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Quantification of interactions between drug leads and serum proteins by use of "binding efficiency"

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ABSTRACT

To develop efficient and reliable methods for prediction of serum protein binding of drug leads, the kinetic characteristics for the interactions between selected compounds and human serum albumin and α_1-acid glycoprotein have been explored using a surface plasmon resonance biosensor. Conventional methods for quantification of interactions (i.e., using rate constants or affinities determined on the basis of a reasonable mechanistic model) were applicable for only a few of the compounds. The affinity of a primary interaction and the contribution of lower affinity secondary interactions could be estimated for some compounds, but the affinity of many compounds could not be quantified by either of these methods. To have a quantification method that could be used for all compounds, independent of affinity and complexity of interaction mechanisms, the concept of "binding efficiency," analogous to "catalytic efficiency" used for enzymes, was developed. It allowed the quantification of the binding of compounds interacting with weak affinity and for which saturation is not reached within a concentration range where the compound is soluble or when the influence of interactions with secondary sites makes interpretations difficult. In addition, compounds with large fractional binding can be identified by this strategy and simply quantified relative to reference compounds. This approach will enable ranking and identification of structure–activity relationships of compounds with respect to their serum protein binding profile.

Drug leads often fail during the later stages of the preclinical drug discovery phase as a result of extensive binding to serum proteins [1,2]. Two of the most abundant proteins in blood plasma (serum) are human serum albumin (HSA)[1] and α_1-acid glycoprotein (AGP) [3,4]. The concentrations are estimated at 35–50 g/L and 0.4–1.0 g/L for HSA and AGP, respectively. Both proteins are known to bind a variety of drugs and other compounds in the circulation. The binding is complex due to multiple binding sites as well as ligand-induced conformational changes [4]. The multiple binding sites include well-defined sites with a certain degree of specificity for ligands (e.g., acidic or basic features) as well as hydrophobic surfaces and cavities with few other determinants for specificity.

Because serum proteins are present at relatively high concentrations in blood plasma, they can efficiently transport drugs throughout the body and also serve as a drug reservoir. However, the kinetic profile of the drug–ligand interaction is critical for drug efficacy because efficient transport requires rapid association rates as well as rapid dissociation rates (resulting in moderate affinity). If the association rates are slow, the drug will not be bound unless very high concentrations are used, whereas if the dissociation rates are slow, the drug will be sequestered. In both cases, high compound concentrations will be required for efficacy, increasing the risk of side effects. The fine balance between optimal transport and sequestration (i.e., facilitating but not withholding the substance from interaction with its target) is a critical factor for drug action.

The characterization of interactions between potential drug leads and serum proteins is very important as part of their pharmacokinetic profiling. However, the early evaluation of serum protein binding is hampered by a lack of efficient and informative experimental methods. A variety of analytical methods are employed for studies of drug–serum protein interactions, including equilibrium dialysis, ultrafiltration, ultracentrifugation, optical biosensors, and micropartitioning, as reviewed recently [5,6]. In addition, various forms of capillary electrophoresis [7,8] and chromatography [9], as well as circular dichroism [10], are employed. These methods differ in throughput, cost, and the quality of information. So, the problem is not the lack of methods as such but rather that essentially all of these methods are equilibrium based and, therefore, provide no kinetic information. The possibility of obtaining relevant structure–activity relationship (SAR) data for protein

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[1] *Abbreviations used:* HSA, human serum albumin; AGP, α_1-acid glycoprotein; SAR, structure–activity relationship; SPR, surface plasmon resonance; HIV-1, human immunodeficiency virus type 1; PBS, phosphate-buffered saline; EDC, *N*-ethyl-*N*′-(3-dimethylaminopropyl)-carbodiimide; MES, [2-(4-morpholino)-ethanesulfonic acid]; NHS, *N*-hydroxysuccinimide; RU, resonance units; PDEA, 2-(2-pyridinyldithio)ethane amine; RI, refractive index; *BE*, binding efficiency; LC-MS/MS, liquid chromatography-tandem mass spectrometry.

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binding for the optimization of compounds, therefore, is limited even when a large number of compounds are evaluated. Moreover, the studies are typically performed at a stage where there are few degrees of freedom available for structural modification and the predictive power of available methods is poor and not well understood. These factors contribute to the difficulties in identifying compounds with suitable serum protein binding characteristics during the early stages of a drug discovery program.

In a previous study, we used a surface plasmon resonance (SPR)-based biosensor to characterize the interactions between a set of taxol analogues and HSA and AGP. It resolved some of the complexities of serum protein interactions with drugs and revealed that the kinetics of ligand interactions may be more characteristic for serum protein binding sites than the affinities [11]. In the current study, the goal was to develop new strategies for efficient characterization of serum protein binding of drug leads. Here data were also obtained using a cell-based system and ultrafiltration. The compounds were inhibitors of cathepsin K, human immunodeficiency virus type 1 (HIV-1) protease, or HIV-1 reverse transcriptase (see Fig. 1a–c in Supplementary material). Compounds with known serum protein binding were used as references (see Fig. 1d in Supplementary material). To overcome some of the inherent challenges of characterizing interactions between drugs and serum proteins, a novel strategy for quantifying interactions with rapid kinetics and weak affinities was developed. It was used as a basis for defining the characteristics of the interactions, allowing both qualitative SAR analysis and quantitative ranking as well as investigation of correlations with serum effects on drug efficacy.

Materials and methods

Materials

Cathepsin K inhibitors (1 and 2), HIV-1 protease inhibitors (atazanavir, 3–13), and HIV-1 reverse transcriptase inhibitors (MIV-160 and MIV-170) [12] were synthesized in-house. Dapivirine, efavirenz, nevirapine, delavirdine, iopanoic acid, glyburide, diclofenac, warfarin, digitoxin, dipyridamole, naproxen, propranolol, alprenolol, pindolol, and quinine were purchased from Sigma (Stockholm, Sweden). HSA (essentially fatty acid and globulin free), AGP (99% purity), and human serum H4522 were purchased from Sigma. RPMI 1640, fetal calf serum, penicillin, and streptomycin were purchased from Thermo Fisher Scientific (Waltham, MA, USA). MT4 cells were a kind gift from N. Yamamoto (Yamaguchi University).

Biosensor analysis

All measurements were performed with a Biacore S51 instrument (GE Healthcare, Uppsala, Sweden). An online degassing system (Degasys Ultimate DU4010, Uniflows, Tokyo, Japan) was coupled to the instrument. Experiments were performed at room temperature with a flow rate of 30 μl/min.

Preparation of plasma protein sensor surfaces and samples

HSA and AGP were immobilized in two detection areas on the same S CM5 sensor chip (GE Healthcare), allowing experiments to be performed simultaneously with both proteins. A third detection area, with the unmodified dextran matrix, was used as a reference surface. Phosphate-buffered saline (PBS, pH 7.4, Sigma) was used as running buffer in the immobilization.

Human serum albumin

HSA was immobilized by amine coupling at 25 °C according to standard Biacore procedures and using standard reagents (GE Healthcare). The dextran polymer was activated with 0.2 M *N*-ethyl-*N*′-(3-dimethylaminopropyl)-carbodiimide (EDC) and 50 mM *N*-hydroxysuccinimide (NHS) for 10 min. A stock solution of 1 mg HSA/ml in PBS (pH 7.4) was diluted to a concentration of 25 μg/ml in 10 mM sodium acetate (pH 5.2) and injected for 7 min. An injection of 1 M ethanolamine (pH 8.5) for 7 min blocked remaining unreacted groups on the surface. Final immobilization levels were between 4000 and 7000 resonance units (RU).

α1-Acid glycoprotein

AGP was immobilized by surface thiol coupling at 25 °C according to standard Biacore procedures and using standard reagents (GE Healthcare). To introduce active disulfide groups on the protein surface before immobilization, 2 mg of AGP was mixed with 6 mg of 2-(2-pyridinyldithio)ethane amine (PDEA) and dissolved in 1 ml of 0.1 M MES buffer [2-(4-morpholino)-ethanesulfonic acid, pH 5.0]. After the addition of 50 μl EDC, the solution was incubated for 10 min at room temperature. Separation of the modified protein from unreacted reagents was done with a HiTrap desalting column (GE Healthcare), and elution was performed with 2 ml of 10 mM citrate buffer (pH 3.6). The reaction was assumed to be quantitative, resulting in a final AGP–PDEA concentration of 1 mg/ml. Aliquots of the modified protein were stored at −20 °C and diluted to 300 μg/ml in 10 mM citrate buffer (pH 3.6) prior to immobilization.

