JOURNAL OF VIROLOGY, Dec. 2007, p. 13845–13851 Vol. 81, No. 24
0022-538X/07/$08.00+0 doi:10.1128/JVI.01184-07

Binding Kinetics of Darunavir to Human Immunodeficiency Virus Type 1 Protease Explain the Potent Antiviral Activity and High Genetic Barrier▽

Inge Dierynck,* Mieke De Wit, Emmanuel Gustin, Inge Keuleers, Johan Vandersmissen, Sabine Hallenberger, and Kurt Hertogs

Tibotec BVBA, Mechelen, Belgium

Received 31 May 2007/Accepted 30 September 2007

The high incidence of cross-resistance between human immunodeficiency virus type 1 (HIV-1) protease inhibitors (PIs) limits their sequential use. This necessitates the development of PIs with a high genetic barrier and a broad spectrum of activity against PI-resistant HIV, such as tipranavir and darunavir (TMC114). We performed a surface plasmon resonance-based kinetic study to investigate the impact of PI resistance-associated mutations on the protease binding of five PIs used clinically: amprenavir, atazanavir, darunavir, lopinavir, and tipranavir. With wild-type protease, the binding affinity of darunavir was more than 100-fold higher than with the other PIs, due to a very slow dissociation rate. Consequently, the dissociative half-life of darunavir was much higher (>240 h) than that of the other PIs, including darunavir's structural analogue amprenavir. The influence of protease mutations on the binding kinetics was tested with five multidrug-resistant (MDR) proteases derived from clinical isolates harboring 10 to 14 PI resistance-associated mutations with a decreased susceptibility to various PIs. In general, all PIs bound to the MDR proteases with lower binding affinities, caused mainly by a faster dissociation rate. For amprenavir, atazanavir, lopinavir, and tipranavir, the decrease in affinity with MDR proteases resulted in reduced antiviral activity. For darunavir, however, a nearly 1,000-fold decrease in binding affinity did not translate into a weaker antiviral activity; a further decrease in affinity was required for the reduced antiviral effect. These observations provide a mechanistic explanation for darunavir's potent antiviral activity and high genetic barrier to the development of resistance.

Since their introduction in 1995, protease inhibitors (PIs) have become a cornerstone of highly active antiretroviral therapy (HAART), with a remarkable decline in morbidity and mortality associated with human immunodeficiency virus type 1 (HIV-1) infection (24). PIs work by blocking the ability of HIV-1 protease to process the viral polypeptides Gag and Gag-Pol into structural and enzymatic proteins during the final stages of viral particle maturation.

Currently, there are nine HIV-1 PIs in clinical use (amprenavir [APV], atazanavir [ATV], darunavir [DRV], indinavir [IDV], lopinavir [LPV], nelfinavir [NFV], ritonavir [RTV], saquinavir [SQV], and tipranavir [TPV]), and additional inhibitors are undergoing clinical trials (19). Despite the success of PIs in HAART, the emergence of multidrug-resistant (MDR) viruses has resulted in a high incidence of cross-resistance between PIs, which limits their sequential use, thereby reducing the treatment options available for HIV patients (4, 26). Thus, there is an ongoing clinical requirement for new antiviral agents with broad-spectrum activity against PI-resistant mutants for the long-term management of HIV infection. DRV, previously known as TMC114, is a very effective PI (3, 28) with high in vitro and in vivo potency against wild-type (WT) and MDR HIV-1. Moreover, DRV appears to have a very high genetic barrier to the development of resistance (2).

Although drug resistance is a complex phenomenon, the most important effect at the molecular level is the introduction of mutations within the protease gene, resulting in an enzyme with significantly decreased binding affinity for the inhibitor, with respect to the protease substrates (11). An inhibitor with a good resistance profile would possess high binding affinity to WT protease which would not be significantly reduced by mutations associated with drug resistance. Inhibition studies and in vitro resistance selection experiments are insufficient for delineating the interactions between HIV-1 protease and its inhibitors, in regard to resistance. Essential information on drug-target interactions can be derived from thermodynamic and kinetic binding studies.

By assessing the binding thermodynamics of DRV, using isothermal titration calorimetry, it has been shown that DRV binds tightly to WT HIV-1 protease with a favorable enthalpy due to the strong interactions between the bis-tetrahydrofuranyl urethane moiety of DRV and the main-chain atoms of Asp29 and Asp30 in the protease-active site (12, 13). This high affinity together with the close fit of DRV within the substrate envelope could explain DRV's high potency and much lower level of resistance selection.

Biosensor-based studies, or surface plasmon resonance (SPR) technology, are an efficient and sensitive methodology used to analyze the binding kinetics of an interaction (21, 25). This type of assay allows the measurement of an association

* Corresponding author. Mailing address: Tibotec BVBA, Gen De Wittelaan L 11B 3, 2800 Mechelen, Belgium. Phone: 32 15 461229. Fax: 32 15 444374. E-mail: idierinc@tibbe.jnj.com.

▽ Published ahead of print on 10 October 2007.

TABLE 1. Genotypic profile of the MDR proteases derived from clinical isolates[a]

Wild-type →	V	Q	L	I	I	Q	K	D	V	L	E	E	M	S	K	K	M	I	I	F	I	K	R	Q	D	I	L	I	I	A	I	V	V	V	N	I	I	N	L	L	Q	I
Position →	3	7	10	13	15	18	20	30	32	33	34	35	36	37	43	45	46	47	50	53	54	55	57	58	60	62	63	64	66	71	72	75	77	82	83	84	85	88	89	90	92	93
PR mutant A (14-5)		K	F	V	V			N	I*	F*		D	I	N				V*			L*			E		V	P			V						V*		D	T	M		
PR mutant B (12-5)	I	K	F	V		H	T	N	I*	F*	N	D	I	N	E	R		V*			M*				N		P		F	T	T	I	I					D	V*	M	K	
PR mutant C (13-4)	I	K							I*	F*		D	I	N	T		I	V*	V*			R	K			V	P			L				I			V			M		L
PR mutant D (11-1)	I	K			V		R		I*			D	I	E			L			L	V						P			L	V	V		T			V			M		
PR mutant E (10-1)	I	K	V		I					F*		D	L	T	T		L				V	R	K			V	P	V			I			T	D							

[a] The number of IAS-USA PI RAMs (x) and the number of PI mutations associated with a diminished susceptibility to DRV (y) are mentioned between parentheses ($x - y$) for each mutant. Primary PI mutations are indicated in pink, PI RAMs are in orange, and DRV RAMs are indicated by an asterisk. The mutants are ranked (A to E) according to a decreasing number of DRV RAMs.

rate (k_{on}) next to a dissociation rate (k_{off}), which are indicators for, respectively, the degree of recognition and the binding stability of the ligand-analyte interaction. The ratio of the two is a measure of the affinity or binding strength of the analyte for the ligand ($K_D = k_{off}/k_{on}$), meaning that a high affinity can be caused by a high association or a slow dissociation rate (7).

