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Novel Method To Assess Antiretroviral Target Trough Concentrations Using *In Vitro* Susceptibility Data

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Durable suppression of HIV-1 replication requires the establishment of antiretroviral drug concentrations that exceed the susceptibility of the virus strain(s) infecting the patient. Minimum plasma drug concentrations (C_{trough}) are correlated with response, but determination of target C_{trough} values is hindered by a paucity of *in vivo* concentration-response data. In the absence of these data, *in vitro* susceptibility measurements, adjusted for serum protein binding, can provide estimations of suppressive *in vivo* drug concentrations. We derived serum protein binding correction factors (PBCF) for protease inhibitors, nonnucleoside reverse transcriptase inhibitors, and an integrase inhibitor by measuring the effect of a range of human serum concentrations on *in vitro* drug susceptibility measured with the PhenoSense HIV assay. PBCFs corresponding to 100% HS were extrapolated using linear regression and ranged from 1.4 for nevirapine to 77 for nelfinavir. Using the mean 95% inhibitory concentration (IC_{95}) for ≥1,200 drug-susceptible viruses, we calculated protein-bound IC_{95} ($PBIC_{95}$) values. $PBIC_{95}$ values were concordant with the minimum effective C_{trough} values that were established in well-designed pharmacodynamic studies (e.g., indinavir, saquinavir, and amprenavir). In other cases, the $PBIC_{95}$ values were notably lower (e.g., darunavir, efavirenz, and nevirapine) or higher (nelfinavir and etravirine) than existing target recommendations. The establishment of $PBIC_{95}$ values as described here provides a convenient and standardized approach for estimation of the minimum drug exposure that is required to maintain viral suppression and prevent the emergence of drug-resistant variants, particularly when *in vivo* concentration-response relationships are lacking.

Fully suppressive antiretroviral therapy (ART) for human immunodeficiency virus type 1 (HIV-1) infection requires the administration of drug combinations that target multiple sites on one or more proteins required for viral replication. Approved antiretrovirals (ARVs) include nucleoside/nucleotide and nonnucleoside reverse transcriptase inhibitors (NRTIs and NNRTIs, respectively), protease inhibitors (PIs), entry inhibitors, and integrase strand-transfer inhibitors (INSTIs). With the exception of the NRTIs, which require intracellular phosphorylation, plasma drug concentrations are correlated with drug efficacy. At the same time, high drug concentrations are associated with excess toxicity.

To durably suppress HIV replication in infected patients, ARV concentrations must reach and be maintained at levels that exceed the susceptibility of the virus to that drug. Treatment response is often hampered by the failure to achieve sufficient drug exposure (i.e., poor adherence and drug interactions), reduced drug susceptibility (i.e., viral drug resistance), or both. Drug concentrations within patients vary over time and, due to ease of sampling, are generally characterized by minimum (trough) concentrations (C_{trough}) immediately prior to administration of the next scheduled dose. Drug concentrations also vary considerably between individual patients as a result of differences in absorption, distribution, metabolism, and excretion. In addition, each drug characteristically binds to human plasma proteins to different extents. Furthermore, the susceptibility of HIV-1 variants, even in patients not previously exposed to drug therapy, varies over a range that is unique to each drug (23, 24, 46).

In vivo clinical pharmacodynamic data are available for some, but not all, ARVs. Efficient collection of these data is difficult and ideally performed early in the drug development process. Alternative methods of incorporating ARV pharmacokinetics into therapeutic decision making are being explored. *In vitro* phenotypic drug susceptibility testing of individual patient viruses is now widely available and generates information that can be used to calculate an inhibitory quotient (IQ), defined as the ratio between the C_{trough} and the drug concentration that inhibits *in vitro* replication by a defined percentage (e.g., 50% or 95% inhibitory concentration [IC_{50} or IC_{95}, respectively]) (27, 35, 43, 56). Derivatives of the IQ, including the genotypic IQ (GIQ; C_{trough} divided by the number of resistance-associated mutations for a given drug) have also been evaluated (36). Several studies have attempted to define the optimal IQ required to produce long-term viral suppression: in some cases, IQ has been retrospectively linked to clinical outcome (15, 34, 41, 42, 55), while in others, direct relationships between IQ and viral load response were not observed (5, 12).

For most ARV drugs, few or no *in vivo* concentration-response data have been generated, or these data are inconsistent with clinical observations. Collectively, there is insufficient agreement in the field regarding the determination of the optimal ARV target trough concentrations in the absence of concentration-response data. We conducted the present study to address this deficiency by (i) assessing the activity of PIs, NNRTIs, and an INSTI in a standardized *in vitro* phenotypic drug susceptibility assay (Phe-

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noSense HIV, Monogram Biosciences) in the presence of human serum (HS), (ii) establishing drug-specific serum protein binding correction factors (PBCFs), and (iii) approximating the optimum target trough concentrations for currently available PIs, NNRTIs, and INSTIs.