The dextran polymer was activated with 0.2 M EDC and 50 mM NHS for 2 min. Thiol groups were introduced on the sensor matrix through an injection of 40 mM cystamine dihydrochloride in 0.1 M sodium borate (pH 8.5) for 3 min. The protein was thereafter injected for 26 min, followed by a 4-min injection of 20 mM PDEA and 1 M sodium chloride in 0.1 M sodium acetate (pH 4.3) to deactivate unreacted groups. Final immobilization levels were between 8000 and 10,000 RU.

Sample preparation

Serial dilutions of compounds were prepared from stock solutions of 5 or 10 mM in 100% Me_2SO and from intermediate solutions of 0.6 mM in 100% Me_2SO. Final compound concentrations of 0–30 μM in PBS (with a constant concentration of 5% Me_2SO) were otherwise identical to the running buffer. Still, solvent correction was performed to compensate for inadequate matching of samples and the running buffer.

Data analysis

Data correction and normalization

The initial processing of data was performed with the evaluation software version 1.2 for Biacore S51 (GE Healthcare). Samples containing buffer alone were injected between the compound injections, allowing correction for unspecific signals from protein surfaces (“blank subtraction”). In addition, correction for nonspecific signals arising from the sensor matrix was performed by subtracting signals from the reference spot on the chip (“reference subtraction”). Finally, corrections were also done to reduce errors due to small differences in the concentration of Me_2SO between the running buffer and the sample solution (“solvent correction”). It involved a refractive index (RI) correction procedure based on a calibration curve obtained by the injection of eight Me_2SO concentrations ranging from 4.5% to 5.8%, typically suitable for signal corrections when using 5% Me_2SO in running buffer and sample solutions.

All data was normalized with respect to compound molecular weight ($MW_{analyte}$), protein molecular weight ($MW_{protein}$), and protein immobilization level ($R_{protein}$):

$$F_{NORM} = \frac{MW_{protein}}{MW_{analyte} \cdot R_{protein}}. \quad (1)$$

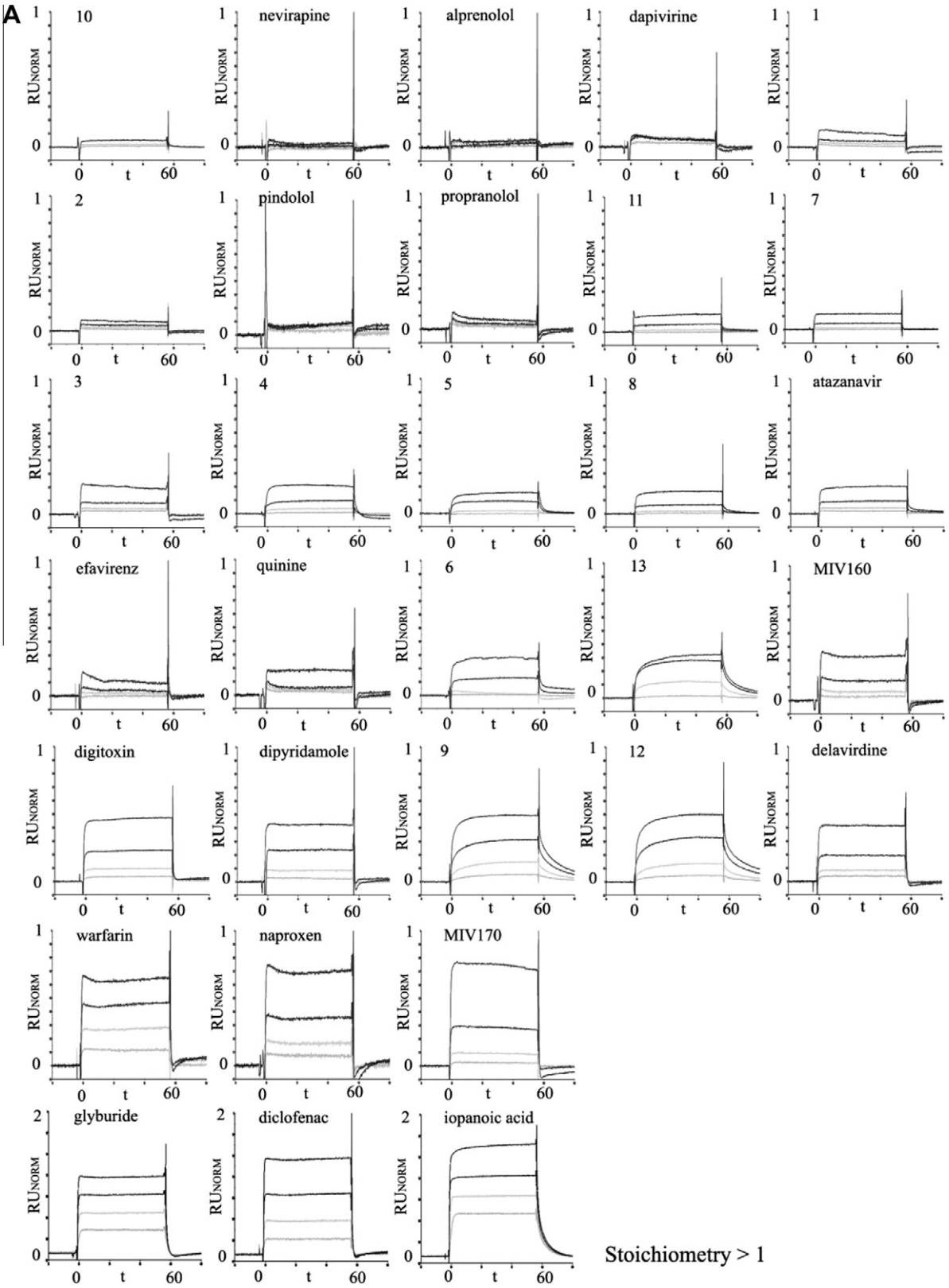

Fig.1. Interactions between inhibitors of cathepsin K, HIV-1 protease, HIV-1 reverse transcriptase, and reference compounds and HSA (A) and AGP (B). The compounds were injected in concentrations from 0 to 30 μM using threefold dilutions (30, 10, 3.3, and 1.1 μM). The injection time (t) was 60 s.

The normalization factor, F_{NORM}, enables a better comparison between compounds of different molecular weights in experiments performed with different immobilization levels of the proteins. Normalized response signals are defined here as RU_{NORM}.

Determination of kinetic parameters (K_D, k_{on}, and k_{off})

Affinities (K_D) and association and dissociation rate constants (k_{on} and k_{off}, respectively) were estimated by globally fitting an equation representing a 1:1 interaction model (Eq. (2)) to normal-