To further establish the molecular basis for DRV's high antiviral potency and broad-spectrum activity, we measured the binding affinities of DRV and other currently approved PIs to WT and MDR proteases by using SPR. The MDR proteases were derived from clinical isolates containing 10 to 14 International AIDS Society-U.S.A. (IAS-USA) PI resistance-associated mutations (RAMs) (6) and included mutants with a decreased susceptibility to DRV. The MDR proteases contained 1 to 5 of the 11 mutations (V11I, V32I, L33F, I47V, I50V, I54L/M, G73S, L76V, I84V, and L89V) identified as those associated with a diminished DRV/ritonavir virologic response and present mostly with a high number of other PI RAMs (S. De Meyer, T. Vangeneugden, E. Lefebvre, H. Azijn, I. De Baere, B. Van Baelen, and M.-P. de Béthune, phenotypic and genotypic determinants of resistance to TMC114: pooled analysis of POWER 1, 2, and 3, presented at the XVth International HIV Drug Resistance Workshop, Sitges, Spain, 13 to 17 June 2006).

MATERIALS AND METHODS

Enzymes and inhibitors. The WT HIV-1 protease containing the Q7K mutation was a gift from C. A. Schiffer (University of Massachusetts Medical School, Worcester, MA). The genotypes of the mutant proteases used in this study are shown in Table 1. Protease genes of mutants A, C, and D were amplified from clinical isolate RNA by reverse transcription-PCR (RT-PCR), while those of mutants B and E were constructed by using synthetic oligonucleotides optimized for *Escherichia coli* codon usage. All mutant proteases included an additional Q7K substitution to prevent autoproteolysis. Plasmid-encoded protease genes of mutants A, C, and D were expressed and purified using standard procedures as described previously (11, 22), where for mutants B and E, an adapted protocol was used. Mutants B and E were expressed as inclusion bodies in *Escherichia coli* BL21(DE3) cells. After protein extraction by disintegration and solubilization in urea-containing buffer, these mutant enzymes were purified using ion-exchange chromatography (Q Sepharose Fast Flow column) and size exclusion chromatography (S75 preparative gel filtration column). The purified mutant B and E enzymes were refolded in 25 mM formic acid, and a final purification was performed with an S30 preparative gel filtration column equilibrated with 0.05 M sodium acetate at pH 5.5, with 10% glycerol and 5 mM dithiothreitol. Purity of the protease preparations was measured by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by Coomassie blue staining. All preparations proved to be >95% pure.

The MDR proteases were derived from clinical isolates with decreased susceptibility to multiple HIV-1 PIs and contained 10 to 14 PI RAMs, based on the IAS-USA fall 2005 guidelines (6).

The PIs ATV, APV, IDV, LPV, NFV, RTV, and SQV were purified from commercial formulations. DRV and TPV were produced as previously reported (28, 29). The purity of all PIs was >95% as determined by liquid chromatography-mass spectrometry.

Viruses. Recombinant HIV-1 isolates derived from clinical samples were constructed as previously described by cotransfection of MT4 cells with sample-derived viral protease and reverse transcriptase coding sequences and an HIV-1 HXB2-derived proviral clone deleted in the protease and reverse transcriptase coding region (5).

Antiviral assay. The antiviral activities of PIs against the laboratory-adapted WT (HIV-1-LAI) isolate and clinical sample-derived recombinant viruses were determined with a cell-based HIV-1 replication assay. MT4 cells equipped with long-terminal repeat-enhanced green fluorescent protein (EGFP) (150,000 cells/ml) were infected with HIV-1 (at a multiplicity of infection of 0.01) in the presence or absence of different inhibitor concentrations. Upon infection by HIV-1, expression of the viral Tat product increased transcription from the HIV-1 LTR promoter, leading to high-level expression of the EGFP reporter. After 3 days of incubation, the amount of HIV-1 replication was quantified by measuring the EGFP fluorescence and expressed as the 50% effective concentration (EC_{50}) value or as the (n-fold) change in susceptibility by dividing the EC_{50} value for the virus tested by the EC_{50} value for the WT virus tested in parallel.

Interaction studies. Measurements of interactions between WT or mutant proteases and inhibitors were performed using a Biacore S51 instrument (Biacore AB, Uppsala, Sweden), essentially as described previously (17). Proteases were immobilized using amine coupling to CM5 sensor chips (Biacore AB) with an extra cross-linking step of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS) for 7 min (WT) or 2 min (MDR) to stabilize the surface. Protease enzymes were dissolved at a concentration range of 2 to 10 μM in acetate buffer at pH 4.5 and immobilized at a flow rate of 10 μl/min and a contact time that varied between 10 and 20 min. In Biacore instruments, the amount of immobilized protein is measured in resonance units (RU), with one RU representing about 1 pg of protein bound per square millimeter of flow cell surface. In our system, we routinely reached a level of 1,500 to 3,000 RU of immobilized protein. Interaction studies were all performed at 20°C with a flow rate of 90 μl/min to minimize mass transport effects. Inhibitors were serially diluted in phosphate-buffered saline containing 5% dimethyl sulfoxide (DMSO) and 3 mM EDTA and supplemented with 0.005% or 0.05% P20, a nonionic surfactant, for studies with the WT and mutant proteases, respectively. The higher P20 level for mutant protease binding studies was needed to obtain binding levels that were high enough to study the weaker interactions of the mutant proteases. Increasing the P20 concentration from 0.005% to 0.05% did not influence PI binding characteristics when applied to WT protease binding studies (data not shown). The inhibitor concentration range was chosen based on the antiviral activity (EC_{50}) of the inhibitor on the corresponding MDR virus. The compounds were injected for a period of 215 s and dissociated for a period of 1,000 s. A duplicate sample and a zero concentration sample were used as a positive and negative control, respectively. A DMSO concentration series was included to eliminate the contribution of a bulk solution to the measured response. Protease immobilization levels of 1,500 to 3,000 RU resulted in binding responses of 5 to 20 RU at the highest compound concentrations. The sensor surface was regenerated in between sample injections by injecting 100% ethylene glycol, and the flow system was washed with 50% DMSO. Buffer injections were conducted to detect possible carryover between samples.

