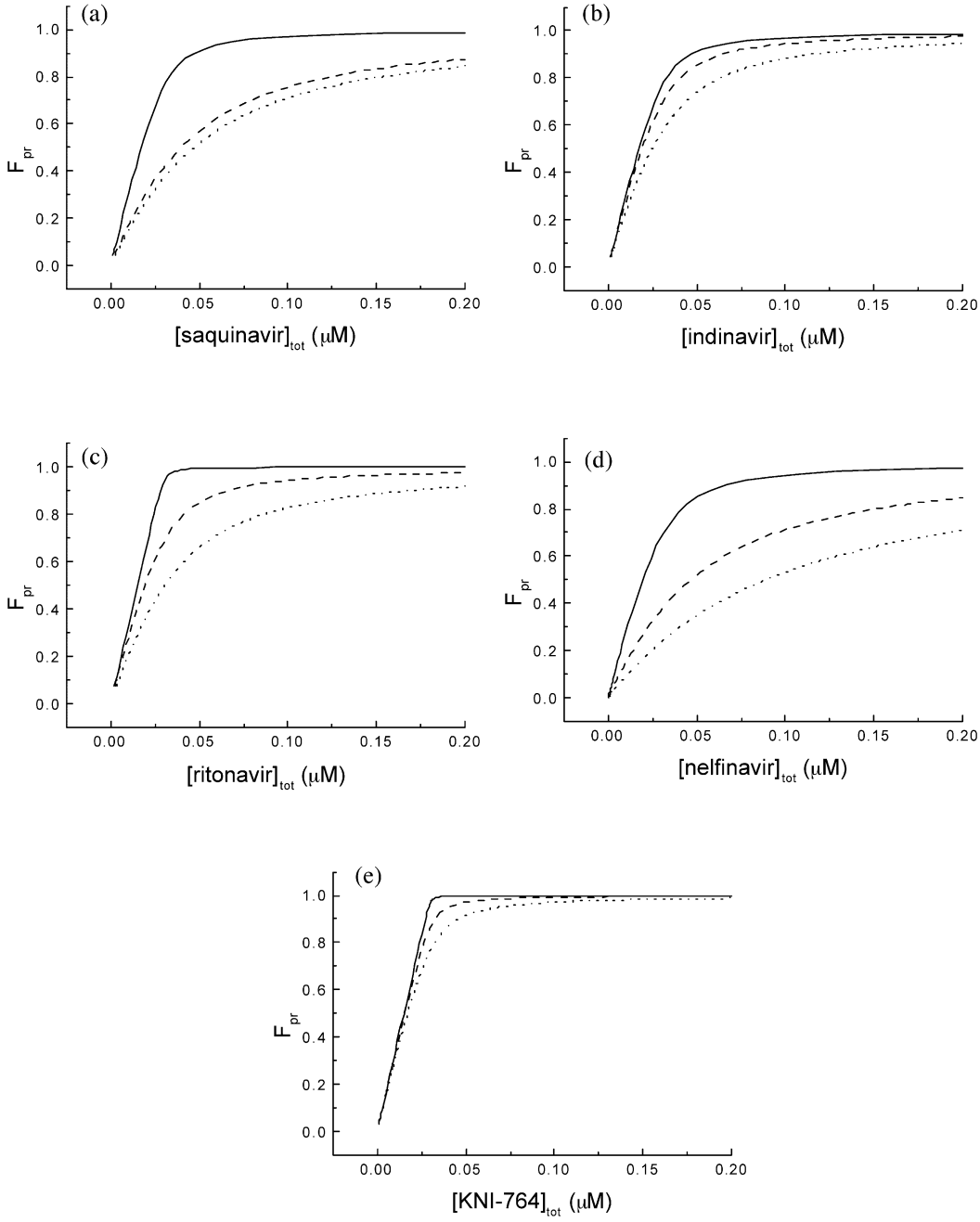


Fig. 3. The degree of protease inhibition as a function of total drug concentration for saquinavir (a), indinavir (b), ritonavir (c), nelfinavir (d) and KNI-764 (e). The solid line represents the degree of inhibition in the absence of serum proteins, the dashed line in the presence of 40 mg/ml HSA and 1 mg/ml AAG, and the dotted line in the presence of 40 mg/ml HSA and 4 mg/ml AAG. The concentration of protease was assumed to be 0.03 μM (see text).

