Resistance to HIV Protease Inhibitors: A Comparison of Enzyme Inhibition and Antiviral Potency

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ABSTRACT: Resistance of HIV-1 to protease inhibitors has been associated with changes at residues Val82 and Ile84 of HIV-1 protease (HIV PR). Using both an enzyme assay with a peptide substrate and a cell-based infectivity assay, we examined the correlation between the inhibition constants for enzyme activity (K_i values) and viral replication (IC_{90} values) for 5 active site mutants and 19 protease inhibitors. Four of the five mutations studied (V82F, V82A, I84V, and V82F/I84V) had been identified as conferring resistance during in vitro selection using a protease inhibitor. The mutant protease genes were expressed in *Escherichia coli* for preparation of enzyme, and inserted into the HXB2 strain of HIV for test of antiviral activity. The inhibitors included saquinavir, indinavir, nelfinavir, 141W94, ritonavir (all in clinical use), and 14 cyclic ureas with a constant core structure and varying P2, P2′ and P3, P3′ groups. The single mutations V82F and I84V caused changes with various inhibitors ranging from 0.3- to 86-fold in K_i and from 0.1- to 11-fold in IC_{90}. Much larger changes compared to wild type were observed for the double mutation V82F/I84V both for K_i (10−2000-fold) and for IC_{90} (0.7−377-fold). However, there were low correlations ($r^2 = 0.017-0.53$) between the mutant/wild-type ratio of K_i values (enzyme resistance) and the mutant/wild-type ratio of viral IC_{90} values (antiviral resistance) for each of the HIV proteases and the viruses containing the identical enzyme. Assessing enzyme resistance by "vitality values", which adjust the K_i values with the catalytic efficiencies (k_{cat}/K_m), caused no significant improvement in the correlation with antiviral resistance. Therefore, our data suggest that measurements of enzyme inhibition with mutant proteases may be poorly predictive of the antiviral effect in resistant viruses even when mutations are restricted to the protease gene.

The replication of the human immunodeficiency virus (HIV)[1] requires its aspartyl protease (HIV PR) to process the virally encoded gag and gag-pol polyproteins (*1, 2*). These cleavage events release enzymes and structural proteins that are essential for the assembly of infectious viral particles (*3, 4*). Inhibition of HIV PR activity in infected cells thus leads to the production of immature, noninfectious virus.

While HIV PR inhibitors can be efficacious against wild-type HIV both in vitro and in man, they also select for HIV variants in the viral population which often display reduced susceptibility to the PR inhibitor (*5*−*9*). Currently, all of the clinically approved inhibitors of HIV PR are peptide mimetics which bind in the active site and adjacent specificity pockets. Mutations both in these regions and in distal sites affect inhibitor and substrate binding by altering the number and/or strength of subsite interactions (*10, 11*). Consequently, in the presence of a PR inhibitor, there is a replicative advantage for HIV-containing mutations that decrease PR inhibitor affinity while retaining sufficient enzyme activity to process the gag and gag-pol polyproteins. Several active site mutations that confer an apparent advantage for viral replication have appeared over time in clinical trials with HIV PR inhibitors (*6, 9, 12*). The current medical challenge is to find combinations of potent antiviral agents which keep plasma viral load at undetectable levels while blocking the development of drug resistance. Clearly, inhibitors which are efficacious toward many of the known active site mutations of HIV PR would be desirable.

Although development of clinical resistance is a measure of the efficacy of HIV PR inhibitors, how mutations in the protease and elsewhere influence the viability of the virus is not thoroughly understood. There is evidence that the viral maturation process is an ordered series of events which requires protease activity before or during budding to sequentially hydrolyze the eight cleavage sites in the gag and gag-pol polyproteins (*13, 14*). Viral mutations in the protease gene and polyprotein cleavage sites affect the extent of substrate cleavage which in turn can alter the rate-limiting step and/or the processing order in the virion (*15*). These combined interactions may cause subtle perturbations in the viral maturation events that are not observable with an in vitro enzymatic assay with one enzyme and one peptide substrate. As a consequence, the magnitude of resistance

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[1] Abbreviations: HIV, human immunodeficiency virus; PR, protease; K_i, enzyme inhibition constant; IC_{90}, viral inhibition constant; r^2, the square of the regression coefficient.

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to an inhibitor may not be quantifiable by simple enzyme kinetics.

Selection experiments in vitro with HIV PR inhibitors have identified many of the active site mutations now emerging in the clinic (*16*). The clinical data available on in vivo resistance to HIV PR inhibitors indicate there may be some order to the selective process. Typically, active site mutations emerge first followed by compensatory mutations that seem to improve the efficiency of the protease by increasing in vivo viral replication while in the presence of the inhibitor (*9, 17*). Therefore, finding inhibitors that are less affected by active site mutations may forestall the appearance of compensatory mutations and lead to more effective HIV drug therapy.

Our aim was to study the extent of correlation of enzyme resistance with antiviral resistance for HIV PR inhibitors by comparing the inhibition of both enzyme and viral replication. The residues altered in the mutants (Val82 and Ile84) had been identified by passaging HIV-1 in the presence of increasing concentrations of HIV PR inhibitors (*5, 7, 18*). An enzymatic assay and a whole-cell viral infectivity assay were used to measure the inhibition constants for the wild-type and mutant HIV proteases and viruses. We estimated enzyme resistance to HIV PR inhibitors by measuring the change in the dissociation constants (K_i values) for each mutation compared to wild type. Antiviral resistance to each inhibitor was determined by measuring the change in the viral IC_{90} values from wild type to isogenic mutant virus.