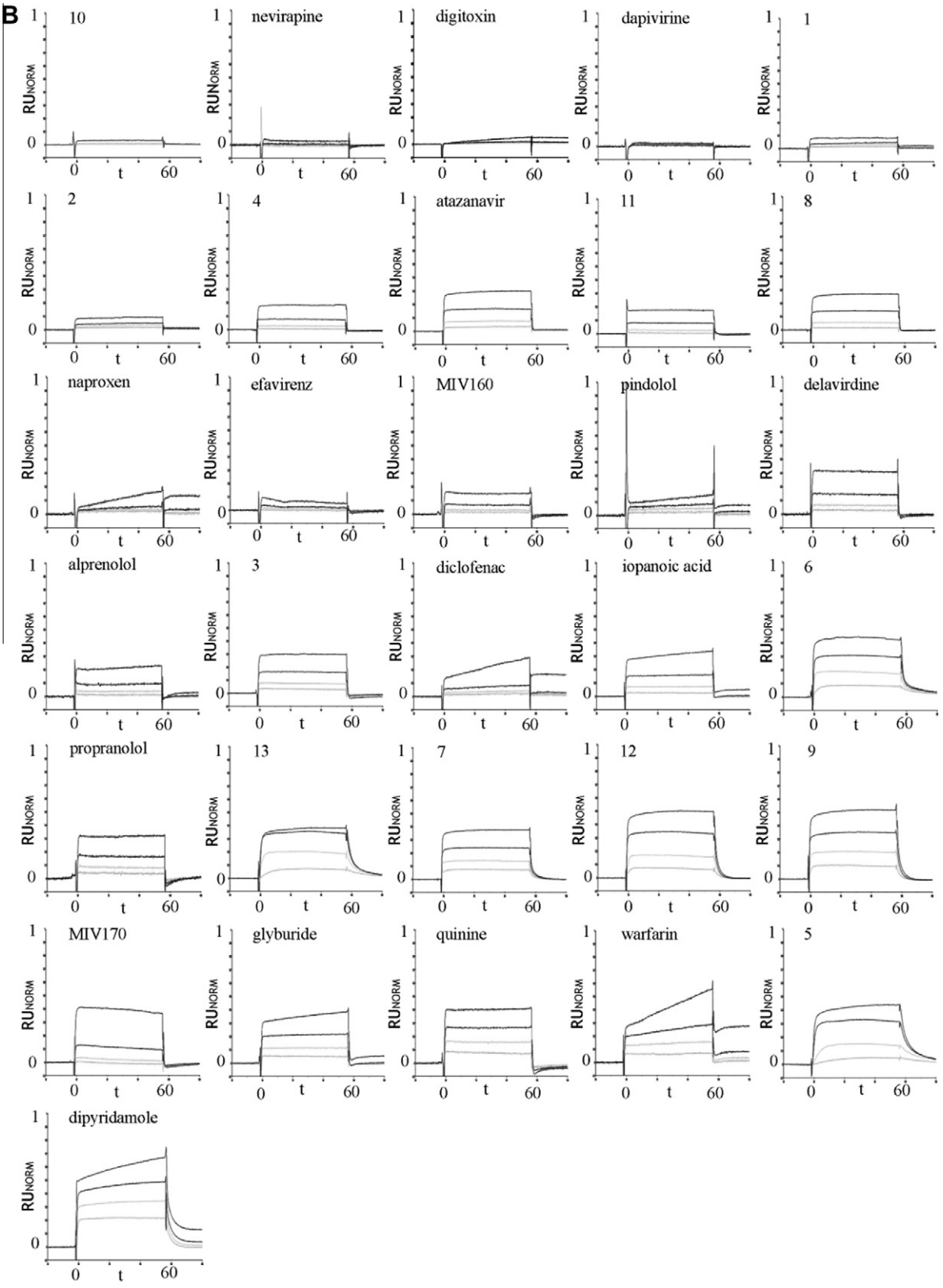

Fig. 1 (*continued*)

ized signal response values (R) for the complete series of compound concentrations (C). These analyses were performed with BIAevaluation 4.0.1 (GE Healthcare):

$$R(t) = R_{\max} \cdot \frac{C \cdot k_{on}}{C \cdot k_{on} + k_{off}} \left(1 - e^{-t(C \cdot k_{on} + k_{off})}\right) \quad (2)$$

Quantification of affinity from equilibrium signals

GraFit 5 (Erithacus Software, Surrey, UK) was used for the extended data analysis involving nonlinear regression analysis of the complete dataset. Apparent affinities (K_D^*) were calculated from the amount of complex (R_{eq}) at different compound concentrations (C), estimated from normalized equilibrium signals extracted 50 s after injection, and by using a 1:1 interaction model described by Eq. (3):

$$R_{eq} = \frac{R_{max} \cdot C}{C + K_D^*}. \quad (3)$$

The same data were also analyzed with a 2:1 interaction model, described by Eq. (4), and used to calculate the two affinities of the primary and secondary interactions (K_{D1} and K_{D2}):

$$R_{eq} = \frac{R_{max1} \cdot C}{C + K_{D1}} + \frac{R_{max2} \cdot C}{C + K_{D2}}. \quad (4)$$

Similarly, the data were also analyzed with a model representing interactions with a primary binding site and secondary interactions with affinities much weaker than can be detected within the range of the experiment. This was performed with Eq. (5), developed from Eq. (4) by assuming that $K_{D2} \gg C$:

$$R_{eq} = \frac{R_{max,prim} \cdot C}{C + K_{D,prim}} + k \cdot C. \quad (5)$$

In this case, the affinity and the maximal response signal for the primary protein interaction were determined as $K_{D,prim}$ and $R_{max,prim}$, respectively, and the amount of secondary binding was estimated from the slope of the interaction at high C where $k = R_{max2}/K_{D2}$.

Definition of binding efficiency

The concept of binding efficiency (*BE*) was developed from Eq. (3). By reorganizing the equation to

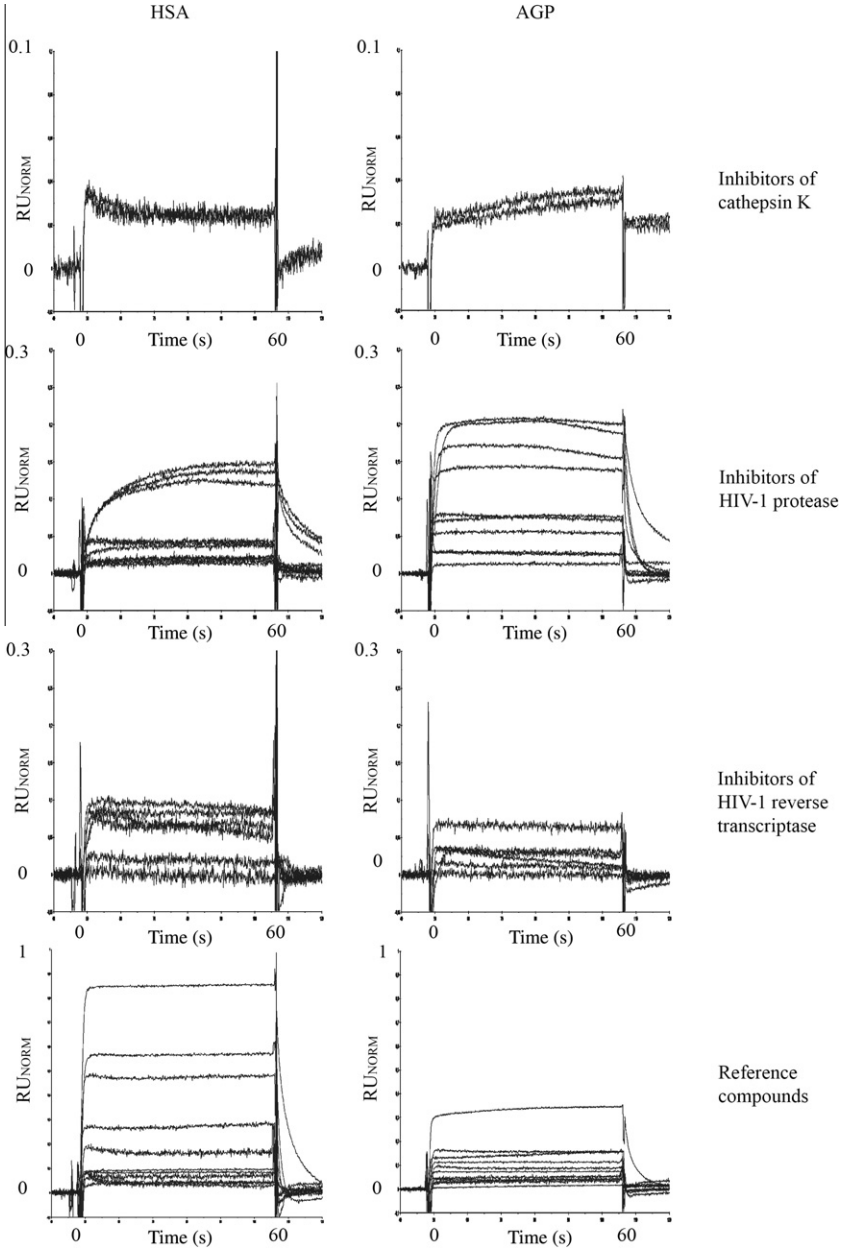

Fig.2. Overlay of single-concentration sensorgrams for inhibitors of cathepsin K, HIV-1 protease, HIV-1 reverse transcriptase, and reference compounds interacting with HSA and AGP. All compounds (Table 1) were injected at 3.3 μM.

$$R_{eq} = \frac{\frac{R_{max}}{K_D} \cdot C}{\frac{C}{K_D} + 1}, \quad \text{or } R_{eq} = \frac{BE \cdot C}{\frac{C}{K_D} + 1}, \tag{6}$$

where *BE* is defined as

$$BE = \frac{R_{max}}{K_D}. \tag{7}$$

By rearranging Eq. (3) into Eq. (6), BE can be obtained directly as a parameter by nonlinear regression analysis of the complete data set using Eq. (6) rather than by calculating BE from two separately determined parameters. In the procedure used here, K_D was also set to be a free variable and the starting value was taken from the K_D value obtained from the regression analysis using Eq. (3). The value of BE represents the slope of the initial linear part of the saturation curve described by Eq. (3) because this equation is reduced to a linear equation at low concentrations ($C \ll K_D$):

$$R_{eq} = \frac{R_{max}}{K_D} \cdot C. \tag{8}$$

Fig. 5 illustrates how BE relates to the complete saturation curve.