MATERIALS AND METHODS

Determination of drug activity in the presence of human serum. The PhenoSense HIV assay (Monogram Biosciences, South San Francisco, CA) was performed as described previously (47), with the following modifications. For all PIs except darunavir and atazanavir, each drug was prepared at 10 times the final concentration using complete medium containing 10% fetal bovine serum (FBS) without HS. Fifteen microliters of the 10× drug stocks was mixed with 85 μl of complete medium containing 10% FBS and 0%, 25%, 50%, or 75% pooled HIV-negative HS or 90% HS plus 10% FBS in 96-well plates. Fifty microliters of trypsinized, transfected (virus-producing) cells was added to the plates containing 100 μl drug and medium, which had been resuspended in the corresponding medium (i.e., with or without HS at 25 to 90%). Thus, the concentration of HS present during virus particle formation was 22.5%, 45.0%, 67.5%, or 81%; FBS was present at 10% for all conditions. Viral stocks were harvested approximately 48 h after transfection, and 100 μl was used to infect fresh 293 cell cultures (target cells) that had been plated in a volume of 50 μl in medium containing 10% FBS. During the period of time when darunavir and atazanavir were evaluated, several modifications to the PhenoSense assay were implemented, resulting in subtle differences in final HS concentrations compared to the procedure described above. These changes resulted in final HS concentrations during virus production of 21.7%, 43.5%, 65.2%, or 76.5%. For the purposes of this report, these minor differences (3 to 6%) in final HS concentration were ignored during analysis. For NNRTIs and raltegravir, virus produced in the presence of the same range of HS concentrations used for most of the PIs, but in the absence of drugs, was used, and the drugs were added at the time of infection. Three lots of HS (Intergen, Purchase, NY) were used during the course of these experiments. The HS contained an average of 0.77 mg/ml α-1 acid glycoprotein and 44.6 mg/ml albumin. This level of α-1 acid glycoprotein is consistent with values previously reported in HIV-negative subjects (29).

Approximately 72 h after infection, target cells were lysed and luciferase activity was measured to assess virus replication in the presence or absence of drugs at each concentration of HS. IC_{50} and IC_{95} values were determined as described previously (47). Between 2 and 32 (mean, 8.5) replicates of susceptibility assays were performed for each virus, using either a drug-sensitive reference virus (NL4-3) or a well-characterized multidrug-resistant virus (MDRC-4) that exhibits reduced susceptibility to all licensed reverse transcriptase and protease inhibitors.

Calculation of PBCFs and target trough concentrations. A PBCF corresponding to 45% HS was defined as the fold increase in experimentally determined IC_{50} in the presence of 45% HS compared to normal complete medium (10% FBS). The PBCF was calculated within each batch and then averaged across replicates from multiple batches. The PBCF for 100% HS was derived by linear regression analysis (Prism 5.0; GraphPad, San Diego, CA) based on fold changes in IC_{50} in the presence of 22.5%, 45.0%, 67.5%, or 81% HS versus 0% HS; the PBCF was extrapolated from the y intercept when $x = 100$. r^2 values for the linear regression ($\log_{10}$ IC_{50} versus % HS) were between 0.82 and 0.99, except for saquinavir (0.59) and tipranavir (0.61). Second- and third-order polynomial fittings were inferior for all drugs (data not shown).

Mean IC_{50} and IC_{95} values of wild-type viruses for each drug were derived from the susceptibility measurements of clinical isolates tested between 2009 and 2010, as compiled in the Monogram Biosciences database. An isolate was defined as wild type in protease (PR) and reverse transcriptase (RT) if drug-selected mutations in PR and RT as previously described (46) were not detected (n = 6,537). For raltegravir, drug-selected mutations were defined as any change from the wild-type subtype B amino acid sequence at the following positions: H51, T66, E92, F121, G140, Y143, Q146, S147, Q148, S153, N155, and R263 (n = 1,200). The PR and RT genotypes of these viruses were not available. The protein-bound IC_{50} or IC_{95} values ($PBIC_{50}$ or $PBIC_{95}$, respectively) were computed by multiplying the mean IC_{50} or IC_{95} values by the corresponding PBCF for each drug. All C_{trough} values except that for raltegravir are derived from the individual drug manufacturers' prescribing information (1–3, 6–9, 17, 19, 38, 53, 54). The C_{trough} for raltegravir was taken from the intensive PK profiles of patients treated with raltegravir twice daily in the QDMRK study (50).