TABLE 2. Kinetic parameters for the binding of PIs to WT HIV-1 protease[a]

PI	Parameters						
	k_{on} ($M^{-1}s^{-1}$) ± SEM	k_{off} (s^{-1}) ± SEM	K_D (M) ± SEM	$t_{1/2}$ (s)	No. of measurements	K_D reference (M)	EC_{50} (M)
DRV	$(2.2 \pm 0.8) \times 10^6$	**$(7.8 \pm 0.5) \times 10^{-7}$**	**$(4.1 \pm 1.7) \times 10^{-13}$**	**890,000**	5		6.8×10^{-9}
APV	$(2.1 \pm 0.8) \times 10^6$	$(8.6 \pm 1.0) \times 10^{-4}$	$(7.1 \pm 1.6) \times 10^{-10}$	810	7	7.3×10^{-10}	4.8×10^{-8}
ATV	$(3.8 \pm 0.5) \times 10^5$	$(1.4 \pm 0.1) \times 10^{-4}$	$(3.6 \pm 0.3) \times 10^{-10}$	5,000	4	4.0×10^{-10}	1.1×10^{-8}
IDV	$(6.6 \pm 2.3) \times 10^5$	$(7.8 \pm 1.5) \times 10^{-4}$	$(1.9 \pm 0.9) \times 10^{-9}$	890	4	1.7×10^{-9}	4.0×10^{-8}
LPV	$(8.6 \pm 0.8) \times 10^5$	$(1.6 \pm 0.1) \times 10^{-4}$	$(1.9 \pm 0.2) \times 10^{-10}$	4,300	3	1.0×10^{-10}	1.4×10^{-8}
NFV	$(1.3 \pm 0.4) \times 10^5$	$(2.5 \pm 0.2) \times 10^{-4}$	$(2.8 \pm 1.2) \times 10^{-9}$	2,800	4	3.7×10^{-9}	6.9×10^{-8}
RTV	$(5.8 \pm 1.1) \times 10^5$	$(8.1 \pm 1.1) \times 10^{-4}$	$(1.6 \pm 0.3) \times 10^{-9}$	860	6	8.3×10^{-10}	6.4×10^{-8}
SQV	$(1.4 \pm 0.5) \times 10^5$	$(1.5 \pm 0.6) \times 10^{-4}$	$(1.2 \pm 0.3) \times 10^{-9}$	4,600	4	3×10^{-10}	2.6×10^{-8}
TPV	$(2.0 \pm 0.1) \times 10^5$	$(1.1 \pm 0.5) \times 10^{-4}$	$(4.9 \pm 2.3) \times 10^{-10}$	6,300	5		4.4×10^{-7}

[a] Kinetic parameters were determined by kinetic evaluation of interaction studies performed at 20°C on a Biacore S51. Values are given as means ± standard errors of the means (SEM). Values outside the detection limits are in bold type. The EC_{50} values were determined by an antiviral assay as described in Materials and Methods. K_D reference values, measured at 25°C, were obtained from the literature (27).

Data analysis. As it was not possible to reach an equilibrium state in most of the experiments, all analyses were based on the evaluation of binding kinetics (8). Data were analyzed using simultaneous nonlinear regression analysis (global fitting) with Biacore S51 or Biacore T100 BiaEval evaluation software (Biacore AB).

The individual rate constants k_{on} and k_{off} and a derived affinity constant, $K_D = k_{off}/k_{on}$, were determined by a kinetic evaluation of the sensorgrams fitting a 1:1 model or a heterogeneous ligand model. Experimentally, compound concentrations exceeding the K_D value need to be used to allow a reliable analysis. For WT protease, where the K_D value is low, experiments were done with correspondingly low concentrations of compound, and binding effects nonspecific to the protease target were undetectable. Since the evaluated inhibitors were expected to have

TABLE 3. Kinetic parameters of the interaction between MDR HIV-1 protease (PR) mutants and PIs[a]