Serum binding data

Effect of serum on antiviral efficacy. The inhibitory effect of compounds against HIV-1 was based on inhibition of virus-induced cytopathogenicity in acutely infected MT4 cells, as described previously [12,13]. Briefly, MT4 cells were seeded in 96-well assay plates at a density of 2×10^4 cells/well in RPMI 1640 medium supplemented with 10% fetal calf serum, penicillin (50 U/ml), and streptomycin (0.05 mg/ml). Compounds, dissolved in Me_2SO (100%) were fivefold serially diluted in cell culture media and added to the assay plates. Finally, 20–50 $TCID_{50}$ (50% Tissue Culture Infective Dose) HIV-1 IIIb was added to each well except for the cell control wells. After 4 days of incubation, cell viability was determined by the XTT method [14]. The reduction in cytopathogenicity (% inhibition) was calculated from

$$\%\ \text{inhibition} = \left(\frac{OD_{\text{compound}} - OD_{\text{infected cells}}}{OD_{\text{cell control}} - OD_{\text{infected cells}}}\right) \cdot 100. \tag{9}$$

EC_{50} values were determined from plots of percentage inhibition versus compound concentration.

To analyze the effect of human serum on the antiviral activities of the test compounds, assays were performed in 50% human serum added to RPMI 1640 medium containing 10% fetal calf serum, penicillin (50 U/ml), and streptomycin (0.05 mg/ml). Compounds dissolved in Me_2SO (100%) were serially diluted in human serum and added to 96-well assay plates (100 μl/well). Next, 50 μl of cell suspension containing four times the final concentration of cells and 50 μl of virus suspension containing four times the final concentration of virus were added, and the plates were incubated for 4 days and analyzed with the XTT assay as described above. Quantification of the human serum (HS) effect was done by using the concept of fold change, which is defined by

$$\text{fold change} = \frac{EC_{50} + 50\%\ HS}{EC_{50}}. \tag{10}$$

Ultrafiltration. Ultrafiltration was carried out using microcentrifuge Eppendorf tubes (Millipore, Billerica, MA, USA) with Amicon Ultrafree-MC 30-kDa cutoff filter units inserted. All compounds were diluted in acetonitrile to 200 μM (from 10-mM stock solutions in 100% Me_2SO). Next, 1 ml of human serum (diluted 1:4) was mixed with compound to a final concentration of 1 μM, and 200 μl of this mixture was pipetted onto the ultrafilter. The lid was removed from tubes, and the filter was incubated for 1 h in 37 °C with 9.5% CO_2. The sample was centrifuged for 25 min (5000*g*, 37 °C), and 50 μl of the supernatant was analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS).

Correlation analysis. Correlation analysis among biosensor interaction parameters, cell culture, and ultrafiltration data was performed using Statistica 6.0 (StatSoft Scandinavia, Uppsala, Sweden). The significance level was set to $P < 0.05$.

Results

Biosensor analysis

The interactions between a selection of inhibitors of cathepsin K, HIV-1 protease, and HIV-1 reverse transcriptase (see Fig. 1a–c in Supplementary material) and immobilized HSA and AGP were studied. Compounds with known binding to serum, HSA, and/or AGP (see Fig. 1d in Supplementary material) were used as references. Measurements were performed with a range of compound concentrations up to 30 μM (in PBS with 5% Me_2SO), above which limitations in solubility for many compounds were expected to interfere with the interpretation of the data. The sensorgrams for the interactions with HSA and AGP are shown in Fig. 1A and B, respectively. They are arranged in order of increasing affinity for each protein. The data show that all tested compounds bound reversibly to both proteins and that the kinetics were very rapid. Regeneration procedures for the sensor surface, therefore, were not required. The interaction characteristics were rather similar for the compounds within each class, as illustrated by the overlaid sensorgrams for 3.3 μM of each compound (Fig. 2). (Note the different scales on the *y* axis.)

Some of the compounds had a slower dissociation than the others, as easily seen in Fig. 2. These can be identified in Fig. 1 as the HIV-1 protease inhibitors 9, 12, 13, and iopanoic acid for HSA and the HIV-1 protease inhibitors 5, 6, 7, 9, 12, 13, and dipyridamole for AGP.

A qualitative analysis of the complete set of sensorgrams (Fig. 1A) identifies MIV-170 as the compound with the highest steady-state level binding for HSA of the tested compounds, being of the same order as the positive reference compounds. MIV-170 and several other compounds had binding levels of the same order as the positive reference compounds for AGP. Dapivirine showed very weak interactions with both HSA and AGP. However, it has very poor solubility, and so the data may be misleading. Interactions between HSA and certain compounds (glyburide, diclofenac, and iopanoic acid) reached a steady-state signal higher than the theoretical maximum of 1 (Fig. 1A), indicating secondary interactions or conformational changes. Compound 10 was not soluble at the highest concentrations, and so the sensorgrams above 3.3 μM are not shown.

Although the sensorgrams revealed that the kinetics for the compounds differed significantly enough to give rise to these differences in the steady-state levels, they were difficult to quantify. This was due not only to the rapid kinetics but also to the complexity of the interaction. A number of different approaches for quantification were therefore explored.

Determination of kinetic parameters

First, the kinetic parameters were determined by simultaneous global regression analysis of the association and dissociation phases for a complete concentration series using a suitable mechanistic model (Eq. (2)) (results not shown). However, for many of the compounds, the rate constants could not be reliably estimated because the values were faster than the acceptable range for quantification ($10^4\ M^{-1}\ s^{-1} < k_{on} < 10^7\ M^{-1}\ s^{-1}$, $10^{-4}\ s^{-1} < k_{off} < 10^{-1}\ s^{-1}$)

[15]. The problem was more frequent for values of k_{on} than for values of k_{off}. Only few of the compounds interacted slowly enough and with sufficiently high affinity for the rate constants to be estimated by this method (Table 1).

Table 1
Quantitative parameters for compounds interacting with HSA and AGP.

Compound	BE[a] (RU_{NORM}·mM^{-1})	K_D^*[b] (μM)	$K_{D,prim}$[c] (μM)	$R_{max,prim}$[d] (RU_{NORM})	k[e]	k_{off}[f] (s^{-1})
(A) HSA						
1	7 ± 0.5	23	7.4	0.05	0.002	nd
2	12 ± 4	10	2.4	0.03	0.001	nd
Atazanavir	13 ± 4	4	4.1	0.06	0.005	0.01
3	16 ± 1	36	0.7	0.03	0.005	nd
4	11 ± 1	41	32	0.35	0.001	0.30
5	9 ± 2	30	31	0.35	0.003	0.15
6	12 ± 1	430	nd	nd	nd	0.02
7	3 ± 0.1	98	56	0.21	0.002	0.49
8	6 ± 0.3	110	57	0.26	0.003	0.04
9	36 ± 3	120	13	0.73	0.009	0.08
10	6 ± 1	22	20	0.13	0.001	0.63
11	5 ± 0.03	69	64	0.39	0.0003	0.02
12	39 ± 8	13	nd	nd	nd	0.08
13	30 ± 2	7.5	74	nd	nd	0.12
MIV-160	22 ± 6	38	3.4	0.08	0.009	nd
MIV-170	26 ± 0.4	160	30	0.31	0.019	nd
Dapivirine	20 ± 0.8	4.6	nd	nd	nd	nd
Efavirenz	4 ± 0.1	120	20	0.03	0.002	nd
Nevirapine	2 ± 0.2	nd	nd	nd	0.001	nd
Delavirdine	25 ± 5	34	5.3	0.15	0.010	nd
Iopanoic acid	320 ± 25	2.6	0.8	1.00	0.020	0.40
Glyburide	173 ± 18	3.4	2.1	0.90	0.008	nd
Diclofenac	140 ± 17	6.5	4.6	1.03	0.016	0.85
Warfarin	100 ± 20	6.2	4.8	0.64	0.003	nd
Digitoxin	25 ± 3	31	15	0.41	0.007	0.70
Dipyridamole	29 ± 7	23	29	1.00	0.003	nd
Naproxen	36 ± 1	24	6.1	0.36	0.014	nd
Propranolol	16 ± 2	6.2	7.4	0.05	0.002	nd
Alprenolol	9 ± 1	1.1	nd	nd	nd	nd
Pindolol	28 ± 6	1	2.2	0.11	0.002	nd
Quinine	15 ± 2	150	nd	nd	0.006	nd
(B) AGP						
1	10 ± 0.2	9.3	1.4	0.04	0.002	0.51
2	11 ± 0.8	11	1.1	0.03	0.002	0.11
Atazanavir	18 ± 8	18	8	0.23	0.004	nd
3	17 ± 2	20	10	0.25	0.004	nd
4	8 ± 3	67	48	0.35	0.002	nd
5	37 ± 9	9.2	nd	nd	nd	0.14
6	35 ± 7	6.8	4.4	0.38	0.003	0.38
7	37 ± 10	8.4	3.2	0.25	0.005	0.9
8	11 ± 4	27	43	0.98	0.007	nd
9	32 ± 11	7.4	4.2	0.43	0.005	0.05
10	3 ± 1	74	48	0.13	0.001	0.38
11	6 ± 2	56	56	0.50	0.005	nd
12	35 ± 6	10	13	0.85	0.008	0.68
13	45 ± 12	4.5	10	0.89	0.002	0.61
MIV-160	9 ± 2	39	3.7	0.04	0.004	nd
MIV-170	10 ± 4	nd	1026	0.09	0.012	nd
Dapivirine	8 ± 0.8	2.2	nd	nd	nd	nd
Efavirenz	2 ± 0.1	230	68	0.05	0.001	nd
Nevirapine	3 ± 2	nd	nd	nd	nd	nd
Delavirdine	7 ± 0.6	35	6	0.11	0.007	nd
Iopanoic acid	20 ± 3	29	6.9	0.16	0.007	nd
Glyburide	31 ± 6	14	4.9	0.24	0.006	nd
Diclofenac	14 ± 1	nd	35	0.01	0.009	nd
Warfarin	51 ± 3	17	3.1	0.23	0.011	nd
Digitoxin	4 ± 1	8	0.3	0.01	0.001	nd
Dipyridamole	100 ± 17	3.2	1	0.47	0.007	0.50
Naproxen	12 ± 0.5	68	nd	nd	nd	nd
Propranolol	29 ± 5	22	4.5	0.14	0.007	nd
Alprenolol	13 ± 2	59	7.7	0.07	0.006	nd
Pindolol	17 ± 0.05	10	2	0.07	0.003	nd
Quinine	35 ± 7	8	3.9	0.31	0.004	nd

