Inhibition of Wild-Type and Mutant Human Immunodeficiency Virus Type 1 Proteases by GW0385 and Other Arylsulfonamides

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ABSTRACT: The arylsulfonamide derivatives described herein were such potent inhibitors of human immunodeficiency virus type 1 (HIV-1) protease (enzyme, E) that values for the inhibition constants (K_i) could not be determined by conventional steady-state kinetic techniques (i.e., the minimal enzyme concentration usable for the activity assay was much greater than the value of the dissociation constant). Consequently, two alternative methods were developed for estimation of K_i values. The first method employed kinetic determinations of values for k_1 and k_{-1}, from which K_i was determined (k_{-1}/k_1). The second method was a competitive displacement assay used to determine binding affinities of other inhibitors relative to that of GW0385. In these assays, the inhibitor of unknown affinity was used to displace [^{3}H]-GW0385 from E·[^{3}H]GW0385. From the concentration of E·[^{3}H]GW0385 at equilibrium, the concentrations of enzyme-bound and free inhibitors were calculated, and the ratio of the K_i value of the unknown to that of GW0385 was determined ($K_{i,unknown}/K_{i,GW0385}$). The values of k_1 were calculated from data in which changes in the intrinsic protein fluorescence of the enzyme associated with inhibitor binding were directly or indirectly monitored. In the case of saquinavir, the fluorescence changes associated with complex formation were large enough to monitor directly. The value of k_1 for saquinavir was $62 \pm 2\ \mu M^{-1}\ s^{-1}$. In the case of GW0385, the fluorescence changes associated with complex formation were too small to monitor directly. Consequently, the value of k_1 was estimated from a competition experiment in which the effect of GW0385 on the binding of E to saquinavir was determined. The value of k_1 for GW0385 was estimated from these experiments to be $137 \pm 4\ \mu M^{-1}\ s^{-1}$. Because E·[^{3}H]GW0385 was stable in the standard buffer at room temperature for greater than 33 days, the value of the first-order rate constant for dissociation of E·[^{3}H]GW0385 (k_{-1}) could be estimated from the time-course for exchange of E·[^{3}H]-GW0385 with excess unlabeled GW0385. The value of k_{-1} calculated from these data was $(2.1 \pm 0.1) \times 10^{-6}\ s^{-1}$ ($t_{1/2} = 91$ h). The K_i value of wild-type HIV-1 protease for GW0385, calculated from these values for k_1 and k_{-1}, was 15 ± 1 fM. Three multidrug resistant enzymes had K_i values for GW0385 that were less than 5 pM.

Six HIV-1[1] protease inhibitors, including amprenavir, are marketed for the treatment of AIDS (*1*). The targeted enzyme is an aspartyl protease in which the active site is formed by aspartyl groups from each monomer of the catalytically competent homodimer (*2*, *3*). The protease, which catalyses the hydrolysis of selected peptidyl bonds of the viral polyprotein, is required for the production of infective virions. Although protease inhibitors (PIs) have been successful in reducing AIDS-related complications and deaths, drug-resistant viruses arise with mutations in the protease sequence, as well as with mutations in the protease cleavage sites in the polyprotein (*4*−*7*). Because cross-resistance among structurally different PIs occurs (*1*, *8*), new HIV protease inhibitors, such as arylsulfonamides (*9*) that retain activity against drug-resistant as well as wild-type viruses, are needed for continued successful therapy.

The K_i value of an enzyme for an inhibitor is defined by the concentrations of enzyme (E), inhibitor (I), and enzyme·inhibitor (E·I) in solution at equilibrium. Typically, the concentration of HIV protease in a mixture of E and E·I is determined with an activity assay with a fluorescently labeled peptide substrate (*10*, *11*). The sensitivity of this determination is limited by the concentration of enzyme necessary to observe product formation and the requirement that the enzyme concentration not greatly exceed the K_i value. With this activity assay, the lower limit for the value for the inhibition constant (K_i) is ∼10 pM for wild-type HIV protease. The assay is less sensitive with mutant proteases with lower catalytic efficiencies. Alternatively, the K_i value is determined from the ratio of the value of the rate constant for dissociation of E·I (k_{-1}) and the value of the bimolecular

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[1] Abbreviations: HIV-1, human immunodeficiency virus type 1; MES, 2-(*N*-morpholino)ethanesulfonic acid; AIDS, acquired immunodeficiency syndrome; PI, protease inhibitor; E(wt), dimer of HIV-1 protease; E(mut-1), dimer of HIV-1 protease with amino acid substitution I50V; E(mut-2), dimer of HIV-1 protease with amino acid substitutions M46I, L63P, A71V, V82F, and I84V; E(mut-3), dimer of HIV-1 protease with amino acid substitutions L10I, L19Q, K20R, E35D, M36I, S37N, M46I, I50V, I54V, I62V, L63P, A71V, V82A, and L90M; E·I, complex of HIV-1 protease dimer and inhibitor.

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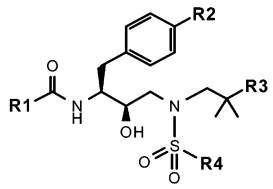

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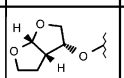
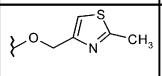
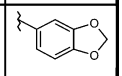
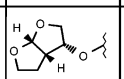
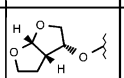
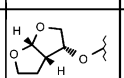
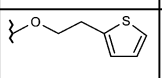
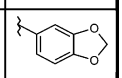
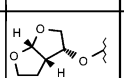
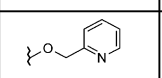
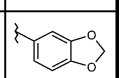
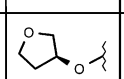
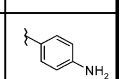
GW0385			H	
2		H		
3		H		
4			H	
5			H	
amprenavir		H	H	

FIGURE 1: Structures of inhibitors of HIV proteases used in the present studies.

rate constant for association of enzyme and inhibitor (k_1). We have used the latter method to determine K_i values for wild-type and mutant HIV proteases. A kinetic competitive displacement assay was developed to determine values for k_{-1} for a series of tight-binding inhibitors. Knowledge of these values and the values for k_1 (measured with a spectrofluorometric assay) allowed calculation of the value of the dissociation constant (K_i). Low femtomolar values for K_i were observed for arylsulfonamide inhibitors of HIV-1 protease.