RESULTS

Drug susceptibility was determined using the PhenoSense assay (47) under standard conditions (10% FBS) and in the presence of 23 to 81% HS. In some cases, because of the cytostatic effects of HS on cultured cells, infectivity (i.e., luciferase signal) was too low to accurately determine the IC_{50} at 81% HS. Assays were performed using a drug-susceptible reference virus (NL4-3) and a well-characterized multidrug-resistant reference virus (MDRC-4). Representative inhibition curves generated using NL4-3 are shown in Fig. 1 for a PI (darunavir) (Fig. 1A), NNRTI (efavirenz) (Fig. 1B), and INSTI (raltegravir) (Fig. 1C). IC_{50}s of all drugs for both the drug-susceptible and -resistant viruses at increasing HS concentrations are plotted in Fig. 2. As expected, the inhibition curves (Fig. 1) shifted toward higher drug concentrations (i.e., to the right) as the percentage of HS increased, although the magnitude of this shift was specific for each drug. For example, the IC_{50}s for saquinavir and tipranavir were between 23 and 81% HS remained relatively constant, while the IC_{50}s for most other drugs increased incrementally. Since the relationship between IC_{50} and HS concentration for most drugs was fairly linear between 23 and 81% HS, the IC_{50} at 100% HS was estimated using linear regression.

The ratio between the estimated IC_{50} at 100% HS (mimicking the *in vivo* situation) and the IC_{50} under standard *in vitro* conditions is referred to as the PBCF. A PBCF for 45% HS can also be calculated using the experimental data. Table 1 lists the PBCF values for 100% HS and 45% HS derived here, along with the 50% HS correction factors derived in a previous study (40), as well as estimations of protein binding derived from *in vitro* biochemistry studies as reported by the various drug manufacturers. Correction factors for 45% HS (this work) and 50% HS (40) were highly correlated for the six drugs where both values were available. The PBCF values that were independently determined in both studies also correlated with the magnitude of protein binding for each drug (Table 1). Previously reported PBCFs for atazanavir and amprenavir (13.4 and 7.4, respectively) are similar to the values reported here, in spite of the fact that these older studies used only purified α-1 acid glycoprotein and human serum albumin and not complete serum (14, 48).

To derive *in vivo* target trough concentrations for patients infected with drug-susceptible virus, PBCFs were applied to mean IC_{50} and IC_{95} values from clinically derived HIV-1 strains. Over 6,500 results from viruses lacking detectable amino acid substitutions that are nonpolymorphic and selected under ARV drug pressure (46) were identified in a database of linked phenotypic and genotypic assay results. Fewer results were available for raltegravir (n = 1,200). The total plasma drug concentration expected to inhibit wild-type virus *in vivo* by 50% ($PBIC_{50}$) or 95% ($PBIC_{95}$) was calculated from these data (Table 2). Although it is not known whether the $PBIC_{50}$ or $PBIC_{95}$ most accurately reflects the optimal target trough concentration for these agents, given the consider-

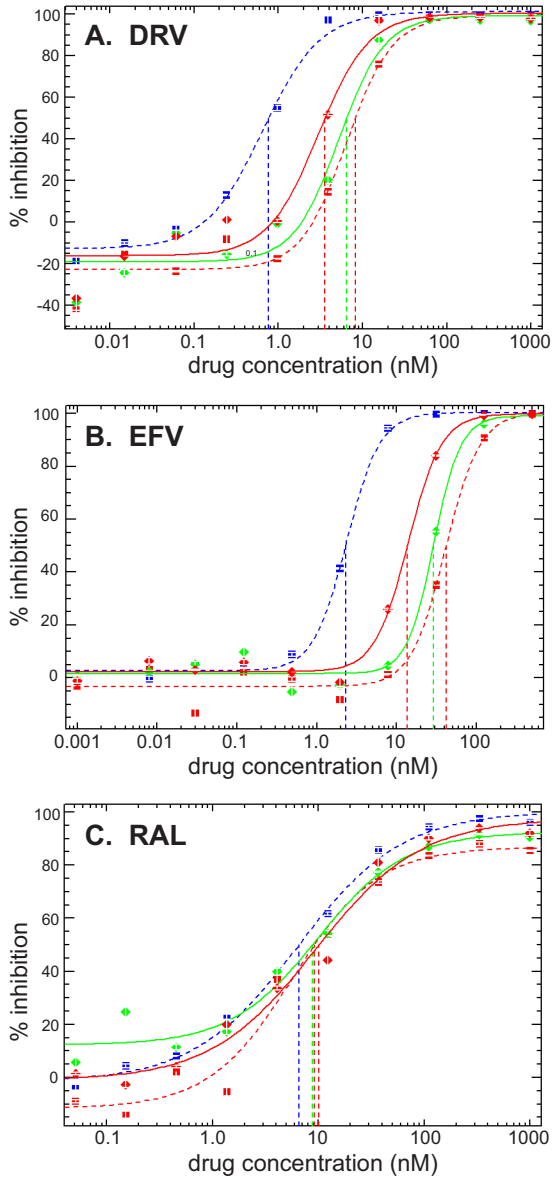

FIG 1 Effect of human serum on *in vitro* drug susceptibility. Shown are representative PhenoSense inhibition curves demonstrating the effect of addition of human serum at 22.5% (red diamonds, solid line), 45.0% (green diamonds, solid line), or 67.5% (red squares, dotted line), compared to standard (10% FBS) conditions (blue squares, dotted line). The drug-susceptible (NL4-3) reference virus was used. (A) Darunavir (DRV); (B) efavirenz (EFV); (C) raltegravir (RAL). Vertical dotted lines denote the IC_{50}.