A simple enzyme kinetics assay has proven to be a valuable tool in the search for "broad spectrum" inhibitors of HIV PR. However, we, like several other research groups, have found that enzyme resistance generally differs from in vitro antiviral resistance. One approach used to address the disparity with antiviral resistance was to modify the estimate of enzyme resistance by adjusting the K_i values with the catalytic efficiency (k_{cat}/K_m) of the enzymes (*19*). The "vitality values", defined by the ratio $(K_i k_{cat}/K_m)_{mutant}/(K_i k_{cat}/K_m)_{wt}$, were calculated for the inhibitors and mutants. Several groups have suggested that vitality might be predictive of the selective advantage of a mutant over wild type in the presence of an HIV PR inhibitor (*19*−*21*). However, a correlation between vitality values and in vitro antiviral resistance has not been shown. In this report, we examine the inhibition of viral activity and the enzyme kinetics for wild-type HIV PR and 5 active site mutants using a group of 19 protease inhibitors of HIV. Both the virus and enzyme contained identical HIV PR mutations in a HXB2 viral background. We assessed the impact of the mutations on inhibitor potency by calculating the ratio of the mutant to wild-type K_i values, IC_{90} values, and vitality values. Because changes in the K_m and k_{cat} values may not equally affect the viral processing event, we also examined whether mutant/wild-type ratios of K_i adjusted individually with K_m and k_{cat} provided a better indication of the antiviral resistance $[(IC_{90})_{mutant}/(IC_{90})_{wt}]$ for the group of inhibitors.

MATERIALS AND METHODS

Inhibitors. The HIV PR inhibitors indinavir (MK-639) and nelfinavir (AG-1343) were obtained from Merck Research Laboratories and Agouron Research Laboratories, respectively. Samples of the HIV PR inhibitors saquinavir (Ro 31-8959), VX-478 (141W94), and ritonavir (ABT-538) were synthesized at the DuPont Merck Pharmaceutical Co. The cyclic ureas were prepared as described elsewhere (*22*−*26*). All inhibitors were dissolved in dimethyl sulfoxide (DMSO) and stored at −20 °C.

In Figure 1, the inhibitors have been classified according to their structural features. The linear peptidyl mimetic inhibitors saquinavir, 141W94, nelfinavir, ritonavir, and indinavir comprised the first set of inhibitors. Four of the inhibitors of HIV PR (saquinavir, nelfinavir, ritonavir, and indinavir) have been approved by the United States Food and Drug Administration for use in combination therapies for the treatment of HIV.

The second set of inhibitors contained symmetrical seven-membered cyclic ureas that have been arranged into two series according to their substituent groups. In the first series, XK234 has a small cyclopropylmethyl P2, P2′ group while DMP 323 (XM323), DMP 450, XN063, and XP521 contain P2, P2′ benzyl groups with residues at the 3- or 4-positions of the benzyl ring. XR835 and XZ442 have the largest P2, P2′ groups consisting of 5-indazolemethyl moieties.

The second series of cyclic ureas consisted of SB561, SB570, SB571, SD146, XV638, XV643, and XV652. Each inhibitor contains a P3, P3′ group attached by an amide linker to the 3-position of the P2, P2′ benzyl ring (*26*).

Substrate. The synthesis of the fluorescent substrate 2-aminobenzoyl-ATHQVYF(NO_2)VRKA-OH has been described elsewhere (*27*). Stock solutions were prepared in DMSO.

Preparation of HIV Proteases. The synthetic genes for the six HXB2 strain HIV-1 proteases were constructed with mutagenic oligonucleotide primers (containing a unique *Eag*I site) using PCR (*11*). The constructed mutant genes were then inserted into the translation vector pET11C and expressed in *Escherichia coli* BL21(DE-3) as previously described (*27*). The recombinant enzymes were solubilized with 6 M guanidine hydrochloride and isolated from inclusion bodies. The enzymes were purified by use of two sizing columns (Pharmacia Superdex 200 and Superdex 75) and refolded on a hydrophobic interaction column. The eluted proteases were determined to be homogeneous based on SDS−PAGE analysis. The protein concentrations were estimated by the method of Bradford (*28*). Aliquots of the enzymes were frozen at −70 °C.

HIV Protease Assays. HIV PR activity was measured using the discontinuous HPLC assay described previously as assay system C (*29*). Briefly, the activity of HIV proteases was measured in the absence and presence of four different concentrations of inhibitor at a fixed concentration of both enzyme and substrate. The HIV proteases (concentration ranged from 0.05 to 2.0 nM) were preincubated 5 min at ambient temperature (20−25 °C) with inhibitors at concentrations ranging from 0.1 to 12 500 nM. Substrate was then added (concentration ranged from 5 to 100 μM) and incubated at 20 ± 1 °C for 60 min. The reaction was terminated with 0.1 M ammonium hydroxide, and the extent of hydrolysis was determined using ion-exchange HPLC. A Pharmacia FPLC mono Q HR 5/5 column eluted at 1.0 mL/min with 0−30% buffer B for 5 min was used to separate the fluorescent cleavage product (retention time = 3.4 min) from the fluorescent substrate. The mobile phase buffer A contained 20 mM Tris-HCl, 0.02% sodium azide, and 10%