Note: All values are mean values. Some values could not be determined for various reasons. nd, not determined.
[a] Binding efficiency, determined by nonlinear regression analysis using the complete dataset and Eq. (6). Standard deviations are based on triplicate measurements.
[b] Apparent affinities, determined from Eq. (3) by using steady-state values for a series of inhibitor concentrations (0–30 μM).
[c] Affinity for the primary interaction, determined from Eq. (5).
[d] Maximal response for the primary interaction, determined from Eq. (5).
[e] Secondary interaction(s), determined from Eq. (5).
[f] Dissociation rate constant, determined from Eq. (2).

Determination of affinities

Due to the difficulties in the determination of kinetic rate constants, the concentration dependence of steady-state values (R_{eq}) was used to estimate apparent affinities (K_D^* values). Only few of the compounds showed saturation within the concentration range that could be reliably used (Fig. 3), indicating that the affinities for HSA and AGP were generally low. Because a 1:1 interaction model

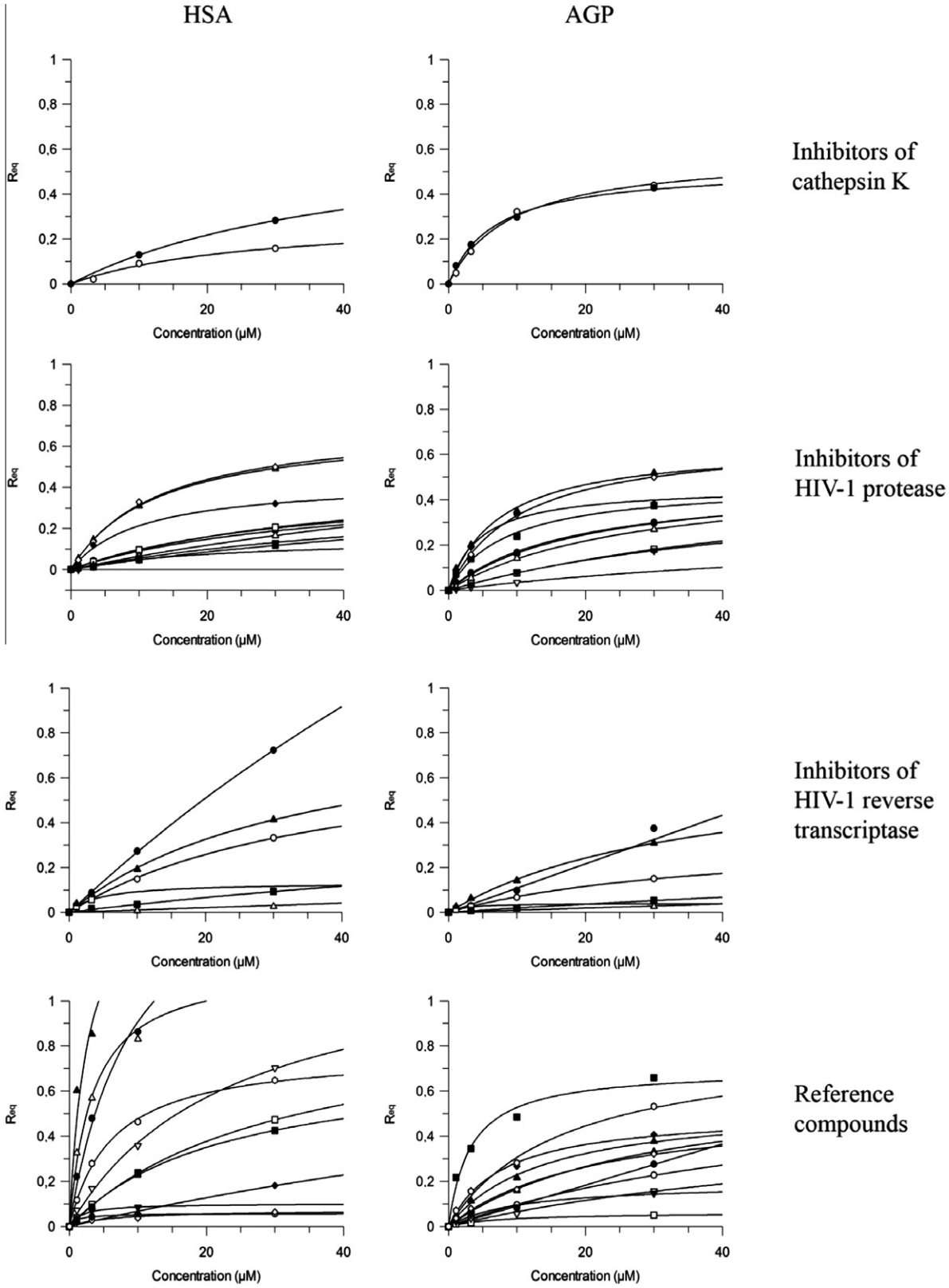

Fig.3. Concentration dependence of steady-state values (R_{eq}) for the binding of the compounds shown in Fig. 1 to HSA and AGP. Solid lines represent theoretical curves corresponding to 1:1 interactions obtained from nonlinear regression analysis of steady-state values (after a 50-s injection) at concentrations up to 30 μM. Signals were normalized.

could not generally be used to describe the data, the lack of discernible saturation was not simply a matter of low affinity but most likely also a consequence of the involvement of multiple binding sites. These may be well-defined secondary binding sites or hydrophobic cavities or surfaces that bind compounds in a less specific manner. Three of the compounds (glyburide, diclofenac, and iopanoic acid) had a stoichiometry higher than 1 for HSA (also evident from Fig. 1A), suggesting that they bind to more than one site.

To determine the possible contribution of secondary interactions, also for compounds not exhibiting a stoichiometry higher than 1, equations describing two alternative models were fitted to the data. The principle for the fitting of the equations is shown for the interaction between iopanoic acid and HSA in Fig. 4. The standard equation, describing a simple 1:1 interaction model (Eq. (3)), gave an estimate of the overall apparent affinities (K_D^*) for the serum protein interactions (Table 1). Poor fits and large differences in the values determined in two separate experiments illustrated the weakness in the procedure and the low reliability of the values (especially for high K_D values). The 2:1 interaction model (Eq. (4)), assuming two independent interactions with affinities of a magnitude equal to or below the highest concentration used in the experiments, was not better (data not shown). However, the equation describing a 1:1 interaction model in combination with a linear term for weaker binding with one or several secondary sites (Eq. (5)) was most suitable for describing the serum protein interactions. It enabled the separation of the primary interaction from weaker secondary interactions and the determination of the affinity ($K_{D,prim}$) and maximal response ($R_{max,prim}$) for the primary interaction (Table 1). The *y* axis intercept of the extrapolated line describing the unspecific interaction could be used to visually estimate $R_{max,prim}$ for the primary serum protein interaction, as illustrated in Fig. 4C. Interestingly, the slope of the binding curve at high concentrations was parallel for many of the compounds (see Fig. 3 and for parameter *k* in Table 1, e.g., HIV protease inhibitors and AGP), indicating that the amounts of secondary interactions were similar. This was seen for both HSA and AGP. For some compounds, the "saturation curve" was linear in the whole concentration range, showing that the concentration range was not enough for the quantification of even a primary interaction. For these compounds, $K_{D,prim}$, $R_{max,prim}$, and *k* could not be determined (shown as "nd" in Table 1).