able inter- and intrapatient variability in C_{trough}, a conservative approach is to use the highest of these (i.e., the $PBIC_{95}$). The corresponding optimal IQ_{95} (C_{trough} divided by $PBIC_{95}$) for an effective ARV drug should thus be 1 or greater. In fact, this is the case for all of the drugs that we examined except nelfinavir (IQ_{95} = 0.26). For comparison, the currently proposed target drug concentrations from the Department of Health and Human Services (DHHS) guidelines (45) are also listed in Table 2.

DISCUSSION

The results generated in this study add to the current body of knowledge relating ARV drug target trough concentrations to drug efficacy and more specifically demonstrate the importance of serum protein binding when relating *in vitro* drug susceptibility measurements to plasma drug concentrations. As expected, the ARV drugs that exhibit the lowest levels of protein binding (indinavir, amprenavir, atazanavir, nevirapine, and raltegravir) have the smallest PBCFs (Table 1). Notably, lopinavir also has a relatively low PBCF, despite reports of high (98 to 99%) serum protein binding. This apparent discrepancy has been reported by others and may be attributed to differential binding properties of bovine serum proteins in the tissue culture medium (26, 28).

The PBCFs reported here agree well with values previously published by Molla et al. for the subset of PIs that were tested in both studies (40). This earlier study employed a multiple-replication-cycle inhibition assay using MT4 cells, HIV-1 IIIB, and a colorimetric readout for cytopathic effects. In contrast, our study was conducted using a single-cycle inhibition assay, replication-defective recombinant viruses, and a luciferase reporter readout (47). Our methodology permits assessments of protein binding effects on drug susceptibility measurements at higher HS concentrations than replication-competent systems because of the shorter period of time that cells are in contact with high HS concentrations (2 to 3 days). In most cases, we were able to assess drug susceptibility in the presence of 81% HS, although the luciferase signal was considerably reduced at this high concentration.

Our results for several PIs are also consistent with previously derived *in vivo* pharmacokinetic/pharmacodynamic relationships, particularly amprenavir, indinavir, and saquinavir. In a dose-finding study of amprenavir, a fitted sigmoid maximum effect (E_{max}) curve between amprenavir C_{trough} and HIV-1 RNA response demonstrated a significant relationship (51). The plasma concentration required to produce 50% of the maximum response (EC_{50}) was 87 ng/ml. By using the reported Hill coefficient, we were able to estimate the EC_{95} (320 ng/ml) value as well. Current DHHS guidelines recommend an amprenavir target C_{trough} of 400 ng/ml (45). Using the PBCF determined in this study, our estimate of amprenavir $PBIC_{95}$ is 358 ng/ml (Table 2), which is strikingly similar to the recommended C_{trough} value.

In a small subset of pediatric patients receiving indinavir, stavudine, and didanosine, a significant inhibitory E_{max} relationship between the indinavir C_{trough} and change in HIV-1 RNA from baseline to week 24 was described (16). The maximum reduction in HIV-1 RNA (2.0 $\log_{10}$ copies/ml) corresponded to an EC_{50} of 80 ng/ml. In a separate study in adult patients treated with indinavir in combination with zidovudine and lamivudine, the indinavir C_{trough} levels at week 4 were significantly associated with changes in HIV-1 RNA from baseline to week 4 (42). Based on an E_{max} model, an EC_{90} was estimated to be 110 ng/ml. Current DHHS guidelines recommend a target C_{trough} of 100 ng/ml (45). We estimated the indinavir $PBIC_{95}$ to be 73 ng/ml (Table 2), which again is similar to *in vivo* estimations.

A significant correlation between saquinavir C_{trough} and sustained viral load suppression has been observed in children (21). The minimum mean saquinavir C_{trough} associated with durable viral load suppression was 200 ng/ml. Additional support for this target comes from the modeling of saquinavir pharmacokinetics/pharmacodynamics following saquinavir monotherapy in HIV-positive patients (18). Following administration of 1,200-mg saquinavir three times daily (soft gel formulation), both area under the curve (AUC) and C_{trough} measures were related to peak reduction in plasma HIV-1 RNA. The median 24-h AUC (AUC_{24})