MATERIALS AND METHODS

Materials. ULTRA MES and Sephadex G-10 were purchased from Sigma Chemicals. The syntheses of GW0385, **2**, **3**, **4**, and **5** (Figure 1) have been described (*12*, *13*). Amprenavir and saquinavir were from GlaxoSmithKline compound stores.

Synthesis of [^{3}H]GW0385. [^{3}H]GW0385 was prepared by exposing a stirred solution of 2 mg of bromo-GW0385 (monobrominated at the 3 position of the alkoxyphenyl ring) and 2 mg of sodium acetate trihydrate in tetrahydrofuran to tritium gas (1 atm) in the presence of 2 mg of 10% palladium on charcoal for a period of 5 h. The crude tritiated product was purified by reverse-phase HPLC.

Preparation of HIV Proteases. Preparation of HIV-1 protease (E(wt)) and I50V HIV-1 protease (E(mut-1)) were as previously described (*14*). HIV proteases E(mut-2), having amino acid substitutions M26I, L63P, A71V, V82F, and I84V, and E(mut-3), having amino acid substitutions L10I, L19Q, K20R, E35D, M36I, S37N, M46I, I50V, I54V, I62V, L63P, A71V, V82A, and L90M, were prepared similarly.

General Methods. Enzymatic activity was measured at 30 °C in assay buffer (0.1 M MES−Na^+, 0.4 M NaCl, 0.02% poly(ethylene glycol)-8000, pH 5.5) plus 5% DMSO. The fluorescence increase resulting from hydrolysis of 10 μM substrate (2-aminobenzoyl-Thr-Ile-Nle-Phe(p-NO_2)-Gln-Arg-NH_2, S) was monitored (*10*) with $\epsilon_{ex} = 340$ nm and $\epsilon_{em} = 420$ nm. Values for the steady-state kinetic parameters for substrate hydrolysis were determined by analysis of the complete time course for substrate hydrolysis (14). The concentration of E(wt) was determined by titration of enzymatic activity with amprenavir the concentration of which was determined spectrophotometrically ($\epsilon_{265} = 16.7$ mM^{-1} cm^{-1} in 0.1 N NaOH). Enzyme concentrations were expressed in terms of amprenavir binding sites. Concentrations of inhibitor solutions other than amprenavir were determined by titration of 7 nM HIV-1 protease. Concentrations of E(mut-1), E(mut-2), and E(mut-3) were determined by titration with compound **3** at 70, 2, and 36 nM enzyme, respectively. Enzyme solutions were stored as 100 μL samples at −80 °C in 50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), pH 7.0, in 10% glycerol, 5% ethylene glycol, 175 mM imidazole, and 1 mM 2-mercaptoethanol. Dimethyl sulfoxide solutions of inhibitors were stored in glass vials at −20 °C.

G-10 Chromatography to Isolate Protein-Bound GW0385 from an Assay Mix. G-10 resin (12 g) was equilibrated overnight at room temperature in 30 mL of assay buffer. Swollen resin was stored at 4 °C for up to 2 weeks. G-10 columns (0.4 cm × 3.5 cm) were prepared in 1.5 mL column supports (Burdick and Jackson no. 9446) immediately prior to use. After sample application (50 μL), protein and protein-bound inhibitors were eluted at ambient pressure from the resin with 800 μL of assay buffer into a liquid scintillation vial. Radioactivity was counted with a Beckman liquid scintillation counter after addition of 2 mL of Wallac Optiphase scintillant to the vial.

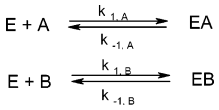
Kinetic Competitive Displacement Assay. HIV-1 protease and excess [^{3}H]GW0385 were incubated in assay buffer at room temperature to form E·[^{3}H]GW0385. After 10 minutes ($t = 0$), nonlabeled inhibitor was added to the solution such that the final volume was 2 mL with 4% DMSO. Concentrations were 50 nM HIV-1 protease, 80 nM [^{3}H]GW0385, and 320 nM nonlabeled inhibitor unless stated otherwise. This mixture was incubated in a sealed glass vial at room temperature in the dark. At selected times, E·[^{3}H]GW0385 was separated from free label with a G-10 column (see above). From the time course for E·[^{3}H]GW0385 disappearance, the value of the rate constant for dissociation of E·[^{3}H]GW0385 was determined (see below).

Calculation of k_{-1} Values from the Time Course of a Displacement Experiment. The association of HIV-1 protease with an inhibitor is usually described by a one-step kinetic mechanism (Scheme 1). In this scheme, k_{-1} is the rate constant for dissociation of the enzyme−inhibitor complex and k_1 is the bimolecular rate constant for association of dimeric enzyme (E) with inhibitor (I). When two inhibitors, A and B, are present in solution with E, they compete to form their respective E·I complexes E·A and E·B (Scheme 2). If A and B are two isotopic forms of the same inhibitor, then $k_{1,A} \approx k_{1,B}$ and $k_{-1,A} \approx k_{-1,B}$. For example, if A is [^{3}H]-

Scheme 1: One-Step Mechanism for Binding of E (HIV Protease Dimer) to I (Inhibitor)

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EI$$

Scheme 2: Competition of A and B for E.



GW0385 and B is nonlabeled GW0385, the value of $k_{-1,A}$ can be determined from a displacement experiment. As described above (kinetic competitive displacement assay), E was added to [^{3}H]GW0385 to form E·[^{3}H]GW0385 (E·A). To this mixture, excess nonlabeled GW0385 (B) was added. The concentration of E·[^{3}H]GW0385 was measured as a function of time. Dynafit (*15*) was used to calculate k_{-1} from these data according to Scheme 2 with a measured value for k_1 (see below). When B was a compound other than GW0385, $k_{-1,B}$ (the dissociation rate constant for E·B) was calculated with Dynafit using known values for $k_{-1,A}$ and $k_{1,A}$ and an estimate of 100 s^{-1} μM^{-1} for $k_{1,B}$.