Binding efficiency analysis

To overcome the problems of quantifying interactions that do not become saturated within the range of available concentrations (i.e., with low affinities), the concept of binding efficiency was introduced (Eq. (7)). The new parameter was defined as the slope of the saturation curve at low compound concentrations, as illustrated in Fig. 5. Thus, it represents how much compound will be bound as a function of concentration without any assumptions of the mechanism of binding, number of binding sites, and so forth. *BE* values could be determined by nonlinear regression analysis

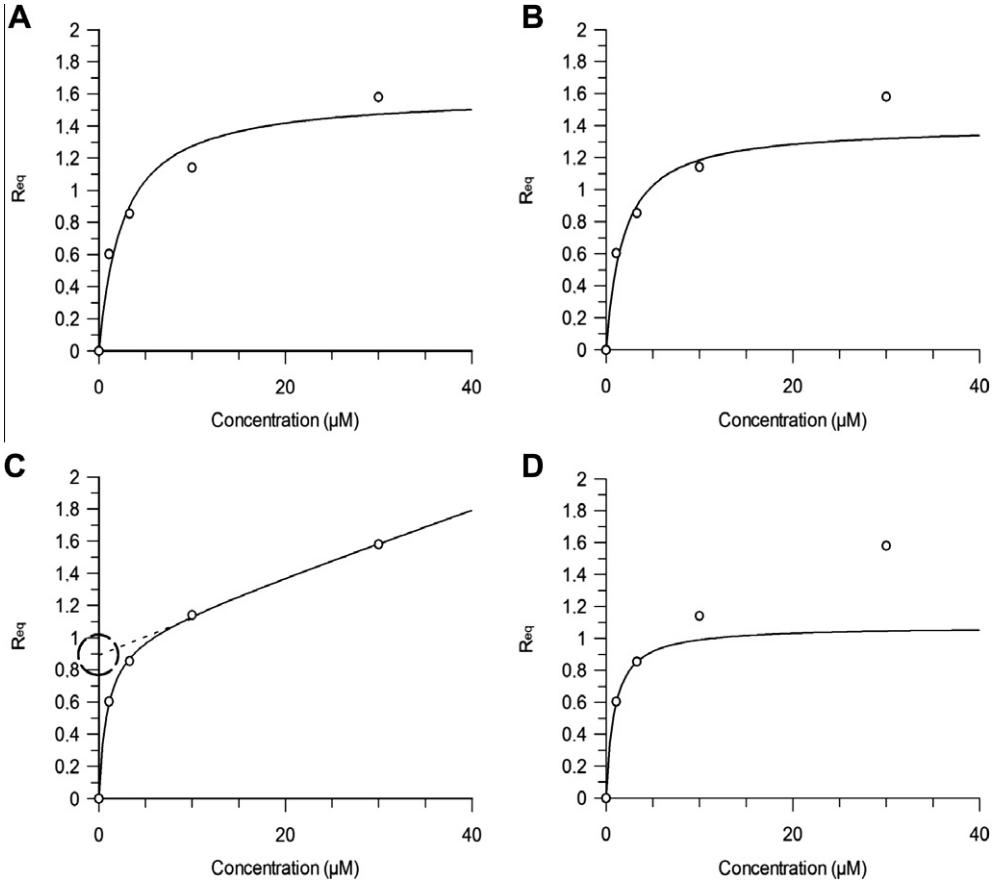

Fig.4. Concentration dependence for the interaction between iopanoic acid and HSA with different theoretical models fitted to the experimental data: (A) 1:1 interaction model (Eq. (3)); (B) 2:1 interaction model (Eq. (4)); (C) model describing a primary 1:1 interaction in combination with low-affinity secondary interaction(s) (Eq. (5)). (D) The theoretical primary interaction with HSA was estimated by using $R_{max,prim}$ and Eq. (5). The maximal response for the specific protein binding was further estimated by the *y* axis intercept of the continuous black line (dashed line in panel C), as indicated with a circle. The slope of the line for high concentrations (10–30 μM) in panel C indicates the amount of secondary interaction(s). Signals were normalized.

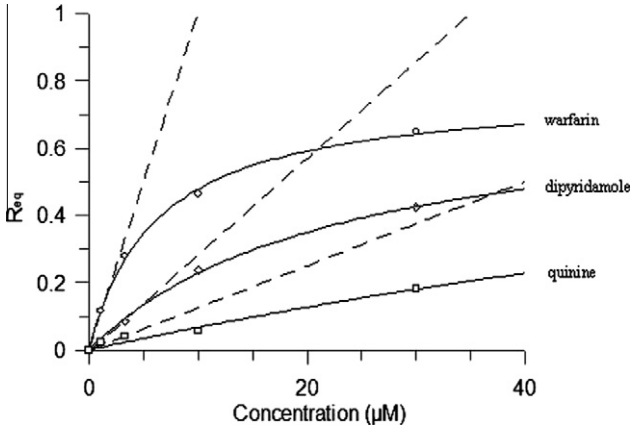

Fig.5. Illustration of the concept of binding efficiency. Saturation curves for the interaction between HSA and warfarin (circles), dipyridamole (stars), and quinine (squares). The solid curves represent the theoretical curves obtained with a 1:1 interaction model (Eq. (6)), whereas the dashed lines correspond to the binding efficiency (*BE*), also obtained by nonlinear regression analysis of the complete dataset using Eq. (6). These represent the slope of the saturation curve at low compound concentrations, as defined by Eq. (8). Signals were normalized to F_{NORM}.

of the complete dataset for all compound interactions, and the results were reproducible. *BE* values for all studied compounds are summarized in Table 1.

To get an idea of the binding of the inhibitors of cathepsin K, HIV-1 protease, and HIV-1 reverse transcriptase to HSA and AGP, their *BE* values were ranked relative to reference compounds (Fig. 6). All inhibitors showed low to moderate *BE* values in comparison with the reference compounds.

Serum interaction analysis

As a means of estimating the serum binding propensity of the compounds in a physiologically relevant context, the inhibitory effect (EC_{50} values) of protease and reverse transcriptase inhibitors against replication of HIV-1 in cell culture were determined both with and without the addition of human serum (Table 2). For all compounds, the fold change (Eq. (10)) was calculated as a pragmatic quantification of serum effects (Table 2). In addition, ultrafiltration was also used to determine the unbound fraction (% free) in serum for a selected set of compounds (Table 2).

Correlation analysis

To identify experimental descriptors from the interaction analysis that have a high predictive value for serum protein binding in cellular systems, a correlation analysis with all parameters for the different HIV-1 inhibitors and HSA and AGP (Tables 1 and 2) was performed. No notable correlation was identified by the statistical approach using the full dataset. Nevertheless, it was observed that, among the compounds with slow dissociation from AGP, two were HIV-1 protease inhibitors (7 and 13) that became much less effective in the presence of serum (i.e., had a large fold change) (Table 2).