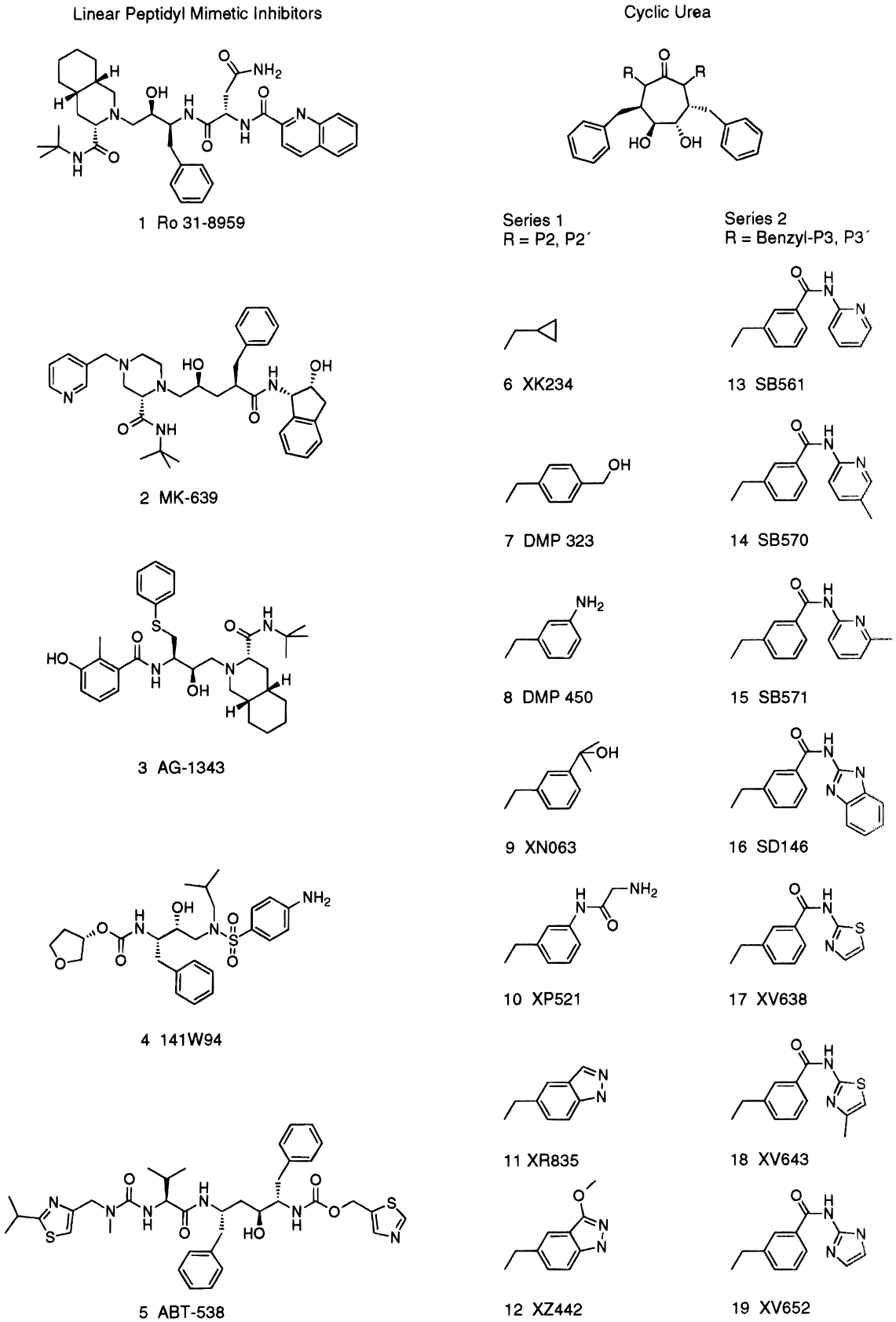

FIGURE 1: Structures of the inhibitors tested. The first set of inhibitors consist of the linear peptidyl mimetics Ro 31-8959 (saquinavir), VX-478 (141W94), AG-1343 (nelfinavir), ABT-538 (ritonavir), and MK-639 (indinavir). The second set are symmetrical cyclic ureas. Series 1 consists of compounds with various P2, P2′ groups. Series 2 cyclic ureas are larger molecules containing P3, P3′ groups attached by an amide linker to the 3-position of the P2, P2′ benzyl group.

acetonitrile at pH 9.0 while buffer B consisted of buffer A plus 0.5 M ammonium formate at pH 9.0. The mono Q column was washed with 100% buffer B at 5−9 min and then stepped down to 0% buffer B from 9 to 12 min to recycle the gradient for the next injection. The cleavage product Abz-ATHQVY−COO^- was measured at excitation

and emission wavelengths of 330 and 430 nm, respectively. With the equation $K_i = I/\{[(K_m + S - f_aS)/f_aK_m] - 1\}$, K_i values were determined from single point calculations using the observed fractional activity ($f_a = v_i/v_o$) and known K_m, [S], and [I]. Fractional activities ranging from 0.1 to 0.8 relative to the uninhibited control were averaged to calculate the K_i value for the inhibitor. Typically, the K_i value was determined from assays at two different [I] values for a compound.