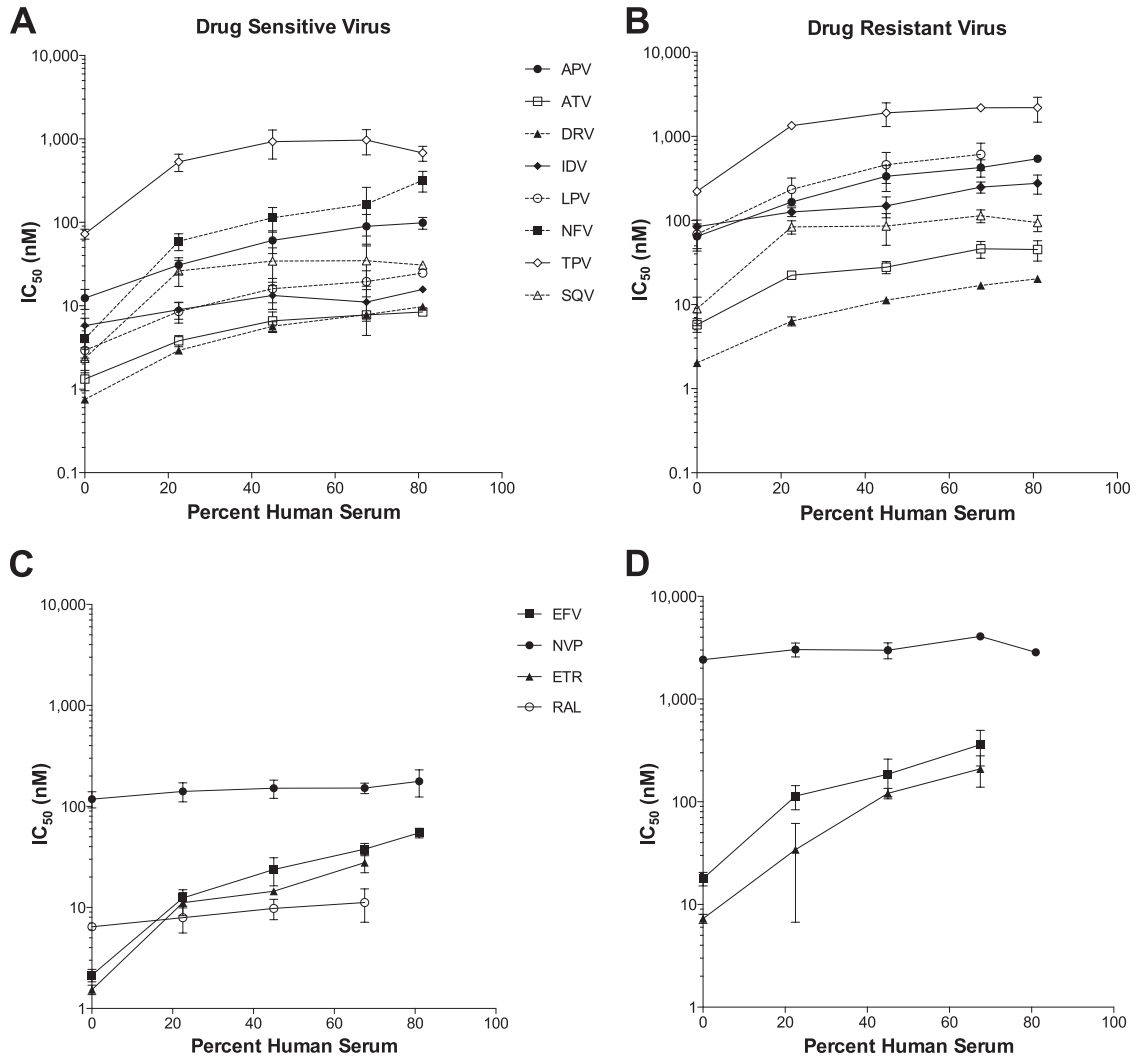

FIG 2 Relationship between ARV drug IC_{50} and human serum concentration. Mean IC_{50}s from replicate susceptibility assays ± SD are shown. (A) Susceptibility of the drug-susceptible (NL4-3) reference virus to protease inhibitors (PIs); (B) susceptibility of the multidrug-resistant (MDRC-4) reference virus to PIs. Results for nelfinavir with MDRC-4 are not shown since human serum concentrations shifted the IC_{50} beyond the dynamic range of the assay. APV, amprenavir (closed circles, solid line); ATV, atazanavir (open squares, solid line); DRV, darunavir (closed triangles, dotted line); IDV, indinavir (closed diamonds, solid line); LPV, lopinavir (open circles, dotted line); NFV, nelfinavir (filled squares, dotted line); SQV, saquinavir (open triangles, dotted line); TPV, tipranavir (open diamonds, solid line). (C) Susceptibility of the drug-susceptible (NL4-3) reference virus to NNRTIs and raltegravir; (D) susceptibility of the multidrug-resistant (MDRC-4) reference virus to NNRTIs. Since the multidrug-resistant virus contains the wild-type integrase region, the results for this virus with raltegravir are included in panel C. EFV, efavirenz (filled squares); ETR, etravirine (filled triangles); NVP, nevirapine (filled circles); RAL, raltegravir (open circles).

was 20 μg · h/ml, which corresponded to 85% of the maximum effect (EC_{85}); the C_{trough} at this dose was 216 ng/ml (18). Current DHHS guidelines recommend a target C_{trough} of 100 to 250 ng/ml (45). We calculated the saquinavir $PBIC_{95}$ to be 234 ng/ml (Table 2), which is highly concordant with clinically established values.