Calculation of K_i Values from the End Points of a Displacement Experiment. The K_i's of E for A and B (Scheme 2) are defined by eqs 1 and 2. The ratio of the K_i's is described by eq 3. The experimentally determined value

$$K_{i,A} = \frac{k_{-1,A}}{k_{1,A}} = \frac{[E][A]}{[E\cdot A]} \quad (1)$$

$$K_{i,B} = \frac{k_{-1,B}}{k_{1,B}} = \frac{[E][B]}{[E\cdot B]} \quad (2)$$

$$r = \frac{K_{i,B}}{K_{i,A}} = \frac{[B][E\cdot A]}{[E\cdot B][A]} \quad (3)$$

of r was used to calculate $K_{i,B}$ when $K_{i,A}$ was known. The value of r was determined as follows: E, [^{3}H]GW0385, and nonlabeled inhibitor B were mixed as in the displacement assay described above ([[^{3}H]GW0385] > [E] and [B] > [E·[^{3}H]GW0385]). After the equilibrium was established, the concentration of E·[^{3}H]GW0385 (E·A) was measured (see G-10 chromatography). The concentrations of A, B, and E·B at equilibrium were calculated from the measured concentration of E·A and conservation of mass. The value of r was calculated by eq 3. The K_i value of inhibitor B was calculated from r and $K_{i,GW0385}$.

Assay to Monitor the Association of HIV Protease and Inhibitor. Fluorescence increases observed upon mixing HIV protease and substrate or inhibitor have been attributed to binding of compound to the protein (*16*−*18*). The formation of enzyme−inhibitor complex (E·I) when I was amprenavir was monitored in a stopped flow spectrophotometer (Applied Photophysics SX.17MV, Leatherhead, U.K., λ_{ex} = 280 nM and λ_{em} > 320 nm). The reaction was initiated by mixing an equal volume of enzyme (0.4 μM in assay buffer) with an equal volume of inhibitor (1−3 μM in assay buffer). According to Scheme 1, the dependency of the observed rate constant, k_{obs}, on inhibitor concentration ([I] ≫ [E]) is described by eq 4.

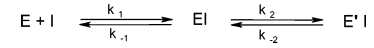
$$k_{obs} = k_1[I] + k_{-1} \quad (4)$$

When formation of E·I could be observed by the difference in fluorescence between E and E·I (for example, with amprenavir or saquinavir), k_1 was determined from the slope of a plot of k_{obs} versus [I]. This method was not used with GW0385 and analogues because the fluorescence changes associated with the formation of E·I were too small. Instead, we monitored the formation of E·GW0385 indirectly, as follows. Formation of E·I with saquinavir leads to a relatively large fluorescence decrease when monitored as above (*16*). The pseudo-first-order rate constant (k_{obs}) describing the equilibration of E with saquinavir is increased in the presence of a competitive inhibitor, whereas the amplitude is decreased. The rate of association of enzyme and GW0385 was determined as described above for amprenavir except that the inhibitor solutions contained a fixed concentration of saquinavir (2 μM for E(wt) and E(mut-1) and 10 and 5 μM, respectively, for E(mut-2) and E(mut-3)) with varying concentrations of GW0385 (0.4−30 μM). Values for the association rate constants were determined from pre-steady-state kinetic data collected with a stopped flow spectrofluorometer. For Scheme 2 with A = saquinavir and B = GW0385, $k_{1,B}$ is obtained from the slope of a plot of k_{obs} as a function of [B] at a constant [A], as described by eq 5.

$$k_{obs} = k_{1,A}[A] + k_{1,B}[B] + k_{-1,A} + k_{-1,B} \quad (5)$$

Determination of Kinetic Parameters for a Two-Step Mechanism for Inhibitor Binding to E. A two-step mechanism in which E·I isomerizes to form E′·I describes the binding of some inhibitors to HIV protease (*16*). The time course of the observed change in fluorescence is described by a double-exponential function ($k_{1,obs}$ and $k_{2,obs}$, Scheme 3, eqs 6 and 7). The value of the overall dissociation constant (K_i) is given by eq 8.

Scheme 3: Two-Step Mechanism for Binding of E (HIV Protease Dimer) to I (Inhibitor).



$$k_{1,obs} = k_1[I] + k_{-1} + k_2 + k_{-2} \quad (6)$$

$$k_{2,obs} = \frac{k_1[I](k_2 + k_{-2}) + k_{-1}k_{-2}}{k_1[I] + k_{-1} + k_2 + k_{-2}} \quad (7)$$

$$K_i = \frac{[E][I]}{[E\cdot I] + [E'\cdot I]} = \frac{k_{-1}k_{-2}}{k_1(k_2 + k_{-2})} \quad (8)$$

RESULTS

Steady-State Kinetic Parameters for Substrate Hydrolysis. K_m and k_{cat} values for E(wt), which were determined from the time course for hydrolysis of 19 μM substrate, were 13 ± 1 μM and 8.2 ± 0.4 s^{-1}, respectively. Substrate solubility precluded determination of K_m and k_{cat} values for the mutant proteases. However, the ratio of k_{cat} to K_m was estimated from the first-order time course for substrate depletion. These values were 0.376 ± 0.006 (E(mut-1)), 0.058 ± 0.003 (E(mut-2)), and 0.354 ± 0.004 μM s^{-1} (E(mut-3)).