The kinetic constants for HIV proteases were determined by the method of Lineweaver and Burk with fixed concentrations of HIV protease between 0.25 and 2.0 nM and varied substrate concentrations of 20−100 μM, respectively. The k_{cat} values were calculated from the V_{max} values and the active-site concentrations of the HIV proteases (*27*). The active-site concentrations for the six HIV proteases were quantified by titrating with different concentrations of XP954 (P2, P2′ = 3-amidoximebenzyl) and plotting the percent of HIV protease activity with respect to inhibitor concentration. This highly potent cyclic urea inhibitor had K_i values ranging from 0.01 to 1.0 nM for the six HIV proteases.

Preparation of Mutant HIV Virus. To introduce defined sequence alterations into the protease gene, recombinant DNA plasmids containing the 5′ half of the HIV HXB2 genome were subjected to site-directed mutagenesis as previously described (*26*, *30*). Cloned 5′ half HIV plasmids were linearized with *Nco*I, ligated with a complementary *Nco*I-linearized 3′ half HIV plasmid, and used to transfect MT-4 cells by lipofection. The culture was frozen in aliquots after the virally induced cytopathic effect had spread through the culture (typically 7−10 days after lipofection). Viral stocks were not passaged in order to reduce the chance of accumulating additional sequence changes beyond those encoded by the transfected plasmid.

Viral Infectivity Assay. The antiviral potency of compounds was measured as previously described (*26*). Briefly, replication of wild-type and mutant viruses was assessed by measuring the accumulation of viral p24 antigen 3 days after infection of MT-4 cells with HIV-1 HXB2. Cultures of MT-4 cells (2.5×10^5 cells/mL) were infected with appropriate dilutions of each virus stock and cultured for 24 h at 37 °C and 5% CO_2 in RPMI 1640 with 10% fetal calf serum, 2 mM glutamine, and 50 μg/mL gentamycin (Gibco/BRL) without protease inhibitors. After 24 h, infected cultures were washed 3 times in medium and plated into microtiter plate wells at 2.5×10^5 infected cells/mL with various concentrations of each inhibitor. The infected cell cultures with and without added inhibitor were then incubated for 72 h. Appropriate dilutions of each virus stock were determined in preliminary experiments and were selected to result in the accumulation of between 1000 and 4000 ng/mL of p24 antigen at the end of the 4 day culture period. The accumulation of p24 antigen was quantitated in each culture using the DuPont p24 antigen ELISA kit. Based on the untreated culture, the concentration of inhibitor that causes a 90% reduction in viral p24 antigen was defined as the IC_{90} value.

RESULTS

Changes in Catalytic Efficiency for the Mutant and Wild-Type Proteases. The kinetic constants for the six HIV proteases and the peptide substrate are shown in Table 1. The K_m, k_{cat}, and catalytic efficiency for the V82A mutation were not significantly different from those of wild-type enzyme. For the V82F mutation, both K_m and k_{cat} decreased, resulting in an unchanged catalytic efficiency. The catalytic efficiency for the I84V mutation was only 25% of wild type, driven primarily by a 3-fold increase in the K_m. The V82I mutation had the greatest increase in K_m (∼7-fold) with a small decrease in k_{cat} resulting in a catalytic efficiency that was only 9% of wild type. The double mutation V82F/I84V was the least active of the five mutations with a catalytic efficiency of just 2% of wild type. Unlike the single mutations, the combination of the V82F and I84V mutations caused a 6-fold increase in K_m and a substantial decrease in the k_{cat} (approximately 0.1 the rate of wild type).

Table 1: Comparison of the Enzyme Kinetic Constants for Wild-Type and Mutant HIV-1 Protease[a]

HIV PR	K_m (mM)	k_{cat} (s^{-1})	catalytic efficiency, k_{cat}/K_m (mM^{-1} s^{-1})
Wt	0.035 ± 0.008	1.04 ± 0.17	29.9 ± 3.8
V82F	0.012 ± 0.005	0.44 ± 0.16	35.4 ± 3.8
V82A	0.039 ± 0.002	1.33 ± 0.27	34.2 ± 6.8
I84V	0.102 ± 0.025	0.78 ± 0.17	7.6 ± 0.2
V82I	0.245 ± 0.030	0.67 ± 0.19	2.7 ± 0.5
V82F/I84V	0.224 ± 0.067	0.11 ± 0.02	0.5 ± 0.1

[a] All kinetic constant values are the mean and standard deviations of three or more independent experiments.

Changes in the Activity of the Wild Type and Mutants toward Different Classes of Inhibitors. Table 2 shows the IC_{90} and K_i for wild-type enzyme with the 19 inhibitors, and the resistance values (the ratio of mutant/wild type) for the IC_{90}, K_i, and vitality values with the 5 mutations. It is apparent that the wild-type viral IC_{90} values were greater than the corresponding K_i values in all cases shown. The ratio of IC_{90}/K_i, known as "translation values" (*31*), may reflect cell penetration or subcellular localization of the compounds. The ratio varied from 26 to 10 000 for these compounds. The series 2 cyclic ureas have desirable low translation values.

Collectively, the 19 compounds were potent inhibitors of the wild-type protease and virus. The linear peptidyl mimetic inhibitors had K_i values ranging from 0.1 to 0.28 nM and viral IC_{90}'s ranging from 16 to 137 nM. The first series of cyclic ureas containing only P2, P2′ groups had a broad range of activity toward the wild type; the K_i values ranged from 0.03 to 5.8 nM, and IC_{90}'s range from 11 to 2100 nM. Overall, the cyclic ureas from the second series were the most potent of the panel of inhibitors against the wild type. The K_i values were from 0.05 to 0.14 nM, and the IC_{90}'s varied from 2.4 to 20 nM.