Table 2
Effect of serum proteins on inhibitor binding to HIV-1 protease[a]

| Inhibitor | $K_{PR}$ ($M^{-1}$) | $C_{95}$ ($\mu M$) | $C_{95}^{serum}$ ($\mu M$) | $C_{95}^{serum}/C_{95}$ | $EC_{50}^{HS}/EC_{50}$ |
|---|---|---|---|---|---|
| KNI-764 | $3.1\times10^{10}$ | 0.03 | 0.07 | 2.3 | NR |
| Indinavir | $4.5\times10^{8}$ | 0.08 | 0.24 | 3.0 | 1.5 |
| Saquinavir | $4.5\times10^{8}$ | 0.08 | 0.74 | 9.2 | 16 |
| Ritonavir | $7.9\times10^{9}$ | 0.03 | 0.33 | 11.0 | 18 |
| Nelfinavir | $2.3\times10^{8}$ | 0.11 | 1.3 | 11.8 | 29 |

[a] $K_{PR}$ is the association constant of the inhibitor to HIV-1 protease. $C_{95}$ is the concentration of inhibitor required for 95% binding to protease in the absence of serum and $C_{95}^{serum}$ is the corresponding concentration of drug in presence of 40 mg/ml HSA and 4 mg/ml AAG. $EC_{50}^{HS}/EC_{50}$ is the ratio of $EC_{50}$ values in the presence and absence of 50% human serum as determined in the in the literature for MT4 cells infected with a wild type strain of HIV-1 [21]. The values for $K_{PR}$ have been published elsewhere [6,10]. NR = not reported.

generation inhibitor KNI-764 has a lower $C_{95}^{serum}/C_{95}$ value, mainly due to a higher affinity for HIV-protease ($3.1\times10^{10}$ $M^{-1}$) than a lower affinity for serum proteins. For comparison, the effect of 50% human serum on $EC_{50}$ values for first generation protease inhibitors on MT4 cells infected with wild type form of HIV-1 are also shown in Table 2 [21]. In this case $EC_{50}$ is the drug concentration corresponding to 50% reduction of the cytopathological effect of the infected cells.

### *3.4. The effect of drug resistant mutations*

One of the most important side effects associated with antiretroviral therapies is the appearance of viral strains that carry protease molecules with a lower affinity towards protease inhibitors. Because inhibition of these mutant proteases requires higher inhibitor concentrations, the effects of non-specific binding to serum proteins are more pronounced. This point can be illustrated with the double mutation V82F/I84V, which is known to affect all clinical protease inhibitors [22–24]. This mutation has a particularly strong effect on ritonavir, lowering its affinity for the protease by a factor of 370 [10]. The affinities of indinavir, nelfinavir, saquinavir and KNI-764 are reduced by a factor of 80, 18, 11 and 26, respectively [6,10]. The degree of drug-resistant protease inhibition by saquinavir, indinavir, ritonavir and nelfinavir in the absence and presence of serum proteins is shown in Fig. 4 a–d. These figures indicate a dramatic increase in the concentrations of inhibitor necessary to elicit any given degree of inhibition. Table 3 summarizes the $C_{95}^{serum}$ values for each inhibitor against the drug resistant mutant. It is evident that the drug concentrations required for 95% inhibition of the V82F/I84V protease are significantly higher than the maximum plasma concentrations reported for any of the clinical inhibitors (minimum and maximum plasma concentrations ($C_{min}$ and $C_{max}$) measured in patients [25] are also shown in Table 3). For ritonavir and nelfinavir only 75 and 80% inhibition of the V82F/I84V protease would be achieved with their maximum plasma concentrations. The corresponding values for saquinavir and indinavir are 89 and 93%, respectively. It must be noted, however, that maximal plasma concentrations only occur transiently and are sustained only for one hour or less per day during highly active antiretroviral therapy even if the drug is given three times per day as estimated from the half-life of the drugs [2]. On average, an even lower degree of inhibition is expected.

These results underscore some of the problems created by drug binding to serum proteins, especially when target mutations lead to diminished binding affinities. At inhibitor concentrations in which the wild type protease is 95% inhibited, the V82F/I84V mutant is only 67, 24, 5.5 and 51% inhibited by saquinavir, indinavir, ritonavir and nelfinavir, respectively. Under these conditions, the virus carrying the mutant protease becomes better fit to survive and multiply, leading to an increase in its population and the onset of drug resistance.

These studies demonstrate that a thermodynamic analysis of drug binding to serum proteins can be