There is controversy regarding the optimum C_{trough} for efavirenz. The reported therapeutic range (lower to upper exposure targets) for efavirenz is 1,000 to 4,000 ng/ml (37). These data come from a study that explored the relationships between EFV exposure, efficacy, and development of central nervous system (CNS) side effects. Since the discordance between this pharmacodynamic study and our *in vitro* results is considerable, it is worth noting that this was an exploratory field trial in which all patients received the same EFV dose and was not a well-controlled, broad-dose-ranging study. In addition, EFV concentrations were determined using blood samples that were collected on average 14 h postdosing (range of 8 to 20 h postdosing). Although EFV has a relatively long half-life, actual C_{trough} values were not determined or estimated. Furthermore, treatment adherence was not considered, which may account for the broad range of EFV concentrations (125 to 15,230 ng/ml) that were observed. Mutations in cytochrome P450 (CYP) 2B6 are also associated with EFV exposure (22), but in the absence of race/ethnicity data, it is not possible to infer the contribution of genetics to the interpatient variability in EFV concentrations in the study. Also, as discussed by the authors, there was considerable overlap in EFV concentrations among patients that experienced treatment failure versus those that did not. Finally, drug concentrations were determined 3 to 18 months after initiation of EFV therapy—well past the time at which EFV-associated neurologic symptoms have been correlated to plasma concentra-

TABLE 1 Protein binding correction factors

Drug[a]	PBCF with:			% bound[e]
	45% HS[b]	100% HS[c]	50% HS[d]	
APV	4.8 ± 2.1	11 ± 0.6	8.5 ± 3.3	90
ATV	5.0 ± 1.1	9.3 ± 0.2	ND	86
DRV	6.5 ± 1.3	14 ± 0.2	ND	95
IDV	2.0 ± 0.7	3.5 ± 0.07	2.2 ± 1.1	60
LPV	5.5 ± 1.8	9.9 ± 1.0	8.8 ± 4.3	98–99
NFV	27 ± 14	77 ± 8.5	39 ± 11	>98
RTV	12 ± 5.9	20 ± 3.9	25 ± 10	98–99
SQV	14 ± 10	17 ± 6.8	34 ± 14	98
TPV	11 ± 4.2	15 ± 2.7	ND	>99.9
EFV	11 ± 3.6	27 ± 1.2	ND	99.5–99.75
ETR	13 ± 4.2	33 ± 2.7	ND	99.9
NVP	1.3 ± 0.3	1.4 ± 0.1	ND	60
RAL	1.5 ± 0.4	2.1 ± 0.03	ND	83

[a] APV, amprenavir; ATV, atazanavir; DRV, darunavir; IDV, indinavir; LPV, lopinavir; NFV, nelfinavir; SQV, saquinavir; TPV, tipranavir; EFV, efavirenz; ETR, etravirine; NVP, nevirapine; RAL, raltegravir.
[b] The 45% HS values (actual) represent averages ± standard deviations (SD) derived from 3 to 6 assays (this work).
[c] The 100% HS values (extrapolated) represent averages ± standard errors (SE) derived from linear regression (this work).
[d] Mean ± SD ratios between 50% HS and the control for all viruses listed in Table 1 from reference 40. ND, not done.
[e] All percent bound values are from the product prescribing information for each drug (1–3, 6–9, 17, 19, 38, 39, 53, 54).

tions (10). Our estimation of the $PBIC_{95}$ for efavirenz is 126 ng/ml, which is well below the average C_{trough} of 1,800 ng/ml in patients receiving 600 mg once daily and below the proposed EFV lower concentration threshold (1,000 ng/ml). The decay rate of EFV relative to its $PBIC_{95}$ in one patient who discontinued treatment with EFV, stavudine, and lamivudine has been described (49). In this patient, plasma HIV-1 RNA level was <50 copies/ml at the time of treatment discontinuation and viral rebound was detected 21 days later—coincident with the time that the EFV plasma concentration fell below 50 to 100 ng/ml. Although these data represent a single patient, they suggest that the target EFV C_{trough} may be well below 1,000 ng/ml and are consistent with our estimation of EFV $PBIC_{95}$ described here. In light of these findings, reexamination of the generally accepted therapeutic concentration range of 1,000 to 4,000 ng/ml for EFV seems warranted.