The effect of the V82F mutation on the antiviral potency of the inhibitors was mixed: many of the inhibitors maintained wild-type activity, and a few (e.g., saquinavir, SB570, SB571, and SD146) were more potent. Ritonavir and the cyclic ureas XK234, DMP 450, XN063, and DMP 323 had viral IC_{90} values increased 4−7-fold whereas XZ442 increased nearly 16-fold. There were quite different effects on enzyme inhibition. For instance, the three inhibitors 141W94, XV643, and SB570 each had low K_i ratios of ∼0.3, the result of a decrease in the K_i value for the V82F mutation. The K_i ratio for saquinavir, on the other hand, indicated the

Table 2: Inhibition of Wild-Type Protease and Virus, and Resistance Values for Mutant Proteases and Viruses[a]

		inhibition of wild type		relative resistance														
				V82F			V82A			V82I			I84V			V82F/I84V		
		IC_{90} (nM)	K_i (nM)	IC_{90} ratio	K_i ratio	vital	IC_{90} ratio	K_i ratio	vital	IC_{90} ratio	K_i ratio	vital	IC_{90} ratio	K_i ratio	vital	IC_{90} ratio	K_i ratio	vital
							Linear Peptide Mimetic Inhibitors											
1	saquinavir	16.0	0.15	0.25	3.33	3.89	0.51	1.67	1.89	(1.21)	7.3	0.66	0.67	10.67	2.70	0.73	19	0.31
2	indinavir	27.2	0.14	2.43	0.46	0.53	2.25	6.36	7.20	(0.99)	5.4	0.48	0.92	2.57	0.65	8.1	79	1.26
3	nelfinavir	25.8	0.28	−	0.82	0.96	−	4.29	4.86	−	17.5	1.58	−	3.46	0.88	−	86	1.37
4	141W94	74.2	0.11	2.01	0.35	0.40	1.20	1.73	1.96	(2.40)	3.3	0.29	1.79	2.73	0.69	12.3	100	1.60
5	ritonavir	137	0.17	(5.86)	0.82	0.96	(4.7)	11.2	12.7	(2.43)	14.7	1.32	3.69	11.18	2.83	(18.2)	700	11.2
							Series 1 Cyclic Ureas, P2, P2′ Groups											
6	XK234	2063	5.80	3.75	1.05	1.23	1.26	6.55	7.43	(1.12)	26.7	2.41	6.36	86.2	21.8	(25.2)	2025	32.4
7	DMP 323	93.7	0.85	7.21	0.55	0.65	1.01	8.71	9.87	1.74	23.5	2.12	10.6	20.0	5.07	377	1016	16.3
8	DMP 450	125	0.41	4.55	0.88	1.02	1.79	10.73	12.16	0.94	36.6	3.29	6.89	29.3	7.41	104	1256	20.1
9	XN063	22.2	0.08	(3.80)	1.36	1.58	2.70	7.04	7.98	−	24.7	2.22	3.26	21.0	5.32	(261)	1494	23.9
10	XP521	283	0.03	(1.83)	1.07	1.25	−	6.79	7.69	−	17.9	1.61	2.88	8.93	2.26	(23.9)	1036	16.6
11	XR835	10.9	0.04	0.53	1.05	1.23	1.03	2.39	2.71	−	6.58	0.59	0.88	10.0	2.53	20.6	789	12.6
12	XZ442	11.6	0.18	15.8	0.42	0.49	(2.29)	3.28	3.71	−	5.11	0.46	10.1	23.3	5.91	(347)	1144	18.3
							Series 2 Cyclic Ureas, P3, P3′ Groups											
13	SB561	2.4	0.09	(0.25)	0.43	0.50	(1.05)	0.78	0.88	−	1.28	0.11	1.85	1.81	0.46	74	22	0.36
14	SB570	(3.9)	0.08	(0.17)	0.32	0.37	(0.97)	0.61	0.69	−	1.32	0.12	(2.60)	2.50	0.63	(63)	42	0.67
15	SB571	3.1	0.09	(0.13)	0.36	0.42	(0.71)	0.66	0.75	−	1.54	0.14	1.13	2.53	0.64	41	20	0.32
16	SD146	4.4	0.10	(0.19)	0.52	0.60	(0.50)	1.24	1.40	−	0.85	0.08	(0.58)	2.58	0.65	4.8	38	0.61
17	XV638	4.3	0.11	0.88	0.51	0.59	1.67	1.09	1.24	−	1.64	0.15	1.12	1.09	0.28	27	25	0.39
18	XV643	5.7	0.14	0.36	0.32	0.38	1.31	0.50	0.57	−	0.93	0.08	0.67	1.79	0.45	13	21	0.34
19	XV652	19.7	0.05	(1.19)	0.47	0.55	(2.17)	2.16	2.44	−	3.14	0.28	(0.55)	2.35	0.60	(0.9)	10	0.16

[a] Relative resistance values are the ratios of mutant/wild type for K_i, IC_{90}, or $K_i k_{cat}/K_m$ (vitality). All K_i values are the mean of at least three or more independent experiments. Most IC_{90} values are the averages of two or more determinations. Calculations based on a single value are shown in parentheses.

K_i value for the V82F mutation increased ~3-fold. Interestingly, the V82F antiviral resistance values for saquinavir, ritonavir, XK234, DMP 450, XN063, DMP 323, and XZ442 were not predicted by the V82F K_i ratios. However, both the K_i ratios and the antiviral resistance values indicated that the cyclic urea inhibitors with P3, P3′ groups retained wild-type potency toward the V82F mutation.