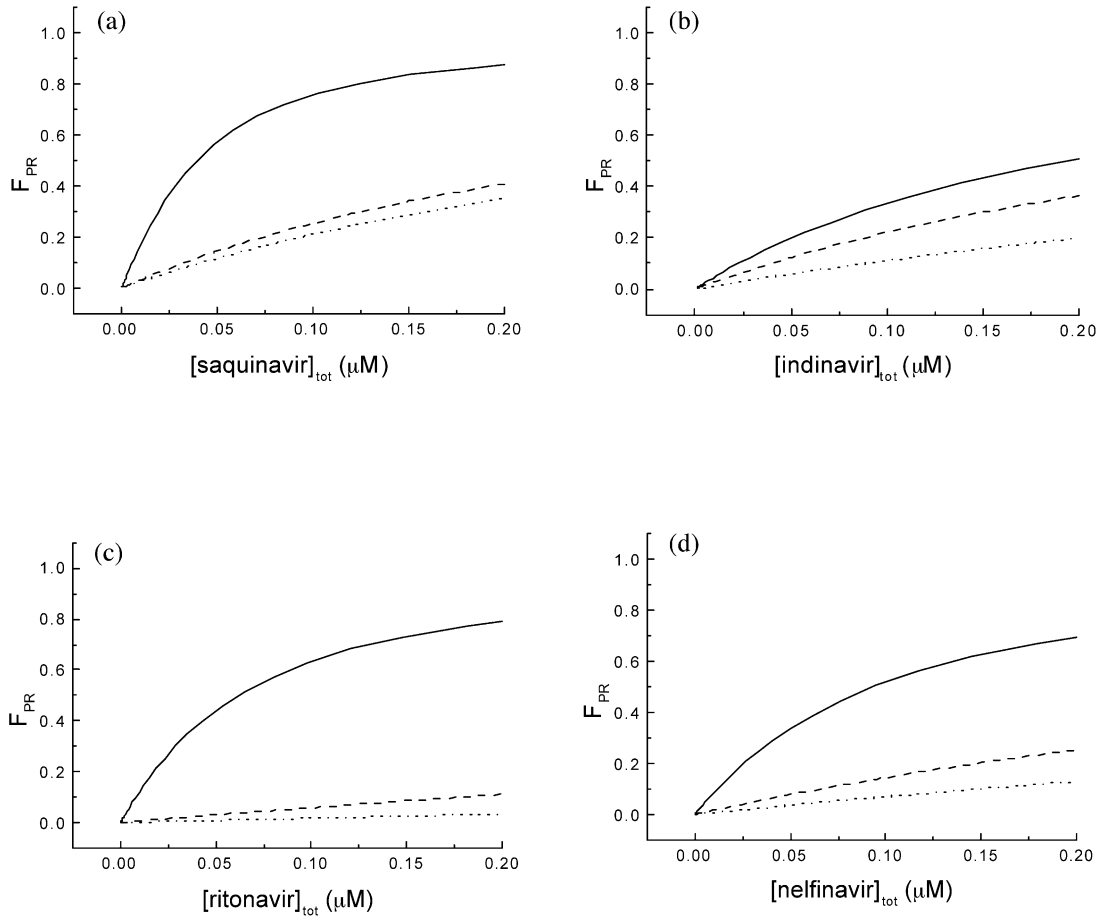


Fig. 4. The degree of inhibition of the drug resistant mutant protease V82F/I84V as a function of the total concentration of saquinavir (a), indinavir (b), ritonavir (c) and nelfinavir (d). The solid line represents the degree of inhibition in the absence of serum proteins, the dashed line in the presence of 40 mg/ml HSA and 1 mg/ml AAG, and the dotted line in the presence of 40 mg/ml HSA and 4 mg/ml AAG. The concentration of protease was assumed to be 0.03 μM (see text).

Table 3
Effect of serum proteins on inhibitor binding to drug resistant mutant HIV-1 protease V82F/I84V[a]

| Inhibitor | $K_{PR,\ V82F/I84V}$ ($M^{-1}$) | $C_{95}$ (μM) | $C_{95}^{serum}$ (μM) | $C_{min}$ (μM) | $C_{max}$ (μM) |
|---|---|---|---|---|---|
| Indinavir | $5.6\times10^6$ | 3.2 | 12.7 | 0.2 | 10.8 |
| Saquinavir | $4.1\times10^7$ | 0.53 | 7.0 | 0.3 | 2.7 |
| Ritonavir | $2.1\times10^7$ | 1.0 | 55.4 | 4.2 | 15.5 |
| Nelfinavir | $1.3\times10^7$ | 1.6 | 16 | 2.2 | 5.3 |

[a] $K_{PR,\ V82F/I84V}$ is the association constant of the inhibitor to HIV-1 protease V82F/I84V. $C_{95}$ is the concentration of drug required for 95% saturation of protease from the resistant mutant V82F/I84V. The minimum and maximum in vivo plasma concentrations ($C_{min}$ and $C_{max}$) were taken from the literature [25]. The values for $K_{PR,\ V82F/I84V}$ have been published elsewhere [10].

used to obtain advanced indication of the effective drug concentration that will be available for the intended target. This information, combined with the additional thermodynamic parameters ($\Delta H$, $\Delta S$, $\Delta C_p$) provided by microcalorimetry and their correlation to structural parameters [26,27], should prove to be important for the development of new optimization algorithms that maximize binding affinity against the selected target while simultaneously minimize binding to unwanted molecules.

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