PI	Virus	Parameter					
		k_{on} ($M^{-1}s^{-1}$) ± SEM	k_{off} (s^{-1}) ± SEM	K_D (M) ± SEM	$t_{1/2}$ (s)	No. of measurements	FC
DRV	WT	$(2.2 \pm 0.8) \times 10^6$	**$(7.8 \pm 0.5) \times 10^{-7}$**	**$(4.1 \pm 1.7) \times 10^{-13}$**	**890,000**	5	1.0
	PR mutant A	$(1.5 \pm 0.5) \times 10^6$	$(5.6 \pm 2.6) \times 10^{-2}$	$(3.6 \pm 0.6) \times 10^{-8}$	12	2	170
	PR mutant B	$(1.6 \pm 0.1) \times 10^6$	$(4.2 \pm 0.1) \times 10^{-2}$	$(2.6 \pm 0.1) \times 10^{-8}$	17	2	140
	PR mutant C	$(2.1 \pm 1.7) \times 10^5$	$(3.5 \pm 0.06) \times 10^{-2}$	$(4.7 \pm 3.7) \times 10^{-7}$	20	2	110
	PR mutant D	$(2.8 \pm 0.06) \times 10^6$	$(8.0 \pm 0.1) \times 10^{-3}$	$(2.9 \pm 0.1) \times 10^{-9}$	87	2	4.7
	PR mutant E	$(5.6 \pm 0.03) \times 10^5$	$(2.2 \pm 0.3) \times 10^{-4}$	$(3.8 \pm 0.5) \times 10^{-10}$	3,200	2	1.0
APV	WT	$(2.1 \pm 0.8) \times 10^6$	$(8.6 \pm 1.0) \times 10^{-4}$	$(7.1 \pm 1.6) \times 10^{-10}$	810	7	1.0
	PR mutant A	**KL**	**KL**	**KL**	**KL**		350
	PR mutant B	**KL**	**KL**	**KL**	**KL**		380
	PR mutant C	$(2.6 \pm 1.8) \times 10^5$	**$(1.2 \pm 0.05) \times 10^{-1}$**	**$(8.8 \pm 8.8) \times 10^{-7}$**	**5.8**	2	210
	PR mutant D	$(4.4 \pm 2.3) \times 10^4$	$(2.0 \pm 1.9) \times 10^{-2}$	$(3.2 \pm 2.6) \times 10^{-7}$	34	2	30
	PR mutant E	$(3.4 \pm 0.8) \times 10^5$	$(1.4 \pm 0.4) \times 10^{-2}$	$(3.9 \pm 0.4) \times 10^{-8}$	51	3	16
ATV	WT	$(3.8 \pm 0.5) \times 10^5$	$(1.4 \pm 0.1) \times 10^{-4}$	$(3.6 \pm 0.3) \times 10^{-10}$	5,000	4	1.0
	PR mutant A	$(4.0 \pm 2.6) \times 10^5$	$(9.5 \pm 2.9) \times 10^{-2}$	$(3.2 \pm 1.3) \times 10^{-7}$	7.3	2	500
	PR mutant B	$(3.1 \pm 1.6) \times 10^6$	$(1.7 \pm 0.4) \times 10^{-3}$	$(6.5 \pm 1.9) \times 10^{-10}$	4,100	2	65
	PR mutant C	$(5.5 \pm 0.9) \times 10^5$	$(9.8 \pm 7.8) \times 10^{-5}$	$(1.6 \pm 1.1) \times 10^{-10}$	7,100	2	5.0
	PR mutant D	$(2.5 \pm 0.5) \times 10^4$	**$(2.8 \pm 0.6) \times 10^{-1}$**	**$(1.2 \pm 0.4) \times 10^{-5}$**	**25**	2	380
	PR mutant E	$(3.1 \pm 1.6) \times 10^5$	$(1.3 \pm 0.5) \times 10^{-2}$	$(4.5 \pm 0.7) \times 10^{-8}$	53	2	55
LPV	WT	$(8.6 \pm 0.8) \times 10^5$	$(1.6 \pm 0.1) \times 10^{-4}$	$(1.9 \pm 0.2) \times 10^{-10}$	4,300	3	1.0
	PR mutant A	**KL**	**KL**	**KL**	**KL**		200
	PR mutant B	**KL**	**KL**	**KL**	**KL**		260
	PR mutant C	$(9.1 \pm 5.8) \times 10^5$	$(1.7 \pm 1.3) \times 10^{-2}$	$(1.6 \pm 0.4) \times 10^{-8}$	41	2	40
	PR mutant D	$(1.1 \pm 0.1) \times 10^4$	$(2.9 \pm 0.7) \times 10^{-2}$	$(2.5 \pm 0.3) \times 10^{-6}$	24	2	52
	PR mutant E	$(3.2 \pm 0.02) \times 10^6$	$(7.7 \pm 0.2) \times 10^{-2}$	$(2.4 \pm 0.1) \times 10^{-8}$	9.0	2	69
TPV	WT	$(2.0 \pm 0.1) \times 10^5$	$(1.1 \pm 0.5) \times 10^{-4}$	$(4.9 \pm 2.3) \times 10^{-10}$	6,300	5	1.0
	PR mutant A	$(2.0 \pm 0.2) \times 10^5$	$(5.5 \pm 2.3) \times 10^{-4}$	$(2.7 \pm 0.8) \times 10^{-9}$	1,300	2	3.0
	PR mutant B	$(2.5 \pm 0.01) \times 10^5$	$(6.6 \pm 1.5) \times 10^{-4}$	$(2.6 \pm 0.6) \times 10^{-9}$	11,000	2	5.6
	PR mutant C	**NDT**	**NDT**	**NDT**	**NDT**		1.0
	PR mutant D	**NDT**	**NDT**	**NDT**	**NDT**		8.0
	PR mutant E	$(1.7 \pm 1.1) \times 10^4$	$(5.2 \pm 0.6) \times 10^{-3}$	$(5.7 \pm 3.5) \times 10^{-7}$	130	2	22

[a] Kinetic parameters were determined by the kinetic evaluation of interaction studies performed at 20°C on a Biacore S51. Values outside the detection limits are in bold type. KL, kinetic limitations due to fast-on, fast-off components. NDT, not determinable due to low response levels and inaccurate fitting. FC, fold change in EC_{50} compared with that of WT HIV-1. Values are given as means ± standard errors of the means (SEM).

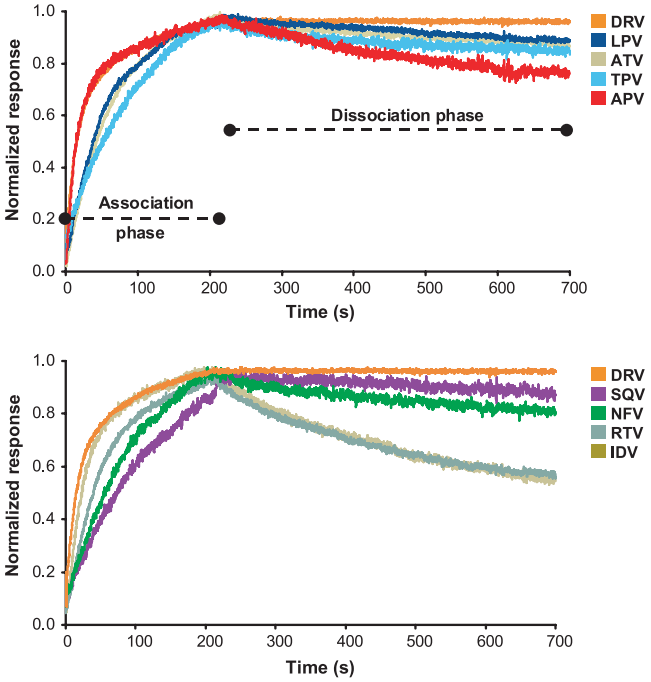

FIG. 1. Overlay of the experimental sensorgrams of the interaction between WT protease and its inhibitors at a concentration of 40 nM (ATV, IDV, LPV, NFV, RTV, SQV, and TPV) or 50 nM (APV and DRV). The sensorgrams were normalized to a maximum response of 1. For clarity, sensorgrams are spread over two graphs, with DRV included in both graphs as a reference point.