Association of HIV Protease with Inhibitors. The time course for binding of E and amprenavir was monitored by the associated increase in fluorescence upon formation of E·amprenavir (λ_{ex} = 280 nm and λ_{em} > 320 nm). The values of the rate constants (k_{obs}) were linearly dependent on amprenavir concentration with each of the HIV proteases studied (Figure 2). The results are described by Scheme 1

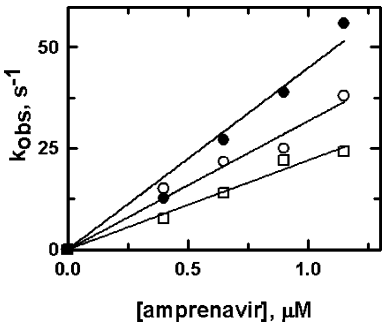


FIGURE 2: Association of HIV proteases with amprenavir. Values for the pseudo-first-order rate constants for the equilibration of 0.2 μM E with amprenavir were estimated from the fluorescence changes associated with complex formation (λ_{ex} = 280 nm; λ_{em} >320 nm). The dependence of k_{obs} values on the concentration of amprenavir is illustrated for E(wt) (●), E(mut-1) (○), and E(mut-3) (□). The slopes of the lines (k_1) were 45 ± 2 (E(wt)), 32 ± 1 (E(mut-1)), and 22.0 ± 0.8 μM^{-1} s^{-1} (E(mut-3)).

and eq 4 with k_1 = 45 ± 2 (E(wt)), 22.0 ± 0.8 (E(mut-3)), and 32 ± 1 μM^{-1} s^{-1} (E(mut-1)). These results indicated that the rates of association of amprenavir with the wild-type and mutant proteases were very similar, which suggested that the decreased affinity of the mutant proteases to amprenavir was primarily a result of differences in the rates of dissociation of E•I. The values of k_{-1} were too small to be determined accurately by this technique (k_{-1} < 1 s^{-1}).

The association of HIV-1 protease with saquinavir results in a decrease in fluorescence (*16*). We observed that the values of the rate constants describing these reactions (data not shown) were linearly dependent upon saquinavir concentration for E(wt), E(mut-1), and E(mut-2) with slopes of 62 ± 2, 52 ± 2, and 3.5 ± 0.1 μM^{-1}, respectively. Under similar conditions, mixing E with GW0385 led to no measurable change in fluorescence. However, GW0385 affected the rate of association of E with saquinavir. At a fixed concentration of saquinavir (I > E) and variable concentrations of GW0385, the observed rate constants were linearly dependent upon the concentration of GW0385 (Figure 3). The data were analyzed according to Scheme 2 and eq 5 with A = saquinavir and B = GW0385. The values of the bimolecular rate constants ($k_{1,B}$) for association of GW0385 with E(wt), E(mut-1), and E(mut-2) were 137 ± 4, 327 ± 3, and 28 ± 2 μM^{-1} s^{-1}, respectively (Table 1).

The fluorescence change resulting from the association of E(mut-3) and saquinavir was biphasic ($k_{1,obs}$, $k_{2,obs}$). The dependence of the two rate constants on saquinavir concentration is illustrated in Figure 4. The data were analyzed according to Scheme 3 and eqs 6 and 7 to obtain values of k_1, k_{-1}, k_2, and k_{-2} equal to 9 ± 1 μM^{-1} s^{-1}, 70 ± 7 s^{-1}, 23 ± 3 s^{-1}, and 2.3 ± 0.5 s^{-1}, respectively. According to eq 8, K_i was calculated to be 0.73 μM. This value is close to the K_i value (0.23 μM) determined by inhibition of enzymatic activity (data not shown).

Fluorescence changes associated with the binding of 0.2 μM E(mut-3) to 2.5 μM saquinavir are shown in Figure 5. The effect of GW0385 on this interaction is illustrated in the upper curve. Dynafit was used to fit the model of Scheme 4 simultaneously to the data obtained in the absence (lower curve) or presence (upper curve) of 0.1 μM GW0385. The fitted value for $k_{1,B}$, the bimolecular rate constant for association of E(mut-3) and GW0385, is 500 ± 70 μM^{-1} s^{-1} (Table 1).

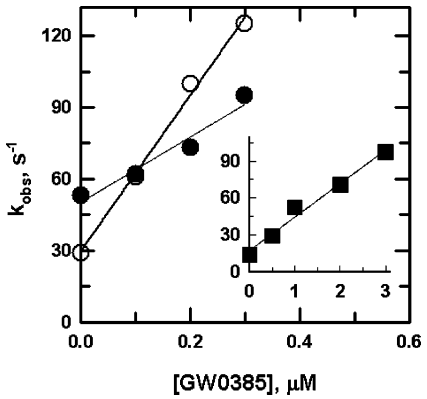


FIGURE 3: Association of HIV proteases with GW0385 in the presence of saquinavir. Values of pseudo-first-order rate constants for equilibration of 0.2 μM E to saquinavir were determined from the fluorescence changes associated with complex formation (λ_{ex} = 280 nm; λ_{em} > 320 nm). In the absence of GW0385, the pseudo-first-order rate constants were linearly dependent upon the concentration of saquinavir (data not shown). At a fixed concentration of saquinavir, the rate constants increased with increasing concentrations of GW0385. Data were obtained with 1 μM saquinavir and (●) 0.2 μM E(wt) or (○) E(mut-1) (main figure) or with 5 μM saquinavir and 0.2 μM E(mut-2) (■, inset). The slopes of the lines, which correspond to bimolecular rate constants for the association of E and GW0385, were 137 ± 4 (E(wt)), 327 ± 3 (E(mut-1)), and 28 ± 2 μM^{-1} s^{-1} (E(mut-2)).

Behavior of HIV-1 Proteases and [^{3}H]GW0385 on G-10 Resin. The recovery of HIV-1 protease from G-10 columns was determined from measurements of enzyme activity in control samples without inhibitor. In these experiments, 50 μL of a 1 μM solution of enzyme in assay buffer was applied to a G-10 column. After the sample was absorbed into the column bed, the column was developed with 100 μL applications of assay buffer. Column eluant was collected (100 μL fractions), and enzymatic activity was measured. Ninety-five percent of the enzymatic activity was recovered in the first 300 μL, and 100% of the activity was recovered in 800 μL. The protein thus behaved as expected on this sizing resin: it was recovered in the excluded volume. In control experiments with [^{3}H]GW0385 in the absence of protease, less than 1% of the radiolabel was recovered in 800 μL of column eluant. This result was not expected based on the total column volume of 440 μL (excluded + included volume). No significant radioactivity was recovered with an additional 10 mL of assay buffer. To determine where the radioactive material was on the column, it was allowed to dry overnight at room temperature after which the resin was expelled from the column by the application of air pressure to the column outlet. The empty column support as well as the expelled resin were sliced and counted in Wallac Optiphase in the liquid scintillation counter. Radioactivity was detected only in the resin from the top 0.5 cm of the column bed. It was not detected immediately after mixing of resin and scintillation cocktail but was detected after several hours, suggesting that it was slowly released from the resin. Thus the G-10 resin acted as an affinity resin for GW0385. In contrast to the behavior of free GW0385 on the resin, if [^{3}H]GW0385 was mixed with excess HIV-1 protease before application to the G-10 resin, all the radioactivity was recovered in the protein fraction (the first 800 μL).