In vivo exposure-response relationships for a number of the ARVs are not well defined. Suggested C_{trough} targets for some of these agents are based either on *in vitro* susceptibility data alone or studies that compare plasma drug concentrations to virologic outcomes (30, 31). For example, in the HIV-NAT 017 study, virologic outcome was assessed in PI-naïve children treated with lopinavir/ritonavir (230/57.5 mg/m^2) plus saquinavir (50 mg/kg) twice daily (4). The 2 children with a lopinavir C_{trough} of <1,000 ng/ml both experienced virologic failure (HIV RNA of >400 copies/ml) at week 24, compared to a single virologic failure among the remaining 17 children with a lopinavir C_{trough} of >1,000 ng/ml. This finding suggests that the target C_{trough} of lopinavir is approximately 1,000 ng/ml in PI-naïve patients. Similarly, plasma drug concentrations and the risk of virologic failure were evaluated in a cohort of 189 HIV-infected, NNRTI-naïve patients treated with standard dose nevirapine in combination with other agents (13). In multivariate analysis, a nevirapine plasma concentration of less than 3,000 ng/ml was associated with a 5-fold increased risk of

TABLE 2 Protein-bound IC_{95} and IQ_{95} values

Drug[a]	Dose (mg)[b]	Molar mass (g/mol)	C_{trough} (ng/ml)[c]	PBCF[d]	WT IC[e]				PBIC (ng/ml)[g]		DHHS target (ng/ml)[h]	IQ_{95} (C_{trough}/$PBIC_{95}$)
					nM[f]		ng/ml					
					50%	95%	50%	95%	50%	95%		
APV	700/100 b.i.d.	505	2,120	11	10	62	5.3	31	60	358	400	5.9
ATV	300/100 q.d.	705	800	9.3	2.4	9.2	1.7	6.5	15.9	60	150	13
DRV	600/100 b.i.d.	548	3,500	14	0.71	3.4	0.4	1.9	5.3	25	NA	138
IDV	800/100 b.i.d.	614	1,300	3.5	7.0	34	4.3	21	15.2	73	100	18
LPV	400/100 b.i.d.	629	5,500	10	5.0	27	3.1	17	31	168	1,000	33
NFV	1,250 b.i.d.	664	1,000	77	16	75	11	50	819	3,865	800	0.26
SQV	1,000/100 b.i.d.	767	400	17	4.7	18	3.6	14	60	234	100–250	1.7
TPV	500/200 b.i.d.	603	15,670	15	88	433	53	261	796	3,902	NA	4.0
EFV	600 q.d.	316	1,800	27	4.2	15	1.3	4.7	36	126	1,000	14
ETR	200 b.i.d.	435	297	33	2.2	8.2	0.9	3.5	31	116	NA	2.6
NVP	400 q.d.	266	4,500	1.4	120	952	32	253	46	366	3,000	12
RAL	400 b.i.d.	444	114	2.1	8.0	100	3.6	44	7.5	94	NA	1.2

[a] APV, amprenavir; ATV, atazanavir; DRV, darunavir; IDV, indinavir; LPV, lopinavir; NFV, nelfinavir; SQV, saquinavir; TPV, tipranavir; EFV, efavirenz; ETR, etravirine; NVP, nevirapine; RAL, raltegravir.
[b] Shown are the prescribed dose and frequency (b.i.d., twice daily; q.d., once daily). For protease inhibitors other than NFV, the amount coadministered with ritonavir is indicated after the slash.
[c] All C_{trough} values are taken from the individual drug manufacturer's prescribing information (1–3, 6–9, 17, 19, 38, 53, 54), except for those for raltegravir, which were from reference 50.
[d] Protein binding correction factor at 100% HS, extrapolated from the experimental data described above.
[e] 50% and 90%, IC_{50} and IC_{95}, respectively.
[f] The wild-type (WT) virus IC_{50} and IC_{95} data are the means of a large collection of independent determinations from clinical samples lacking drug-selected mutations in PR or RT (for PIs and NNRTIs; $n = 6,537$) or in IN (for RAL; $n = 1,200$).
[g] 50% and 95%, $PBIC_{50}$ and $PBIC_{95}$, respectively.
[h] For details, see reference 45. NA, not applicable because no target drug concentration has been determined for PI-naïve patients. For PI-experienced patients, the recommended C_{trough} values for darunavir, tipranavir, and etravirine are 550, 20, 500, and 52 ng/ml, respectively (45).

virologic failure, suggesting that a nevirapine C_{trough} of 3,000 ng/ml or greater should be maintained to obtain optimum virologic outcomes. Using similar approaches, target C_{trough} values of 150 ng/ml and 20,500 ng/ml, respectively, have been proposed for ritonavir-boosted atazanavir (atazanavir/r) and tipranavir (tipranavir/r) (20, 44); however, neither study was sufficiently powered to define threshold therapeutic concentrations. These values are higher than the $PBIC_{95}$ measures that we determined in this study (60 ng/ml and 3,902 ng/ml, respectively) (Table 2). One possible explanation is related to the use of cohort studies, which are likely to include intermittently adherent patients, to define minimum effective C_{trough} values for antiretroviral drugs. Unlike carefully conducted, wide-dose-ranging pharmacokinetic/pharmacodynamic studies, target drug concentrations estimated from cohort studies are based on variations in interpatient concentrations for a given dose administered to many individuals. Consequently, this approach generates insufficient data to accurately populate the low end of a true concentration-response curve and will tend to overestimate the threshold drug concentrations.