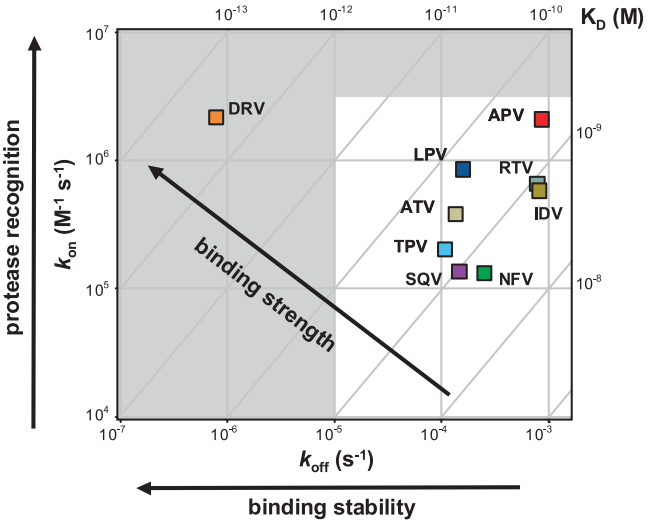

FIG. 2. WT protease interaction kinetic map for all currently used PIs shown as k_{on} and k_{off} values and the combinations of k_{on} and k_{off} that result in the same K_D values (diagonal lines). The area beyond the detection limits due to biophysical and instrument limitations is shaded.

only one binding site on the protease, the 1:1 Langmuir binding model was used (15). This model, assuming a single type of target binding site, was found to accurately fit the binding. For mutant proteases, the 1:1 model mismatched kinetic data due to additional binding components, particularly at high compound concentrations. Experiments using immobilized HIV-1 gp120 protein (data not shown) revealed that the additional binding components were not specific to the protease target but reflected the propensity of the tested PIs to bind nonspecifically to proteins. Therefore, the heterogeneous ligand model, which is essentially the sum of two independent 1:1 binding models (two k_{on}, k_{off} pairs), was used (10, 20). This model is a strong simplification, as nonspecific binding involves many different locations with many different binding constants, resulting in a binding level that does not saturate with increasing compound concentrations. However, the existing software did not allow more accurate modeling. Of the two sets of kinetic parameters generated, only one was considered relevant to compound activity. Components that occurred at the highest concentrations, that possessed unstable binding characteristics (high k_{off} rates), and that did not saturate as a function of increasing concentration were categorized as nonspecific binding, but coverage of the 0.1 to 10× K_D range was assured. The applied fit models accounted for limited mass transport effects.

To prevent suboptimal fitting, we required all important features of the data to be represented by the model and verified that no component of the model was dependent on minor features that could be due to experimental deviations. Every analysis was performed on at least two independent experiments, and it was verified that the fit values for k_{on}, k_{off}, or K_D were reproduced within 1 order of magnitude.

Detection limits. Instrument limitations resulted in detection limits of 10^4 to 5×10^6 $M^{-1}s^{-1}$ for the k_{on} value and 10^{-1} to 10^{-5} s^{-1} for the k_{off} value (R. Karlsson, personal communication; 23). Values outside these detection limits (Table 2 and Table 3, bold type) should be interpreted as censored values, indicating kinetics that were slower or faster than the instrument was able to measure. The term kinetic limitation is used in Table 3 if the k_{on} and k_{off} values were so far outside the detection range that even an approximate determination was not possible. The fast kinetics of PI binding on MDR mutant proteases was the main reason for performing our measurements at the relatively low temperature of 20°C. Although a higher temperature would allow a more accurate determination of the DRV k_{off} value for WT protease (k_{off} value above the lower detection limit), it would compromise the measurements of mutants (k_{off} value beyond the upper detection limit).

RESULTS

Kinetics of inhibitor binding to WT protease. Figure 1 shows an overview of kinetic binding studies of the different PIs with WT protease, with an overlay of the normalized sensorgrams obtained at a PI concentration of 40 or 50 nM. The kinetic parameters of these interactions are listed in Table 2. Values outside the detection limits are in bold and should be interpreted as censored values, indicating kinetics that are slower or faster than the instrument can measure. Owing to the relatively high standard-error-of-measurement values compared to the point values, only point value differences of at least 10-fold were deemed relevant.

DRV demonstrated a fast association but a very slow dissociation from WT HIV-1 protease compared with those of other PIs. The association of DRV to WT protease was 1 order of magnitude faster than that of other PIs, except for DRV's structural analogue APV, which established a comparable association rate (k_{on} of $\sim 2 \times 10^6$ $M^{-1}s^{-1}$). The dissociation of DRV from WT protease was extremely slow, up to 1,000 times slower than the dissociation for the other PIs tested, including APV. In fact, the k_{off} value of the DRV-WT interaction was too slow to be reliably measured (k_{off} of $<10^{-5}$ s^{-1}). This interaction profile of DRV resulted in a very tight binding to WT protease, with an affinity that was more than 2 orders of magnitude higher ($K_D < 10^{-12}$ M) than that for the other PIs. The extremely high stability of the binding of DRV to WT protease is also illustrated in the interaction kinetic diagram (Fig. 2, detection limits of the assay determined by biophysical and instrument limitations are shaded) (R. Karlsson, personal communication; 23), in which the diagonal lines represent different combinations of k_{on} and k_{off} values, yielding identical affinities. The binding affinity of TPV to WT protease was in

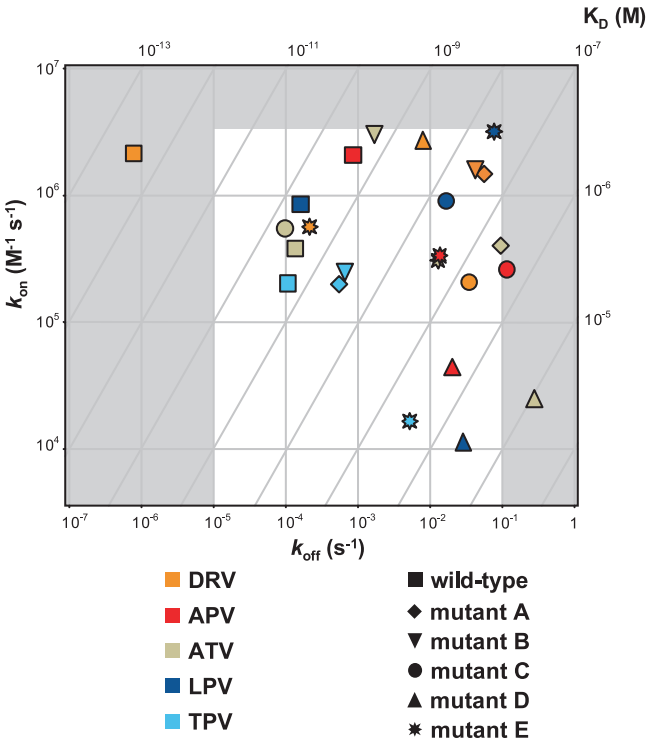

FIG. 3. Interaction kinetic map of binding studies between APV, ATV, DRV, LPV, and TPV on WT and MDR proteases. The area beyond the detection limits due to biophysical and instrument limitations is shaded.