Table 1: Kinetic Parameters for Binding of Amprenavir and GW0385 to HIV Proteases

inhibitor	protease	k_1, μM^{-1} s^{-1}	k_{-1}, s^{-1}	K_I, pM
amprenavir	HIV-1	45 ± 2[a]	*g*	57 ± 4[i]
	mut-1	32 ± 1[a]	*g*	$4\,900 \pm 200$[i]
	mut-2	*g*	*g*	$2\,300 \pm 600$[i]
	mut-3	22 ± 0.8[a]	*g*	$58\,000 \pm 15\,000$[i]
GW0385	HIV-1	137 ± 4[b,c]	$0.000\,002\,1 \pm 0.000\,000\,1$[h]	0.015 ± 0.001[j]
	mut-1	327 ± 3[b,d]	$0.000\,65 \pm 0.000\,05$[h]	2.0 ± 0.2[j]
	mut-2	28 ± 2[b,e]	$0.000\,021 \pm 0.000\,002$[h]	0.75 ± 0.09[j]
	mut-3	500 ± 70[b,f]	$0.001\,72 \pm 0.000\,02$[h]	3.4 ± 0.4[j]

[a] Calculated from spectofluorometric data. [b] Calculated from spectrofluorometric data in the presence of saquinavir. [c] $k_{1,\text{saquinavir}} = 62 \pm 2\ \mu M^{-1}\ s^{-1}$. [d] $k_{1,\text{saquinavir}} = 52 \pm 2\ \mu M^{-1}\ s^{-1}$. [e] $k_{1,\text{saquinavir}} = 3.5 \pm 0.1\ \mu M^{-1}\ s^{-1}$. [f] $k_{1,\text{saquinavir}} = 9 \pm 1\ \mu M^{-1}\ s^{-1}$. [g] Not determined. [h] Calculated from the time course for displacement of [^{3}H]GW0385 from E·[^{3}H]GW0385 by nonlabeled GW0385. [i] Determined with the fluorometric activity assay. [j] $K_i = k_{-1}/k_1$.

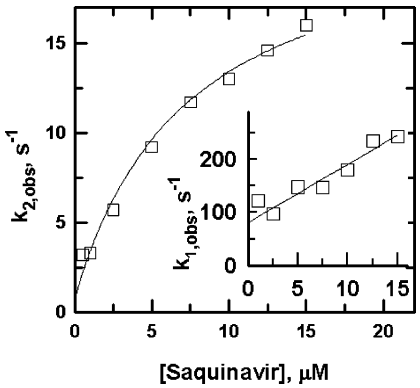


FIGURE 4: Determination of rate constants for the association of E(mut-3) with saquinavir. Two rate constants describing the binding of 0.2 μM E(mut-3) to saquinavir were determined from the fluorescence changes associated with complex formation (λ_{ex} = 280 nm; λ_{em} > 320 nm). The dependence of the two rate constants on saquinavir concentration is illustrated. The lines are the theoretical curves described by eqs 6 and 7 with $k_1 = 9 \pm 1\ \mu M^{-1}\ s^{-1}$, $k_{-1} = 70 \pm 7\ s^{-1}$, $k_2 = 23 \pm 3\ s^{-1}$, and $k_{-2} = 2.3 \pm 0.5\ s^{-1}$.

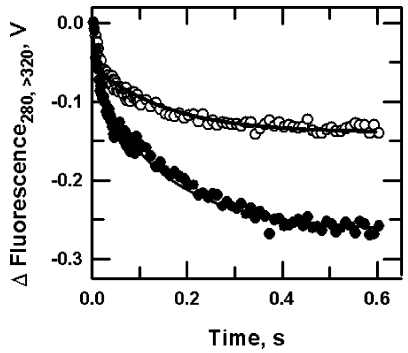
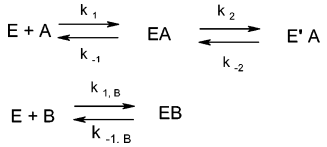


FIGURE 5: Association of E(mut-3) with saquinavir with or without GW0385. The time courses for association of E(mut-3) with saquinavir were monitored by the fluorescence changes associated with complex formation in a stopped-flow spectrofluorometer (λ_{ex} = 280 nm; λ_{em} > 320 nm). The fluorescence of 0.2 μM enzyme solution without inhibitor was 4 V. The reactions were initiated by mixing an equal volume of enzyme solution (0.4 μM) with an equal volume of inhibitor solution (5 μM saquinavir (●) or 5 μM saquinavir with 0.2 μM GW0385 (○)). The data were analyzed with Dynafit according to Scheme 4 with a two-step mechanism for the binding of saquinavir and E(mut-3) and a one-step mechanism for the binding of GW0385 and E(mut-3). The values of k_1, k_{-1}, k_2, and k_{-2} were 9 $\mu M^{-1}\ s^{-1}$, 70 s^{-1}, 23 s^{-1}, and 2.3 s^{-1}, respectively (Figure 4). The value of $k_{-1,GW0385}$ was $1.7 \times 10^{-3}\ s^{-1}$ (see Figure 7). The solid lines are the fits of the model to the data. The value of $k_{1,GW0385}$ determined from these data is $500 \pm 70\ \mu M^{-1}\ s^{-1}$.