The V82A mutation caused a 4−11-fold increase in the K_i values for nelfinavir, indinavir, and ritonavir. Likewise, most of the first series of cyclic ureas increased 3−11-fold. However, saquinavir, 141W94, and XR835 were relatively unchanged. The K_i values for the second series with P3, P3′ groups were also relatively unaffected by the V82A mutation. The IC_{90} values for the viral V82A mutation remained similar to the wild-type virus for most of the inhibitors whereas ritonavir had about a 5-fold increase in the IC_{90} value for this mutation.

The V82I mutation increased the K_i values of the linear peptidyl mimetics and the cyclic ureas with P2, P2′ groups. A small change in the K_i value was observed for 141W94 (~3-fold) while the K_i's for indinavir, saquinavir, ritonavir, and nelfinavir increased 5−18-fold. The first series of cyclic ureas had the greatest loss of affinity ranging from 5- to 37-fold. Within the second series of cyclic ureas, only XV652 increased in K_i value (~3-fold) while the rest of the inhibitors retained wild-type activity for the V82I mutation. Unlike the K_i values, the IC_{90} values for the viral V82I mutation indicate that the peptidyl mimetic inhibitors and XK234, DMP 323, and DMP 450 of the first series of cyclic ureas retained wild-type activity against the virus.

The I84V enzyme mutation showed reduced sensitivity to three of the five peptidyl mimetic inhibitors whereas both indinavir and 141W94 had no significant change in the K_i value. The K_i value for nelfinavir increased ~3-fold whereas both saquinavir and ritonavir increased about 11-fold. Likewise, the first series of cyclic ureas experienced a reduction in activity for the I84V enzyme ranging from 9- to 86-fold. However, there was no significant loss of potency by the second series of cyclic ureas for the I84V enzyme. The IC_{90} values for the viral I84V mutation were similar to wild type for saquinavir, indinavir, and 141W94 whereas the IC_{90} value for ritonavir increases ~3-fold. For the first series of cyclic ureas, XR835 had wild-type activity whereas the rest had a 3−11-fold increase in the IC_{90} value toward the I84V virus. Like the K_i ratios for the I84V mutation, the IC_{90} values for the viral I84V mutation indicate that the second series cyclic ureas had potency similar to wild type.

Toward the double mutation V82F/I84V, both sets of inhibitors had elevated K_i values. Saquinavir was the least affected of the peptidyl mimetic inhibitors with only a 19-fold increase in the K_i value whereas ritonavir incurred the greatest increase (700-fold). Indinavir, nelfinavir, and 141W94 K_i values increased 79-, 86-, and 100-fold, respectively, for the double mutation. Likewise, there was a significant loss of activity (790−2025-fold) for the first series of cyclic ureas for the double mutation. The second series of cyclic ureas were the most potent (10−42-fold increase in K_i) toward V82F/I84V HIV PR. The K_i values ranged from 0.5 to 4.0 nM. The double mutation also caused an increase in the IC_{90} values for most of the peptidyl mimetic inhibitors. The IC_{90} values for indinavir, 141W94, and ritonavir increased 8-, 12-, and 18-fold, respectively, while saquinavir retained wild-type potency toward the V82F/I84V viral mutation. The first series of cyclic ureas had IC_{90} values elevated 21−377-fold over wild type. Most second series inhibitors showed a substantial change in IC_{90} values, increasing 5−75-fold. XV652 and SD146 were least affected by the double mutation; the IC_{90} value for XV652