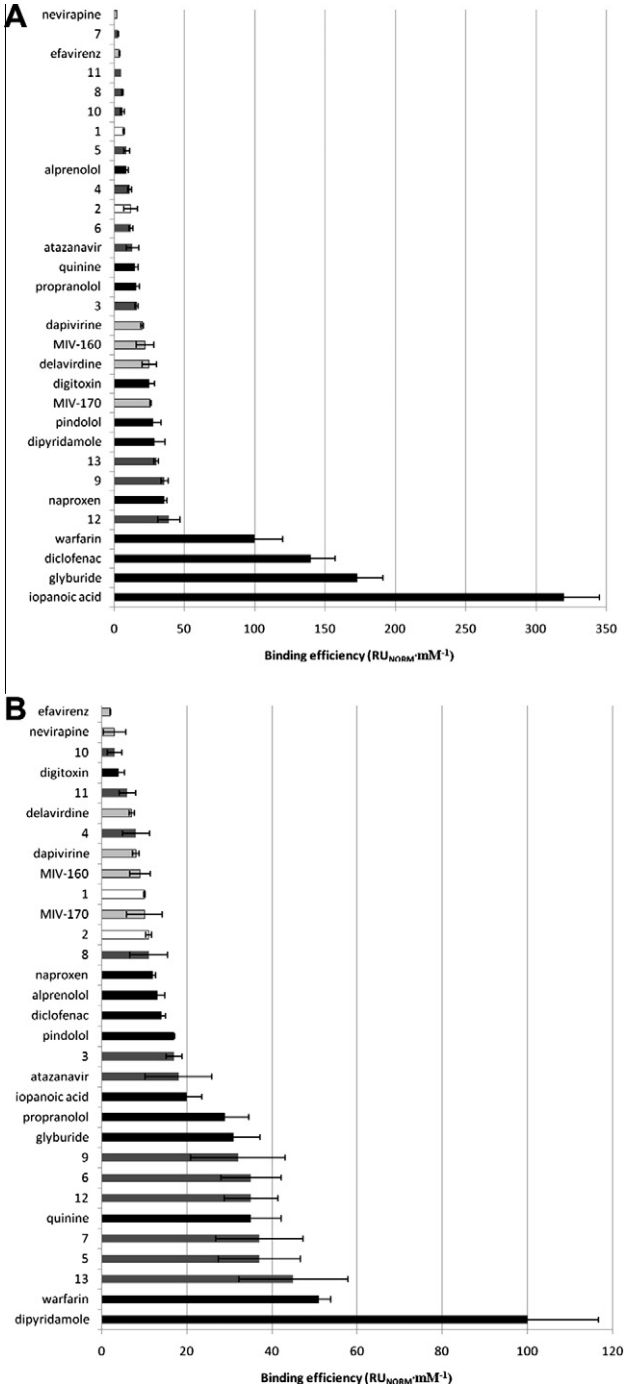

Fig.6. Ranking of compounds based on *BE* values for cathepsin K inhibitors (white), HIV-1 protease inhibitors (dark gray), HIV-1 reverse transcriptase inhibitors (light gray), and reference compounds (black) in their interactions with HSA (A) and AGP (B). Standard deviations obtained from triplicate experiments are indicated as error bars.

Discussion

This study has revealed physiologically relevant characteristics of serum proteins and how they can efficiently transport a variety of compounds in the circulation. By being present at high concentrations and having multiple binding sites, their weak affinities for drug-like compounds, resulting from the rapid kinetics, do not become a limiting factor. Instead, the rapid kinetics ensures that the loading and unloading of the proteins are efficient. However, these characteristics make the characterization of serum protein binding of potential lead compounds very challenging and require methods other than those conventionally used for characterization of lead–target interactions. Nevertheless, characterizing the interactions on a level of "molecular recognition" is expected to be crucial to understand the interactions sufficiently well for SAR analysis. This is required for medicinal chemistry optimization of compounds also from a serum protein binding perspective.

Table 2
Serum binding data determined by a cell culture assay for antiviral replication, where the effect of 50% human serum on inhibition of HIV replication in cell culture (EC_{50}) for HIV-1 protease inhibitors and reverse transcriptase inhibitors was determined, and by ultrafiltration, where the percentage of unbound compound in serum was estimated.

Compound	Type of inhibitor	EC_{50} (μM)	EC_{50} 50% HS (μM)	Fold change	Ultrafiltration (% free)
Atazanavir	PI	0.0075	0.017	2	25
3	PI	0.013	0.035	3	9.7
4	PI	0.083	0.14	2	8.7
7	PI	0.0066	0.87	132	4.3
8	PI	0.0077	0.031	4	17
9	PI	0.009	0.16	18	1.5
10	PI	0.006	0.02	3	6
11	PI	0.008	0.032	4	0.2
12	PI	0.005	0.043	9	1.1
13	PI	0.0014	0.17	121	0.2
MIV-160	RTI	0.0017	0.006	4	nd
MIV-170	RTI	0.0017	0.007	4	nd
Dapivirine	RTI	0.0012	nd	nd	nd
Efavirenz	RTI	0.005	0.045	9	0.4
Nevirapine	RTI	0.17	0.52	3	nd
Delavirdine	RTI	0.11	>4.4	>40	nd

Note: HS, human serum; PI, HIV-1 protease inhibitor; RTI, reverse transcriptase inhibitor; nd, not determined.

Determination of overall apparent affinities is a simple way to evaluate molecular interactions. This was the approach, among others, used in the original studies of ligand–serum protein interactions using SPR biosensor technology [16,17]. However, this neglects the complex situation of multiple binding sites and conformational changes typical for HSA and AGP. This complexity has already been addressed by us [11] and others [18], but the proposed methods do not allow a simple approach for ranking of compounds. In addition, methods involving the determination of affinities are not applicable for low-affinity interactions that do not reach saturation under relevant experimental conditions. Estimated overall apparent affinities, therefore, are not reliable and were found to have low reproducibility in this study. Another strategy in evaluating serum protein interaction characteristics is to determine the interaction kinetic rate constants. But due to fast kinetics and complex interaction mechanisms, serum protein interactions are not easily characterized by this method.

Our experiences with unreliable overall apparent affinities and kinetic rate constants encouraged us to introduce the concept of binding efficiency. Although this novel strategy in evaluation of interactions gives no information about affinities or about individual association and dissociation rate constants, it was shown to be a useful tool for quantifying weak interactions. In cases where experimental conditions limited the analysis by conventional methods, *BE* was a reliable parameter that showed good reproducibility. By avoiding affinity determinations, drug compounds interacting with different affinities (100-fold) to HSA and AGP could easily be compared without using concentrations higher than 30 μM. This ranking of binding to serum proteins was not possible to perform using estimated apparent K_D values because the broad range of affinities for both inhibitors and reference compounds was impossible to estimate under the experimental conditions.

Binding efficiency was determined by the linear relationship of low compound concentrations and the responding equilibrium binding signals, making it a more robust parameter than equilibrium binding signals based on single concentrations. In the evaluation of the novel parameter, *BE* values for the inhibitors of interest and reference compounds were compared with equilibrium binding signals at different concentrations. The comparison indicated that these two parameters are better correlated at low concentrations than high ones, as expected. As low concentrations as possible, therefore, should be used in single concentration hit characterization to avoid problems due to differences in the level of saturation reached for different compounds. Too high concentrations would probably result in misleading ranking results due to the fact that some compounds may have reached saturation, whereas others have not.

We introduced the term "binding efficiency" here because it is comparable to the term "catalytic efficiency" used in enzymology with a similar meaning and mathematical definition. However, during the course of this work, the term binding efficiency turned up in another article [19] but as an alternative to the already established term "ligand efficiency." Because it is more appropriate for the use proposed in this study, we advocate the meaning introduced here but remind the reader that the enzymological term has some limitations [20,21] and that the new term will also need to be used with care.

In addition to binding efficiency, we also divided the serum protein binding of compounds into parts describing primary ($K_{D,prim}$ and $R_{max,prim}$) and secondary (k) interactions. By doing this, it was possible to focus on the primary interactions with high-affinity binding sites on HSA and AGP while still allowing the discrimination of compounds with divergent secondary interactions. By comparing values of $K_{D,prim}$ with values of K_D determined for some of the reference compounds binding to known primary binding sites of serum proteins, the analysis was evaluated. However, previously published data should be interpreted with caution due to methodological differences and inherent difficulties in quantifying this type of interaction. Research data indicate a value of K_D between 2.5 and 6.3 μM for the binding of warfarin to its primary HSA binding site [22–26]. In addition, naproxen has previously been described to bind to its primary HSA binding site with an affinity of approximately 3 μM [27]. We determined the affinities for the primary binding sites to be 5 and 6 μM for warfarin and naproxen, respectively. Affinity data for AGP interactions was not easily found; therefore, similar evaluations as for HSA could not be done.

When determining serum binding characteristics of compounds, percentage binding is most often used as parameter. Working with this parameter, and comparing it with other in vitro and cell culture data, one must keep in mind the complexity of serum proteins, that compounds can bind not only to HSA and AGP, and that this parameter is dependent on both protein and compound concentrations as well as affinities – factors that can vary with time. For example, the conditions (pH, temperature, and ionic strength) influence interaction characteristics depending on the specific forces involved. Moreover, the in vivo concentrations of serum proteins vary depending on the situation, with the concentration of HSA decreasing with age and with the concentration of AGP increasing during acute and chronic infections and

inflammation [3,28]. Previous work concerning the interaction between HIV inhibitors and serum proteins demonstrates that the actual physiological protein concentrations will determine the in vivo distribution of the protein-bound inhibitors [11]. In early studies of serum protein binding, compounds of different characteristics were thought to bind specifically to either HSA or AGP [3]. It was later clarified that this was not the case; the same compound can in fact bind to both proteins. The presence of different amounts of serum proteins, and also the presence of other compounds, may have big effects on the actual in vivo interaction [28]. This makes the interpretations of serum protein interactions even harder. This is probably one reason for the difficulties in identifying clear correlations between different parameters. The complexity of serum should not be underestimated, and it is probable that ligands have many other potential binding partners in serum in addition to HSA and AGP. By extending the strategy to other proteins in blood, it may be possible to refine the picture even further.