Dissociation of E·I. The time-course for dissociation of [^{3}H]GW0385 from E(wt)·[^{3}H]GW0385 is shown in Figure 6 (filled circles). At $t = 0$, the solution contained 50 nM E(wt)·[^{3}H]GW0385. The dissociation of [^{3}H]GW0385 from the complex was monitored after the addition of excess nonlabeled GW0385. The value of the rate constant for dissociation of E(wt)·[^{3}H]GW0385, k_{-1}, was $(2.1 \pm 0.1) \times 10^{-6}\ s^{-1}$. The half-life for dissociation was 91 h. In the absence of unlabeled GW0385 (upper curve, Figure 6), the concentration of [^{3}H] recovered in the protein fraction remained constant throughout the experiment (33 days) indicating that E(wt)·[^{3}H]GW0385 was stable under the assay conditions. The value of K_i, calculated from the ratio of the values for k_{-1} and k_1, was 15 ± 1 fM (Table 1).

The time courses for dissociation of E(wt)·[^{3}H]GW0385 in the presence of other competitive inhibitors were determined (Figure 6). Rate constant values (k_{-1}) calculated from these data are summarized in Table 2. Assuming that the value of the rate constant for association of each alternative inhibitor with the enzyme is equal to that of GW0385, the ratio of $k_{-1,compound}$ to $k_{-1,GW0385}$ for compounds **3**, **4**, and **5** is also the ratio *r*, defined as ($K_{i,compound}/K_{i,GW0385}$). The values for *r* for compounds **2** and amprenavir were determined according to eq 3. The K_i value for amprenavir, determined from *r* and the K_i value of GW0385, was very similar to that determined from inhibition of enzymatic activity, 36 and 57 pM, respectively. Compound **2** was an order of magnitude weaker inhibitor of E(wt) than was GW0385. Compounds **4** and **5** were very similar to GW0385 (K_i = 15 fM).

Scheme 4: Competition of A and B for E with Two-Step Mechanism for Binding of A and E.



Dissociation of [^{3}H]GW0385 from E·[^{3}H]GW0385 with mutant HIV proteases was more rapid than from wild-type enzyme (Figure 7). The data with E(mut-2) (inset) was collected under standard conditions after addition of 320 nM nonlabeled GW0385 to E(mut-2)·[^{3}H]GW0385. To expand the dynamic range of the assay, the concentration of nonlabeled GW0385 was increased to 1.6 μM with E(mut-3) and 3.4 μM with E(mut-1). Values for the rate constants for dissociation of E·[^{3}H]GW0385, $k_{-1,GW0385}$, were determined with Dynafit (*15*) according to Scheme 2. K_i values for GW0385 and the mutant enzymes were between 1 and 3 pM (Table 1).

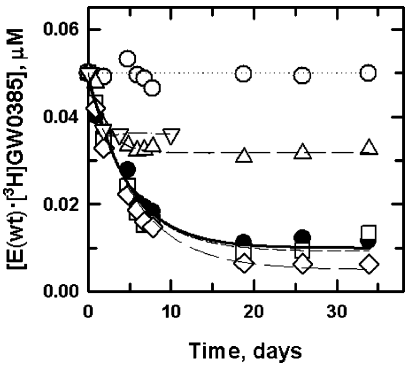


FIGURE 6: Dissociation of [^{3}H]GW0385 from E(wt)·[^{3}H]GW0385 in the presence of competing (nonlabeled) inhibitors. Competitive inhibitors were added to preformed E(wt)·[^{3}H]GW0385. Assay mixtures were incubated in sealed glass vials at room temperature in the dark. The concentration of E(wt)·[^{3}H]GW0385 was determined at various times after removal of non-protein-bound [^{3}H]-GW0385 by G-10 chromatography. Data obtained with 50 nM E(wt)·[^{3}H]GW0385 and 320 nM nonradiolabeled GW0385 ($t = 0$) are represented with filled circles (●). The solid line is the fit of the model of Scheme 2 to these data with $k_1 = 137 \pm 4\ \mu M^{-1}\ s^{-1}$ and $k_{-1} = (2.1 \pm 0.1) \times 10^{-6}\ s^{-1}$. Data obtained with other inhibitors are shown with open symbols. Dashed lines are the fits of the model of Scheme 2 to the data with A = GW0385, $k_{1,GW0385}$ and $k_{-1,GW0385}$ as determined above, and $k_{1,A} = k_{1,B}$. Values of $k_{-1,B}$ obtained from these data are $(2.3 \pm 0.2) \times 10^{-5}\ s^{-1}$ (B = compound **3**, △), $(1.9 \pm 0.4) \times 10^{-6}\ s^{-1}$ (B = compound **4**, □) and $(9.0 \pm 0.2) \times 10^{-7}\ s^{-1}$ (B = compound **5**, ◇). Scheme 2 was fit to the data obtained with compound **2** (▽). Open circles (○) represent data from a control experiment without competing inhibitor, which demonstrated that E(wt)·[^{3}H]GW0385 was stable over the course of the experiment.

Values for the rate constants for dissociation of E(mut-1)·I were determined (Table 2) for compounds **2**, **3**, **4**, and **5** (data not shown). Assuming that the value of the rate constant for association of E(mut-1) with each inhibitor ($k_{1,compound}$) is similar to that of GW0385 ($k_{1,GW0385}$), the ratios of the dissociation constants ($k_{-1,compound}/k_{-1,GW0385}$) can be used to calculate K_i values. Calculated K_i values are low picomolar for each of the compounds for E(mut-1) (Table 2).

Figure 8 illustrates the dissociation of E(mut-2)·[^{3}H]-GW0385 in the presence of competing inhibitors. The data are insufficient to determine values for the rate constants of dissociation for all of the inhibitors. Instead, r, the ratio of $K_{i,compound}$ to $K_{i,GW0385}$ was determined from data collected at equilibrium ($t = 96$ h). The values for r are listed in Table 2 with K_i values calculated from values for r and for the K_i of GW0385 for this mutant enzyme (750 fM, Table 1). As with the wild-type enzyme, the most potent compounds were **4** and **5** ($K_i < 1$ pM).

Of the four proteases studied, dissociation of E·[^{3}H]-GW0385 was most rapid with E(mut-3) (Figure 7). The half-life for dissociation was 0.11 h. The control assays with E(mut-3)·[^{3}H]GW0385 indicated that the complex was stable for at least 2.5 h under assay conditions but was not stable for 24 h. The ratio (r) of the inhibition constants ($K_{i,\ compound}/K_{i,\ GW0385}$) was determined at $t = 1$ h for compounds **2**, **3**, **4**, and **5**. Values for r and calculated K_i values are listed in Table 2. As with the other mutant HIV proteases, K_i values were low picomolar for compounds **3**, **4**, and **5**.