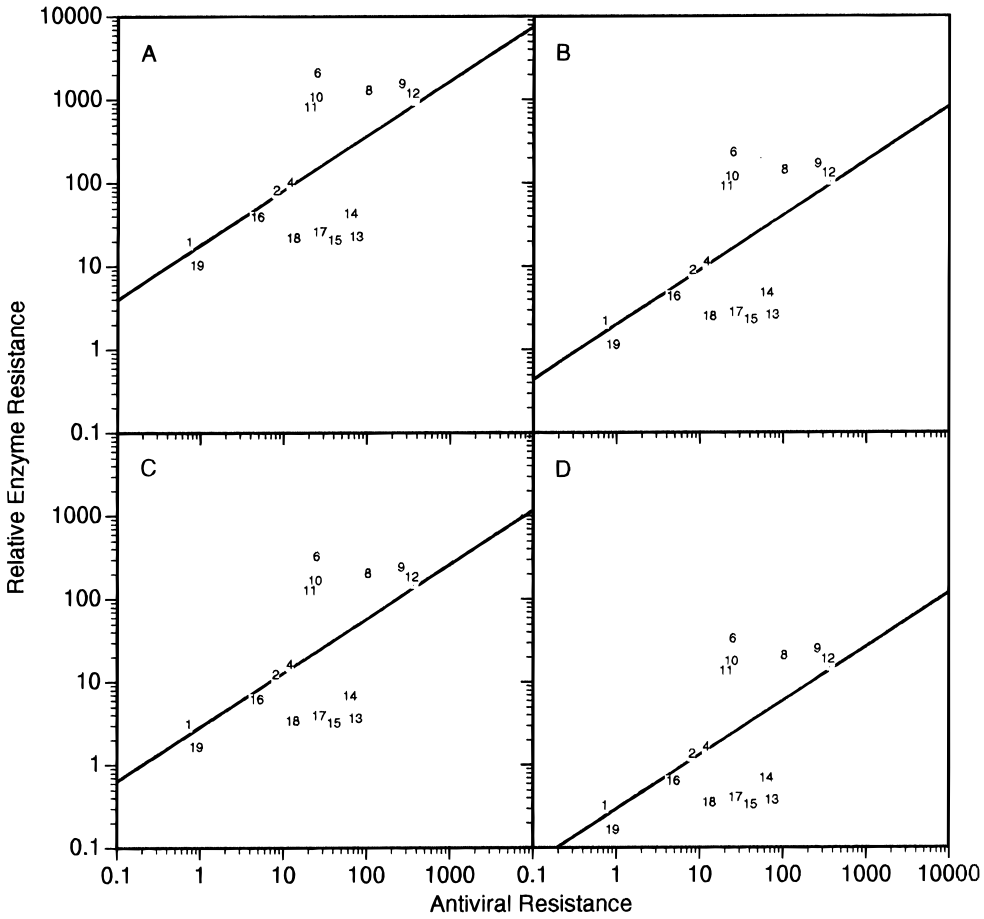

FIGURE 2: Correlation of enzyme and antiviral resistance values for the V82F/I84V double mutation. The enzyme resistance values in the panels are based on (A) K_i ratio, (B) K_ik_{cat} ratio, (C) K_i/K_m ratio, and (D) vitality values. The correlation with antiviral resistance does not significantly change ($r^2 = 0.35 \pm 0.00$). The plotted numbers in each graph correspond to the HIV PR inhibitors in Table 2. Inhibitors 5 and 7 are obscured by 11 and 12, respectively, in each graph.

was equal to wild type, and that for SD146 increased ~5-fold.

Correlation of the Antiviral and Enzyme Resistance Values for the Mutants and Inhibitors. Table 2 shows that enzyme resistance values (K_i ratios or vitality) were not predictive of antiviral resistance. We have plotted in Figure 2 for the double mutant four different comparisons of enzyme resistance values with antiviral resistance values. The double logarithmic graphs show the same low correlation ($r^2 = 0.35$) regardless of whether enzyme resistance values are calculated as mutant/wild-type ratios of K_i, K_ik_{cat}, K_i/K_m, or vitality. Therefore, adjustments of K_i with any combination of k_{cat} or K_m only affect the intercept of the line and do not affect the correlation. This observation is a reflection of the truism that dividing all of a set of values by a constant (k_{cat} or K_m for a given substrate and enzyme) does not change the relationship of the values. The correlation is also low for the other enzymes for the single mutations V82F, V82A, V82I, and I84V (0.017, 0.32, 0.12, and 0.53, respectively).

DISCUSSION

Although currently available HIV PR inhibitors are able to reduce plasma HIV concentration by several orders of magnitude when combined with other drugs, development of resistance to the inhibitors may become a major clinical problem. Since enzyme assays are more convenient than antiviral assays, it obviously would be desirable to use measurement of enzyme activity to predict the potency of an inhibitor against a mutant. The lack of correlation led to the suggestion that adjustment of enzyme inhibition by catalytic efficiency should improve the correlation (*19*−*21*). However, as is evident from Figure 2, using the K_m, k_{cat}, or vitality values does not alter the low correlation of the K_i ratios with antiviral resistance for the five different mutations. These mutant viruses were designed to have only a single or a double amino acid change in the active site region. Naturally occurring mutants may have changes not only in the enzyme but also in the substrate cleavage sites and/or other locations. Mutations in the flaps, dimer interface, and surface loops may also confer resistance (*11*). It appears that prolonged drug exposure may select for mutations at distal sites that partially compensate for the loss of activity associated with the active site changes (*6*, *32*).

It is not known whether the low correlation results we observed were due to cellular factors such as inhibitor penetration or artifacts caused by enzyme assay conditions. With a given set of enzymes, competitive inhibitors, and assay conditions, any peptide substrate should provide similar K_i values, although catalytic efficiency may vary with the substrate and assay conditions. With our peptide substrate, both V82F and V82A had enzymatic activity equivalent to wild type whereas Gulnik et al. reported a 70% reduction for the same mutations with a different peptide substrate. Nevertheless, there is general agreement that both the V82I

and I84V mutations have significantly less enzyme activity than the wild type. We found relative catalytic efficiencies of 9% and 25% for V82I and I84V, respectively, compared to 30% and 40% reported by Gulnik et al.

Although it is not clear why the correlation is low for the panel of inhibitors and active site mutations, there are trends associated with certain inhibitors for the five mutations. For example, most of the second series of cyclic ureas are potent against both enzyme and virus for all tested mutants. The observation that many of the second series of cyclic ureas have favorable translation values suggests that good cellular absorption or subcellular localization of the inhibitors might be critical for predictability. It should be noted that there is apparently no published experimental data on penetration rates or subcellular localization of HIV PR inhibitors. The second series of cyclic ureas with P3, P3′ groups are linked to the core structure with an amide bond, thereby increasing the number of binding interactions formed with the enzyme (*26*). The loss of binding affinity due to the decrease in van der Waals contacts caused by the mutations may therefore be proportionately less (*11*).