The $PBIC_{95}$ calculated for nelfinavir (3,865 ng/ml) in this study is considerably higher than the measured (1,000 ng/ml) or recommended target (800 ng/ml) C_{trough} (45). This discrepancy may be at least partly explained by the observation that nelfinavir is metabolized in the liver, producing metabolites, including M8 (32), which itself exhibits antiviral activity comparable to that of the parent drug (57). M8 concentrations approach 30% of the parent drug concentration, and this metabolite exhibits slightly less serum protein binding than nelfinavir (57). Since nelfinavir is not metabolized to M8 *in vitro*, our estimate of the nelfinavir $PBIC_{95}$ in this study is likely higher than the true *in vivo* value, and thus the effective IQ_{95} is likely to be underestimated.

The IQ_{95} for raltegravir (1.2) is lower than expected for a highly efficacious drug. A recent presentation reported a median plasma C_{trough} in healthy volunteers of 82 ng/ml ($n = 45$), which agrees well with our estimates of $PBIC_{95}$ but is 30% higher than the value reported on the drug label (33). *In vitro* studies have shown that dissociation of raltegravir from the preintegration complex occurs more slowly than dissociation of the preintegration complex itself, meaning that raltegravir binding is essentially irreversible (25). Results from a 48-week once-daily (800-mg) versus twice-daily (400-mg) raltegravir study (50) showed that the once-daily arm was virologically inferior to the twice-daily arm. The average 12-h concentration (C_{12}), derived from intensive pharmacokinetic assessments, was 257 nM (114 ng/ml) in the 400-mg twice daily group versus 40 nM (17.8 ng/ml) in the 800-mg once-daily group. Our results indicate that the raltegravir $PBIC_{95}$ is 94 ng/ml (211 nM), which may in part explain why the once-daily group was inferior. Relationships between C_{trough} and HIV-1 RNA response for two additional INSTI candidates have been demonstrated in wide-dose-ranging early phase monotherapy trials (11, 52).

The application of *in vitro* susceptibility data to *in vivo* $PBIC_{95}$ predictions has several limitations. The HS conditions used in the infectivity assays described here may not accurately mimic the situation *in vivo* (i.e., drug binding in human blood). Inter- and intraindividual variabilities in binding protein concentration encountered *in vivo* are not taken into account using this approach. However, our study was performed using two lots of HS, each pooled from multiple donors, to minimize the possibility of lot-specific artifacts. In addition, *in vitro* determinations of IC_{50} or IC_{95} values for individual drugs do not capture synergistic, antagonistic, or additive effects among different drugs that may occur *in vivo*. Furthermore, the use of a simple linear regression model to extrapolate PBCFs for 100% HS from the experimentally determined IC_{50}s in the presence of 23 to 81% HS may not be equally applicable to all drugs. Most importantly, there is a paucity of *in vivo* pharmacodynamic data with which our results can be validated.

Collectively, the available *in vivo* concentration-response data, which are considered the "gold standard" from the pharmacodynamic perspective, correlate strikingly well with the $PBIC_{95}$ values we have derived from *in vitro* susceptibility and PBCF measurements, with the exceptions of the cohort studies described above. Consequently, we propose that this parameter is an appropriate surrogate measure of target C_{trough} for antiretroviral drugs (excluding those that require intracellular activity [i.e., NRTI]) in lieu of formal early phase pharmacodynamic assessments. Ideally, concentration-response relationships for ARV drugs should be defined early in the development process. Phase III trials (or phase IV postmarketing studies) are not the appropriate time to define drug target concentrations because their design requires that all participants receive either one given dosage or one of several dosages within a narrow range. Late phase I or early phase II trials should incorporate wide dose-finding designs encompassing dosages that exceed 10-fold differences (preferably larger) in order to adequately populate concentration-response curves. In the absence of clearly defined concentration-response relationships, identification of precise target drug exposure is not possible.

The derivation of PBCFs that can be applied to IC_{50} data generated by routine phenotypic assays such as PhenoSense HIV also provides a useful way to relate these *in vitro* concentrations to total *in vivo* drug exposure for applications that include therapeutic drug monitoring and calculation of IQ values for viruses with reduced drug susceptibility.

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