Table 2: Parameters for Binding of Inhibitors to HIV Proteases

protease	compound	k_{-1}, s^{-1}	r[d]	K_I, pM[l]
HIV-1	**2**	*a*	16[e]	0.240
	3	$0.000\ 023 \pm 0.000\ 002$[b]	11[f]	0.165
	4	$0.000\ 001\ 9 \pm 0.000\ 000\ 4$[b]	0.89[f]	0.013
	5	$0.000\ 000\ 9 \pm 0.000\ 000\ 2$[b]	0.41[f]	0.006
	amprenavir	*a*	2400[e,g]	36
mut-1	**2**	0.0087 ± 0.0009[c]	13.3[f]	27
	3	0.002 ± 0.001[c]	2.32[f]	4.6
	4	0.001 ± 0.001[c]	1.98[f]	3.9
	5	0.0008 ± 0.0007[c]	1.28[f]	2.6
mut-2	**2**	*a*	32.8[h]	25
	3	*a*	5.6[i]	4.3
	4	*a*	0.29[j]	0.22
	5	*a*	0.55[j]	0.42
mut-3	**2**	*a*	16[k]	54.4
	3	*a*	0.36[k]	1.2
	4	*a*	0.71[k]	2.4
	5	*a*	0.51[k]	1.7

[a] Not determined. [b] Calculated from the time course for displacement of [^{3}H]GW0385 from the enzyme·[^{3}H]GW0385 complex (0.05 μM) by compound (0.32 μM) with Dynafit using known values for $k_{1,GW0385}$ and $k_{-1,GW0385}$ and an estimated value of $k_{-1,compound}$ of 137 $\mu M^{-1}\ s^{-1}$. [c] Calculated from the time course for displacement of [^{3}H]GW0385 from the enzyme·[^{3}H]GW0385 complex (0.05 μM) by compound (0.32 μM) with Dynafit using known values for $k_{1,GW0385}$ and $k_{-1,GW0385}$ and an estimated value of $k_{-1,compound}$ of 327 $\mu M^{-1}\ s^{-1}$. [d] $r = K_{i,compound}/K_{i,GW0385}$; $r = k_{-1,compound}k_{1,GW0385}/k_{1,compound}k_{-1,GW0385}$. [e] Determined at equilibrium from measured [E·[^{3}H]GW0385] and total concentrations of E, [^{3}H]GW0385, and compound. Total compound was 0.32 or 32 μM (amprenavir). [f] Calculated as $k_{-1,compound}/k_{-1,GW0385}$ assuming that $k_{1,compound} = k_{1,GW0385}$. [g] Determined at equilibrium, day 33. [h] Determined at equilibrium, average of values obtained on days 1, 2, and 4. [i] Determined at equilibrium, average of values obtained on days 2 and 4. [j] Determined at equilibrium, day 4. [k] Determined at equilibrium, 1 h. [l] $K_i = r * K_{i,\ GW0385}$.

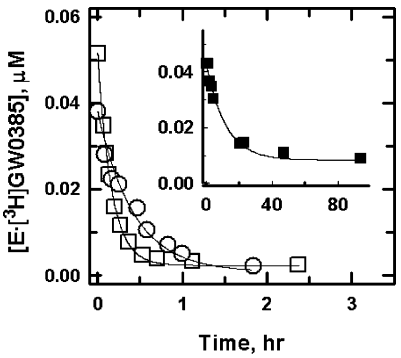


FIGURE 7: Dissociation of [^{3}H]GW0385 from complexes with mutant HIV-1 proteases. Unlabeled GW0385 was added to preformed E·[^{3}H]GW0385. E·[^{3}H]GW0385 was measured as a function of time as described in Materials and Methods. The model of Scheme 2 was fit to the data with Dynafit using k_1 values fixed at 556, 327, and 28 $\mu M^{-1}\ s^{-1}$ and initial E·[^{3}H]GW0385 concentrations of 0.052, 0.038, and 0.043 μM for E(mut-3) (□), E(mut-1) (○), and E(mut-2) (■), respectively. The concentrations of nonradiolabeled GW0385 added at $t = 0$ were 1.6, 3.43, and 0.32 μM, respectively. Values of k_{-1} determined from these data are $(1.72 \pm 0.02) \times 10^{-3}$ (E(mut-3)), $(6.5 \pm 0.5) \times 10^{-4}$ (E(mut-1)), and $(2.1 \pm 0.2) \times 10^{-5}\ s^{-1}$ (E(mut-2)).

DISCUSSION

In this study, we determined inhibition constants for GW0385 with wild-type and mutant HIV proteases from the ratios of k_{-1}/k_1. Because the fluorescence of E and E·[^{3}H]-GW0385 were similar, the value of k_1 was estimated from the effect of GW0385 on the kinetics of formation of E· saquinavir. These studies required a relatively large amount

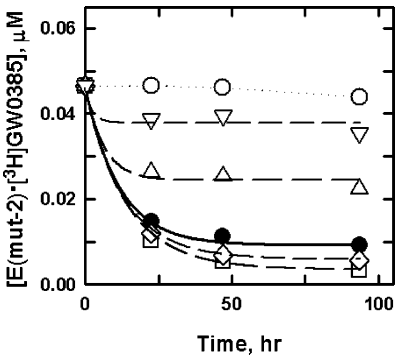


FIGURE 8: Dissociation of [^{3}H]GW0385 from E(mut-2)•[^{3}H]-GW0385 in the presence of competing inhibitors. Inhibitors were added to E(mut-2)•[^{3}H]GW0385. Assay mixtures were equilibrated at room temperature in the dark as described in Materials and Methods. The concentration of E(mut-2)•[^{3}H]GW0385 was measured as a function of time. Data obtained with 46 nM E(mut-2)•[^{3}H]GW0385 and 320 nM nonlabeled GW0385 ($t = 0$) are represented with filled circles (●). The solid line is a simulation according to Scheme 2 with $k_1 = 28\ \mu M^{-1}\ s^{-1}$ and $k_{-1} = 2.1 \times 10^{-5}\ s^{-1}$ (see Figures 3 and 7, respectively). Data obtained with other nonlabeled inhibitors or with the DMSO control are shown with open symbols (compound **3**, △; compound **4**, □; compound **5**, ◇; compound **2**, ▽). The dashed lines are simulations obtained according to Scheme 2 with $k_{1,GW0385} = k_{1,compound} = 28\ \mu M^{-1}\ s^{-1}$, $k_{-1,GW0385}$ as above, and $k_{-1,compound} = K_i k_1$ (Table 2). Open circles (○) represent control assays with no competing inhibitor.

of enzyme, which made it impractical for studying a large number of compounds. Consequently, we used the k_1 value for GW0385 in the calculation of the values of k_{-1} for analogues of GW0385.