By using the more robust parameter of binding efficiency, ranking of compounds relative reference compounds can be done more easily. In addition, the combination of binding efficiency data with data from primary and secondary interactions can further reveal structural information of a compound and how this can be modified to change or improve its serum binding properties. Still, for tight binding inhibitors, it would be possible to resolve the kinetics for enhanced analysis. So, this method has considerable advantages over previous descriptions of how to use SPR biosensors for estimation of ligand binding to serum proteins (see, e.g., Refs. [11,16,17]). In particular, as indicated in this study and previous unpublished work, the inherent difficulties in determining affinities for compounds interacting with weak affinities and/or multiple sites can result in misleading information. Therefore, it is perhaps better to avoid this parameter altogether.

Because k_{off} is known to be of clinical relevance in serum protein interactions, it was of interest to see whether there was any correlation between k_{off} and interactions with serum. Here only few of the compounds exhibited values of k_{off} that could be quantified, but it was possible to visually identify compounds with relatively slow dissociation rates. The analysis suggested correlations between k_{off} and *BE* for AGP, which may be of relevance considering that AGP has an inhibitory effect on the antiviral efficacy of HIV protease inhibitors [29]. However, more data are required for this type of analysis, and so determination of dissociation rates is of interest in future analyses of protein binding of different compounds. Unfortunately, for evaluation of the *BE* value for prediction of serum protein binding in humans and of the physiological effects of kinetics on serum binding, data obtained in suitable human or animal models are required and, therefore, this was outside the scope of the current study.

Conclusions

This study has shown that useful data for predicting serum protein interactions can be obtained using a biosensor-based approach provided that the analysis focuses on binding efficiencies. The dissociation rate was also shown to be of some relevance, and valuable information can be gained for compounds where k_{off} can be determined. The results from this study, therefore, have revealed new characteristics of serum protein interactions of importance for understanding the feature of transport proteins.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ab.2010.10.028.

References

[1] J. Hodgson, ADMET: turning chemicals into drugs, Nat. Biotechnol. 19 (2001) 722–726.
[2] J. Ghuman, P.A. Zunszain, I. Petitpas, A.A. Bhattacharya, M. Otagiri, S. Curry, Structural basis of the drug-binding specificity of human serum albumin, J. Mol. Biol. 353 (2005) 38–52.
[3] Z.H. Israili, P.G. Dayton, Human-1-glycoprotein and its interactions with drugs, Drug Metab. Rev. 33 (2001) 161–235.
[4] T.J. Peters, All about Albumin: Biochemistry, Genetics, and Biomedical Applications, Academic Press, San Diego, 1996.
[5] M.L. Howard, J.J. Hill, G.R. Galluppi, M.A. McLean, Plasma protein binding in drug discovery and development, Comb. Chem. High Throughput Screen. 13 (2010) 170–187.
[6] H. Wan, F. Bergström, High throughput screening of drug-protein binding in drug discovery, J. Liq. Chromatogr. Relat. Technol. 30 (2007) 681–700.
[7] M.H. Busch, L.B. Carels, H.F. Boelens, J.C. Kraak, H. Poppe, Comparison of five methods for the study of drug–protein binding in affinity capillary electrophoresis, J. Chromatogr. A 777 (1997) 311–328.
[8] J. Ostergaard, N.H. Heegaard, Capillary electrophoresis frontal analysis: principles and applications for the study of drug–plasma protein binding, Electrophoresis 24 (2003) 2903–2913.
[9] D.S. Hage, A. Jackson, M.R. Sobansky, J.E. Schiel, M.J. Yoo, K.S. Joseph, Characterization of drug–protein interactions in blood using high-performance affinity chromatography, J. Sep. Sci. 32 (2009) 835–853.
[10] C. Bertucci, M. Pistolozzi, G. Felix, U.H. Danielson, HSA binding of HIV protease inhibitors: A high-performance affinity chromatography study, J. Sep. Sci. 32 (2009) 1625–1631.
[11] S. Cimitan, M.T. Lindgren, C. Bertucci, U.H. Danielson, Early absorption and distribution analysis of antitumor and anti-AIDS drugs: Lipid membrane and plasma protein interactions, J. Med. Chem. 48 (2005) 3536–3546.
[12] M. Hogberg, C. Sahlberg, P. Engelhardt, R. Noreen, J. Kangasmetsa, N.G. Johansson, B. Oberg, L. Vrang, H. Zhang, B.L. Sahlberg, T. Unge, S. Lovgren, K. Fridborg, K. Backbro, Urea-PETT compounds as a new class of HIV-1 reverse transcriptase inhibitors: 3. Synthesis further structure-activity relationship studies of PETT analogues, J. Med. Chem. 42 (1999) 4150–4160.
[13] A. Molla, S. Vasavanonda, G. Kumar, H.L. Sham, M. Johnson, B. Grabowski, J.F. Denissen, W. Kohlbrenner, J.J. Plattner, J.M. Leonard, D.W. Norbeck, D.J. Kempf, Human serum attenuates the activity of protease inhibitors toward wild-type and mutant human immunodeficiency virus, Virology 250 (1998) 255–262.
[14] O.S. Weislow, R. Kiser, D.L. Fine, J. Bader, R.H. Shoemaker, M.R. Boyd, New soluble-formazan assay for HIV-1 cytopathic effects: application to high-flux screening of synthetic and natural products for AIDS-antiviral activity, J. Natl. Cancer Inst. 81 (1989) 577–586.
[15] A. Onell, K. Andersson, Kinetic determinations of molecular interactions using Biacore: minimum data requirements for efficient experimental design, J. Mol. Recognit. 18 (2005) 307–317.
[16] A. Frostell-Karlsson, A. Remaeus, H. Roos, K. Andersson, P. Borg, M. Hamalainen, R. Karlsson, Biosensor analysis of the interaction between immobilized human serum albumin and drug compounds for prediction of human serum albumin binding levels, J. Med. Chem. 43 (2000) 1986–1992.
[17] R.L. Rich, Y.S. Day, T.A. Morton, D.G. Myszka, High-resolution and high-throughput protocols for measuring drug/human serum albumin interactions using BIACORE, Anal. Biochem. 296 (2001) 197–207.
[18] P. Sandblad, R. Arnell, J. Samuelsson, T. Fornstedt, Approach for reliable evaluation of drug protein interactions using surface plasmon resonance technology, Anal. Chem. 81 (2009) 3551–3559.
[19] E. Perola, An analysis of the binding efficiencies of drugs and their leads in successful drug discovery programs, J. Med. Chem. 53 (2010) 2986–2997.
[20] R. Eisenthal, M.J. Danson, D.W. Hough, Catalytic efficiency and k_{cat}/K_M: a useful comparator?, Trends Biotechnol 25 (2007) 247–249.
[21] R.J. Fox, M.D. Clay, Catalytic effectiveness a measure of enzyme proficiency for industrial applications, Trends Biotechnol. 27 (2009) 137–140.
[22] U. Kragh-Hansen, Relations between high-affinity binding sites of markers for binding regions on human serum albumin, Biochem. J. 225 (1985) 629–638.
[23] J.P. Tillement, R. Zini, P. d'Athis, G. Vassent, Binding of certain acidic drugs to human albumin: theoretical and practical estimation of fundamental parameters, Eur. J. Clin. Pharmacol. 7 (1974) 307–313.
[24] G. Sudlow, D.J. Birkett, D.N. Wade, The characterization of two specific drug binding sites on human serum albumin, Mol. Pharmacol. 11 (1975) 824–832.
[25] I. Sjöholm, B. Ekman, A. Kober, I. Ljungstedt-Pahlman, B. Seiving, T. Sjödin, Binding of drugs to human serum albumin: XI. The specificity of three binding

sites as studied with albumin immobilized in microparticles, Mol. Pharmacol. 16 (1979) 767–777.
[26] V. Maes, Y. Engelborghs, J. Hoebeke, Y. Maras, A. Vercruysse, Fluorimetric analysis of the binding of warfarin to human serum albumin: equilibrium and kinetic study, Mol. Pharmacol. 21 (1982) 100–107.
[27] V.K. Cheruvallath, C.M. Riley, S.R. Narayanan, S. Lindenbaum, J.H. Perrin, A quantitative circular dichroic investigation of the binding of the enantiomers of ibuprofen and naproxen to human serum albumin, J. Pharm. Biomed. Anal. 15 (1997) 1719–1724.
[28] J. Woo, Age-related changes in protein binding of drugs: implications for therapy, J. Hong Kong Geriatr. Soc. 9 (1998) 4.
[29] X.Q. Zhang, R.T. Schooley, J.G. Gerber, The effect of increasing α_1-acid glycoprotein concentration on the antiviral efficacy of human immunodeficiency virus protease inhibitors, J. Infect. Dis. 180 (1999) 1833–1837.