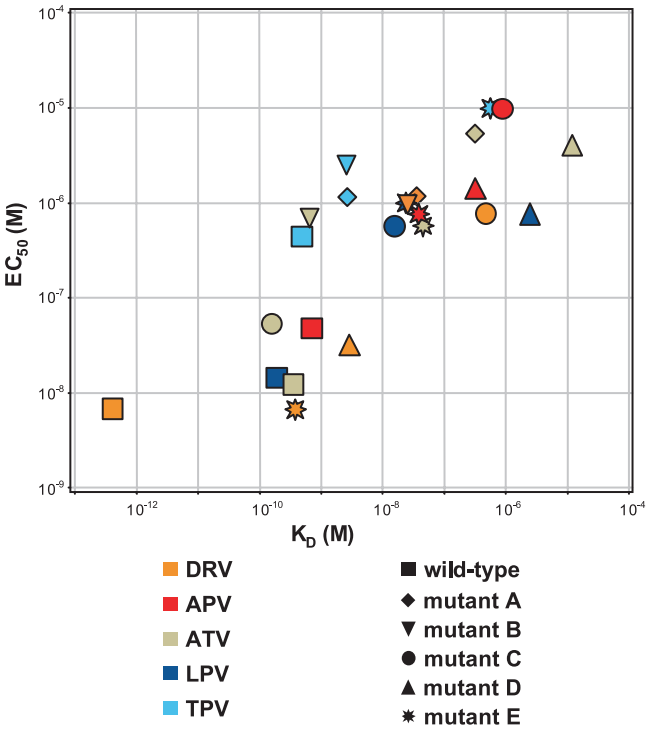

FIG. 4. Relationship between binding affinity (K_D) and antiviral activity (EC_{50}) of PIs to HIV-1 MDR proteases.

the same range as that of APV, ATV, and LPV, whereas its in vitro antiviral activity was at least 10-fold lower. The kinetic parameters determined were in line with previously published results, as indicated in the K_D reference value column in Table 2 (27).

The dissociation rate of a kinetic interaction can be translated into a dissociative half-life [$t_{1/2} = \ln(2)/k_{off}$] that is representative of the interaction time between the protease and its inhibitors. The $t_{1/2}$ of DRV with the WT protease was extremely high (>240 h) in comparison with those of the other PIs because of its slow dissociation rate (Table 2).

Kinetics of inhibitor binding to MDR protease mutants. The binding kinetics of the clinical PIs APV, ATV, DRV, LPV, and TPV with five MDR protease mutants were studied. These protease mutants were derived from clinical isolates with a decreased susceptibility to multiple HIV-1 PIs and contained 10 to 14 PI RAMs, based on the IAS-USA fall 2005 guidelines (6). The genotypes of the mutants are shown in Table 1.

In general, all PIs tested displayed a weaker binding affinity to the MDR proteases than to the WT protease. For DRV, the decrease in binding affinity to MDR proteases compared to that of WT protease was caused mainly by a faster dissociation rate, as the drop in association rate was less than 10-fold (Table 3 and Fig. 3) in all experiments. The association rates of DRV with the MDR proteases were higher than or similar to those of the other tested PIs, except for the binding of LPV to the mutant E (LPV, k_{on} of ~3.2×10^6 $M^{-1}s^{-1}$, versus DRV, k_{on} of ~5.6×10^5 $M^{-1}s^{-1}$). The dissociation rates of DRV from the MDR proteases were more than 1,000-fold faster than that from the WT protease but remained in most cases slower than or similar to those of the other tested PIs. The dissociation rates of ATV and TPV were slower than that of DRV for mutants B and C and mutants A and B, respectively, resulting in a higher binding affinity. The binding affinity of DRV was proportional to the number of mutations associated with a diminished susceptibility to DRV (De Meyer et al.). The mutants A, B, and C, containing four or five of such mutations, displayed the lowest binding affinity and hence a large (*n*-fold) change in antiviral activity. An accurate kinetic characterization of the binding of APV and LPV to mutants A and B was not possible because the interactions were too unstable to be measured, with very high association and dissociation rates (kinetic limitations). ATV and LPV showed a high decrease in binding affinity for mutant D, caused by a change in both the association and dissociation rates. Also for APV and TPV, there was a drop in both the association and the dissociation rates with mutant E, resulting in a decreased binding strength. The binding strength of DRV for mutant D and E proteases was at least 100-fold higher than for the other PIs, including APV, although it was lower than that for the WT protease. The association rates of TPV to the mutants A, B, and E were approximately 10-fold slower than that of DRV. For mutants C and D, extremely low binding levels (mutant D) and an association and/or dissociation rate outside the detection limits (mutants C and D) prevented accurate determination of TPV's kinetic parameters and were scored as not determinable (Table 3). Attempts to increase the immobilization level of mutant D and hence the binding level of TPV were unsuccessful (data not shown).

The observed changes in binding affinity were consistent

with the in vitro antiviral activity data relative to the corresponding MDR viruses for APV, ATV, LPV, and TPV (Fig. 4). A decrease in affinity of the MDR proteases correlated from the onset with a drop in antiviral activity for these PIs. However, for DRV, the PI RAMs present in mutant E (L10V, L33F, M36L, K43T, M46L, I54V, I62V, L63P, and V82T) decreased the binding affinity up to 1,000-fold, to the level of affinity of the other PIs to WT protease, but without affecting antiviral activity of the corresponding viral strain (Table 3). Only a further decrease in affinity correlated with a decreased antiviral effect of DRV.

DISCUSSION

To investigate the molecular basis for the high antiviral efficiency and broad-spectrum activity of DRV, we performed a kinetic binding study with DRV and other approved PIs with the WT and MDR proteases by using SPR.

With the WT protease, the binding affinity of DRV was more than 2 orders of magnitude higher than that of other approved PIs. This was due mainly to the extremely slow dissociation of DRV from WT protease, although its association also compared favorably to that of all tested PIs, with the exception of that of APV, which showed a similar association rate. However, while the association rates of DRV and its structural analogue APV were similar, the more than 100-fold difference in dissociation rates resulted in a much higher binding affinity of DRV to WT protease than with APV. Also, the thermodynamic binding characteristics determined with isothermal titration calorimetry reflect the high binding affinity of DRV to WT protease (12). DRV has been shown to have a significantly higher binding affinity than narrow-spectrum PIs, determined by both favorable enthalpy and entropy changes. In thermodynamic studies, the binding of DRV to WT protease was also approximately 2 orders of magnitude tighter than that of APV, with an extremely favorable enthalpy of binding for DRV compared to that of APV.