The rates of dissociation of E•[^{3}H]GW0385 were readily measured by the displacement assay described herein. The facile separation of enzyme-bound compound from free compound was the key to the success of the assay. The G-10 resin used in this separation behaved both as a molecular sieve, allowing passage of protein and retardation of compounds of lesser molecular weight, and as an affinity resin for GW0385 and amprenavir. It is likely that the G-10 resin also acted as an affinity resin for the other inhibitors, although this was not examined. In the course of assay development, we examined the behavior of amprenavir and HIV-1 protease on other sizing resins. The protease behaved anomalously on both P10 and P30 resins (Pharmacia), making them unsuitable for this study. Utrafiltration was also investigated as a method of separating protein and unbound inhibitor, but absorption of amprenavir and GW0385 to filtration units (Millipore and Amicon) precluded the use of this technique.

The displacement assay was adapted to quantitate binding of nonradiolabeled compounds to HIV proteases through competition with the labeled compound. The concentration of protein-bound label at equilibrium was used to calculate the concentrations of free inhibitors and each E•I. From these numbers, the relative binding affinities of the two compounds were determined. Knowledge of this ratio and the K_i value of GW0385 allowed calculation of K_i values for the nonradiolabeled inhibitors.

Other methods have been described for the determination of protein−ligand binding constants. For example, a surface plasmon resonance (SPR)-based biosensor has been used to determine k_1 and k_{-1} for E•I with $t_{1/2} < 2$ h (19), and isothermal titration calorimetry (ITC) has been used to calculate K_i as low as 100 nM (*20*−*21*). A competitive ligand-displacement assay (*22*) in which a more tightly binding inhibitor is added to a complex of enzyme and weaker inhibitor (displacement isothermal titration calorimetry) has been reported to extend the range of K_i that can theoretically be determined by ITC to about 6 orders of magnitude less than the K_i of the weaker inhibitor. This technique has been used to determine apparent K_i values for inhibitors of HIV-1 protease (*23*) including amprenavir. Using the weak inhibitor acetyl pepstatin ($K_i = 400$ nM) in 10 mM acetate, pH 5, 2% DMSO, the K_i values of E(wt) and E(I50V) for amprenavir were determined to be 200 pM and 30 nM, respectively (23). These values are within an order of magnitude of those reported here, determined in 100 mM MES, pH 5.5, 400 mM NaCl, 4% DMSO.

The effect of ligand binding on thermally or chemically induced protein denaturation (*24*−*27*) has also been used to calculate K_i values. These methods assume that ligand binding stabilizes only the folded conformation. The effect of inhibitors on the urea-induced denaturation (*27*) of HIV-1 protease has been described (*26*). The authors use denaturation curves to calculate free energies of unfolding. The difference in the free energy of unfolding (with and without inhibitor) is used to calculate a K_i value. The method is validated by the observation that the K_i value determined with the urea denaturation assay is similar to the K_i value determined by inhibition of enzyme activity for the relatively weak inhibitor pepstatin A ($K_i = 120$ nM). For stronger inhibitors, K_i values determined by the two methods differ significantly. This may be due to differences in pH and salt concentrations in the assays or the use of assay conditions that promote dissociation of protease dimer (26). Although the use of this model to calculate K_i values for subnanomolar inhibitors of HIV protease awaits further validation, the method should prove useful for studying the relative binding affinities of a related set of ligands with the same protein. The protein and ligand concentrations may limit the use of the urea denaturation assay. For example, these authors used 450 nM protease dimer and 15 μM inhibitors. The aqueous solubility of some of the HIV protease inhibitors described in the present study is less than 1 μM.

The competitive displacement assay described in the present paper uses only 50 nM enzyme (well above the K_i value for all of the inhibitors studied). Consequently, only a slight excess of inhibitor (80 nM) was required to ensure that enzyme was saturated with inhibitor. Because most of the inhibitors studied had affinities for the proteases similar to that of GW0385, similar concentrations of the two inhibitors could be used in the assay. However, GW0385 is several-thousand-fold more potent an inhibitor of HIV-1 protease than is amprenavir, so a large excess of amprenavir (32 μM) was required to displace significant amounts of [^{3}H]-GW0385 from E(wt)•[^{3}H]GW0385. At equilibrium, only 16% of enzyme-bound [^{3}H]GW0385 was replaced by amprenavir. The calculated K_i of 36 pM approaches the upper limit of a K_i value that can be determined by competition with GW0385 for wild-type HIV-1 protease.

The amino acid substitutions of E(mut-1), E(mut-2), and E(mut-3) were chosen for their association with resistance to currently marketed HIV protease inhibitors (*1*, *8*). In the present study, GW0385 was a potent inhibitor of wild-type HIV-1 protease and these clinically relevant mutants. Although the mutant enzymes showed significant differences

in the values of the rate constants for association with GW0385 (up to 20 times that of wild-type enzyme), the major factor contributing to reduced potency against the mutant enzymes was an increase in the values of the rate constants for dissociation of E·I (up to 1000 times that of wild-type enzyme). None the less, GW0385 was a potent inhibitor of each of the mutant enzymes studied ($K_i \leq 5$ pM). Thus GW0385 is a potential "next-generation" therapy to treat patients infected with drug-resistant viruses.

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