The k_{off} value is believed to be a key indicator of in vivo duration of efficacy, more significant than the apparent affinity of the drug for its target, per se (1). The dissociative $t_{1/2}$ value, which can be derived from the dissociation rate, is a direct measure of the interaction time between a drug and its target. DRV's extremely high $t_{1/2}$ value with WT protease (>240 h) suggests that, in vivo, the drug remains bound and thus active throughout its elimination from plasma (terminal elimination half-life in plasma of ~15 h when coadministered with RTV [V. Sekar, S. Spinosa-Guzman, E. Lefebvre, and R. Hoetelmans, clinical pharmacology of TMC114: a new HIV protease inhibitor, presented at the 16th International AIDS Conference, Toronto, Canada, 13 to 18 August 2006]). Thus, the duration of DRV action in vivo may be extended in comparison with that of the other PIs as ATV, LPV, SQV and TPV have a $t_{1/2}$ between 1.2 and 1.8 h, while the interaction time of APV, IDV, and RTV with WT protease was limited to approximately 15 min.

PI resistance resulting from multiple protease mutations could arise as a result of decreases in association rates as well as increases in dissociation rates. In our experiments, the decrease in PI binding affinity with the studied MDR mutants was in general related to a faster dissociation rate. For DRV, a dissociation rate more than 1,000-fold faster than that of WT protease was the main cause of lower affinity as the decrease in association rate was, in all experiments, less than 10-fold. For APV, ATV, LPV, and TPV, the decrease in association rate was, for some mutants, also a contributing factor to the decreased affinity compared to that of WT protease. The association rates of DRV with the MDR proteases remained higher than or in the same range as those of the other PIs. Also the dissociation rates of DRV were slower than or similar to that of the other PIs, except for those mutants containing four to five protease mutations associated with a diminished susceptibility to DRV. For these mutants, ATV and/or TPV showed a slower dissociation rate and hence a higher binding affinity than that of DRV. The binding affinity of DRV to MDR proteases was proportional to the number of DRV RAMs. As such, decreased binding affinity to MDR proteases explains the molecular mechanism of resistance to DRV and the other PIs. DRV displayed a higher binding affinity to the MDR proteases than the chemically related APV. The binding of APV to the MDR mutants with five DRV-associated mutations was even too unstable to be measured. Although the antiviral activity of TPV was significantly lower than that of APV, ATV, and LPV, the binding affinity of TPV to WT protease was in the same range as that of these other PIs. This discrepancy between binding affinity and antiviral activity could be due to a limited permeability of TPV. The association rates of TPV with both WT and MDR proteases were also systematically 10-fold lower than that of DRV.

A more than 1,000-fold decrease in affinity for DRV compared to that of WT protease as observed for mutants A to D was needed to decrease its corresponding antiviral effect. For APV, ATV, LPV, and TPV, a decrease in binding affinity correlated from the onset with a drop in antiviral activity. The large drop in binding affinity of DRV that is sustained without compromising the corresponding antiviral activity provides a mechanistic background for the higher genetic barrier of DRV to development of resistance. The exceptionally high affinity of DRV for WT protease is a prerequisite for making this lag phase in affinity-activity correlation possible.

It has been suggested that DRV may have two binding sites on WT protease and that this would contribute to the high genetic barrier to resistance of DRV (14). Two binding sites with distinct binding kinetics would result from deviations from the 1:1 model on condition that the K_D values for both binding interactions were within our studied concentration range. However, the 1:1 model accurately fitted the binding of all PIs studied, including DRV, to WT protease, indicating that any binding to alternate sites must be at least an order of magnitude weaker. A second binding component did appear in experiments with MDR proteases if higher compound concentrations had to be used, but the characteristics of this component indicated that it corresponded to nonspecific binding and not to binding to a protease target site.

The high impact of the dissociation rate of DRV on its affinity value confirms the relevance of the k_{off} value for evaluation of the efficacy of a compound. As DRV already starts with an extremely high affinity to WT protease, it can sustain a substantial decrease in its binding affinity on MDR mutants without the loss of antiviral activity, allowing it to remain effective, as was shown in the POWER studies (9). The very

high binding affinity of DRV to WT protease necessitates the simultaneous occurrence of multiple mutations before the virus becomes resistant to DRV. Further kinetic characterization will explore the influence of individual mutations on DRV binding to HIV-1 protease.

ACKNOWLEDGMENTS

We thank Lieve Michiels for many helpful discussions on the kinetic experiments, Celia A. Schiffer (University of Massachusetts Medical School, Worcester, MA) for providing WT protease, and Luc Geeraert for help in the preparation of the manuscript.

REFERENCES

1. **Copeland, R. A., D. L. Pompliano, and T. D. Meek.** 2006. Drug-target residence time and its implications for lead optimization. Nat. Rev. Drug Discov. **5:**730–739.
2. **De Meyer, S., H. Azijn, D. L. N. G. Surleraux, D. Jochmans, A. Tahri, R. Pauwels, P. Wigerinck, and M.-P. de Béthune.** 2005. TMC114, a novel human immunodeficiency virus type 1 protease inhibitor active against protease inhibitor-resistant viruses, including a broad range of clinical isolates. Antimicrob. Agents Chemother. **49:**2314–2321.
3. **Ghosh, A. K., J. F. Kincaid, W. Cho, D. E. Walters, K. Krishnan, K. A. Hussain, Y. Koo, H. Cho, C. Rudall, L. Holland, and J. Buthod.** 1998. Potent HIV protease inhibitors incorporating high-affinity P2-ligands and (R)-(hydroxyethylamino)sulfonamide isostere. Bioorg. Med. Chem. Lett. **8:**687–690.
4. **Hertogs, K., S. Bloor, S. D. Kemp, C. Van den Eynde, T. M. Alcorn, R. Pauwels, M. van Houtte, S. Staszewski, V. Miller, and B. A. Larder.** 2000. Phenotypic and genotypic analysis of clinical HIV-1 isolates reveals extensive protease inhibitor cross-resistance: a survey of over 6000 samples. AIDS **14:**1203–1210.
5. **Hertogs, K., M.-P. de Béthune, V. Miller, T. Ivens, P. Schel, A. Van Cauwenberge, C. Van den Eynde, V. van Gerwen, H. Azijn, M. van Houtte, F. Peeters, S. Staszewski, M. Conant, S. Bloor, S. Kemp, B. Larder, and R. Pauwels.** 1998. A rapid method for simultaneous detection of phenotypic resistance to inhibitors of protease and reverse transcriptase in recombinant human immunodeficiency virus type 1 isolates from patients treated with antiretroviral drugs. Antimicrob. Agents Chemother. **42:**269–276.
6. **Johnson, V. A., F. Brun-Vezinet, B. Clotet, B. Conway, D. R. Kuritzkes, D. Pillay, J. M. Schapiro, A. Telenti, and D. D. Richman.** 2005. Update of the drug resistance mutations in HIV-1: fall 2005. Top. HIV Med. **13:**125–131.
7. **Jönsson, U., L. Fägerstam, B. Ivarsson, B. Johnsson, R. Karlsson, K. Lundh, S. Löfas, B. Persson, H. Roos, I. Rönnberg, S. Sjölander, E. Stenberg, R. Stahlberg, S. Urbaniczky, H. Östlin, and M. Malmqvist.** 1991. Real-time biospecific interaction analysis using surface plasmon resonance and a sensor chip technology. BioTechniques **11:**620–627.
8. **Karlsson, R.** 1999. Affinity analysis of non-steady-state data obtained under mass transport limited conditions using BIAcore technology. J. Mol. Recognit. **12:**285–292.
9. **Katlama, C., R. Esposito, J. M. Gatell, J.-C. Goffard, B. Grinsztejn, A. Pozniak, J. Rockstroh, A. Stoehr, N. Vetter, P. Yeni, W. Parys, and T. Vangeneugden.** 2007. Efficacy and safety of TMC114/ritonavir in treatment-experienced HIV patients: 24-week results of POWER 1. AIDS **21:**395–402.
10. **Khalifa, M. B., L. Choulier, H. Lortat-Jacob, D. Altschuh, and T. Vernet.** 2001. BIACORE data processing: an evaluation of the global fitting procedure. Anal. Biochem. **293:**194–203.
11. **King, N. M., L. Melnick, M. Prabu-Jeyabalan, E. A. Nalivaika, S.-S. Yang, Y. Gao, X. Nie, C. Zepp, D. L. Heefner, and C. A. Schiffer.** 2002. Lack of synergy for inhibitors targeting a multi-drug-resistant HIV-1 protease. Protein Sci. **11:**418–429.
12. **King, N. M., M. Prabu-Jeyabalan, E. A. Nalivaika, P. Wigerinck, M.-P. de Béthune, and C. A. Schiffer.** 2004. Structural and thermodynamic basis for the binding of TMC114, a next-generation human immunodeficiency virus type 1 protease inhibitor. J. Virol. **78:**12012–12021.
13. **Koh, Y., H. Nakata, K. Maeda, H. Ogata, G. Bilcer, T. Devasamudram, J. F. Kincaid, P. Boross, Y.-F. Wang, Y. Tie, P. Volarath, L. Gaddis, R. W. Harrison, I. T. Weber, A. K. Ghosh, and H. Mitsuya.** 2003. Novel bis-tetrahydrofuranylurethane-containing nonpeptidic protease inhibitor (PI) UIC-94017 (TMC114) with potent activity against multi-PI-resistant human immunodeficiency virus in vitro. Antimicrob. Agents Chemother. **47:**3123–3129.
14. **Kovalevsky, A. Y., F. Liu, S. Leshchenko, A. K. Ghosh, J. M. Louis, R. W. Harrison, and I. T. Weber.** 2006. Ultra-high resolution crystal structure of HIV-1 protease mutant reveals two binding sites for clinical inhibitor TMC114. J. Mol. Biol. **363:**161–173.
15. **Langmuir, I.** 1916. The constitution and fundamental properties of solids and liquids. Part I. Solids. J. Am. Chem. Soc. **38:**2221–2295.
16. Reference deleted.
17. **Markgren, P.-O., M. T. Lindgren, K. Gertow, R. Karlsson, M. D. Hämäläinen, and U. H. Danielson.** 2001. Determination of interaction kinetic constants for HIV-1 protease inhibitors using optical biosensor technology. Anal. Biochem. **291:**207–218.
18. Reference deleted.
19. **Mastrolorenzo, A., S. Rusconi, A. Scozzafava, and C. T. Supuran.** 2006. Inhibitors of HIV-1 protease: 10 years after. Expert Opin. Ther. Pat. **16:**1067–1091.
20. **Morton, T. A., D. G. Myszka, and I. M. Chaiken.** 1995. Interpreting complex binding kinetics from optical biosensors: a comparison of analysis by linearization, the integrated rate equation, and numerical integration. Anal. Biochem. **227:**176–185.
21. **Myszka, D. G.** 1997. Kinetic analysis of macromolecular interactions using surface plasmon resonance biosensors. Curr. Opin. Biotechnol. **8:**50–57.
22. **Ohtaka, H., A. Schon, and E. Freire.** 2003. Multidrug resistance to HIV-1 protease inhibition requires cooperative coupling between distal mutations. Biochemistry **42:**13659–13666.
23. **Önell, A., and K. Andersson.** 2005. Kinetic determinations of molecular interactions using Biacore: minimum data requirements for efficient experimental design. J. Mol. Recognit. **18:**307–317.
24. **Palella, F. J. J., K. M. Delaney, A. C. Moorman, M. O. Loveless, J. Fuhrer, G. A. Satten, D. J. Aschman, and S. D. Holmberg.** 1998. Declining morbidity and mortality among patients with advanced human immunodeficiency virus infection. N. Engl. J. Med. **338:**853–860.
25. **Rich, R. L., and D. G. Myszka.** 2004. Why you should be using more SPR biosensor technology. Drug Discov. Today **1:**301–308.
26. **Richman, D. D., S. C. Morton, T. Wrin, N. Hellmann, S. Berry, M. F. Shapiro, and S. A. Bozzette.** 2004. The prevalence of antiretroviral drug resistance in the United States. AIDS **18:**1393–1401.
27. **Shuman, C. F., M. D. Hamalainen, and U. H. Danielson.** 2004. Kinetic and thermodynamic characterization of HIV-1 protease inhibitors. J. Mol. Recognit. **17:**106–119.
28. **Surleraux, D. L. N. G., A. Tahri, W. G. Verschueren, G. M. E. Pille, H. A. de Kock, T. H. M. Jonckers, A. Peeters, S. De Meyer, H. Azijn, R. Pauwels, M.-P. de Bethune, N. M. King, M. Prabu-Jeyabalan, C. A. Schiffer, and P. B. T. P. Wigerinck.** 2005. Discovery and selection of TMC114, a next generation HIV-1 protease inhibitor. J. Med. Chem. **48:**1813–1822.
29. **Trost, B. M., and N. G. Andersen.** 2002. Utilization of molybdenum- and palladium-catayzed dynamic kinetic asymmetric transformations for the preparation of tertiary and quaternary stereogenic centers: a concise synthesis of tipranavir. J. Am. Chem. Soc. **124:**14320–14321.