Although several research groups have shown that different substrates yield different vitality values (*20, 21*), changing substrates will not affect the correlation for a panel of inhibitors toward a HIV PR mutation. Only changes in the K_i values, related, for example, to differences in assay conditions, could alter the correlation with the viral IC_{90} values. Substantial differences in the K_i values for the linear peptidyl mimetic inhibitors have been reported in other studies for the wild-type and mutant HIV PR (*16, 19*−*21, 32*−*34*) which may result in a different correlation with antiviral resistance.

CONCLUSIONS

In this study, we have determined that for five HIV-1 PR mutations the mutant/wild-type ratio of K_i values and antiviral resistance have a low correlation. In addition, we have demonstrated that the correlation with antiviral resistance does not change upon calculating the enzyme resistance with K_m, k_{cat}, or vitality values. The low correlation implies there must be other variables that affect the activity of inhibitors toward HIV mutations within the context of the virus.

Overall, the second series of cyclic ureas (which have P3, P3′ groups) has a better resistance profile toward the five HIV PR mutations than the first series of cyclic ureas lacking these groups. Albeit each compound varies in the amount of change with the mutation, both the ratio of K_i values and the antiviral resistance data support that increased binding interactions improve the potency of the cyclic ureas toward V82F, I84V, and the double mutation V82F/I84V. Last, our study illustrates that although a rapid, inexpensive enzyme assay may be sufficient to discover potent inhibitors, it is important to use both an enzyme and a virally based assay to assess the quantitative structural activity relationships of inhibitors toward HIV PR mutations.

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REFERENCES

1. Debouck, C., Gorniak, J. G., Strickler, J. E., Meek, T. D., Metcalf, B. W., and Rosenberg, M. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 8903−8906.
2. Henderson, L. E., Benveniste, R. E., Sowder, R., Copeland, T. D., Schultz, A. M., and Oroszlan, S. (1988) *J. Virol. 62*, 2587−2595.
3. Kohl, N. E., Emini, E. A., Schleif, W. A., Davis, L. J., Heimbach, J. C., Dixon, R. A. F., Scolnick, E. M., and Sigal, I. S. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 4686−4690.
4. Peng, C., Ho, B. K., Chang, T. W., and Chang, N. T. (1989) *J. Virol. 63*, 2550−2555.
5. Otto, M. J., Garber, S., Winslow, D. L., Reid, C. D., Aldrich, P., Jadhav, P. K., Patterson, C. E., Hodge, C. N., and Cheng, Y.-S. E. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 7543−7547.
6. Condra, J. H., Schleif, W. A., Blahy, O. M., Gabryelski, L. J., Graham, D. J., Quintero, J. C., Rhodes, A., Robbins, H. L., Roth, E., Shivaprakash, M., Titus, D., Yang, T., Teppler, H., Squires, K. E., Deutsch, P. J., and Emini, E. A. (1995) *Nature 374*, 569−571.
7. King, R. W., Garber, S., Winslow, D. L., Reid, C., Bacheler, L. T., Anton, E., and Otto, M. J. (1995) *Antiviral Chem. Chemother. 6*, 80−88.
8. Markowitz, M., Mo, H., Kempf, D. J., Norbeck, D. W., Bhat, T. N., Erickson, J. W., and Ho, D. D. (1995) *J. Virol. 69*, 701−706.
9. Molla, A., Korneyeva, M., Gao, Q., Vasavanonda, S., Schipper, P. J., Mo, H., Markowitz, M., Chernyavskiy, T., Niu, P., Lyons, N., Hsu, A., Granneman, G. R., Ho, D. D., Boucher, C. A. B., Leonard, J. M., Norbeck, D. W., and Kempf, D., J. (1996) *Nat. Med. 2*, 760−766.
10. Chen, Z., Li, Y., Schock, H. B., Chen, E., and Kuo, L. C. (1995) *J. Biol. Chem. 270*, 21433−21436.
11. Ala, P. J., Huston, E. E., Klabe, R. M., McCabe, D. D., Duke, J. L., Rizzo, C. J., Korant, B. D., DeLoskey, R. J., Lam, P. Y. S., Hodge, C. N., and Chang, C.-H. (1997) *Biochemistry 36*, 1573−1580.
12. Richman, D. D. (1995) *Nature 374*, 494.
13. Tritch, R. J., Cheng, Y.-S. E., Yin, F. H., and Erickson-Viitanen, S. (1991) *J. Virol. 65*, 922−930.
14. Kaplan, A. H., Manchester, M., and Swanstrom, R. (1994) *J. Virol. 68*, 6782−6786.
15. Doyon, L., Croteau, G., Thibeault, D., Poulin, F., Pilote, L., and Lamarre, D. (1996) *J. Virol. 70*, 3763−3769.
16. Maschera, B., Graham, D., Palú, G., Wright, L. L., Tisdale, M., Myers, R., Blair, E. D., and Furfine, E. S. (1996) *J. Biol. Chem. 271*, 33231−33235.
17. Nijhuis, M., Schuurman, R., de Jong, D., Schipper, P., Danner, S., and Boucher, C. (1997) in *Abstracts of the International Workshop on HIV Drug Resistance, Treatment Strategies and Eradication*, p 61, St. Petersburg, FL, June 25−28.
18. Hodge, C. N., Aldrich, P. E., Bacheler, L. T., Chang, C.-H., Eyermann, C. J., Garber, S., Grubb, M., Jackson, D. A., Jadhav, P. K., Korant, B., Lam, P. Y. S., Maurin, M. B., Meek, J. L., Otto, M. J., Rayner, M. M., Reid, C., Sharpe, T. R., Shum, L., Winslow, D. L., and Erickson-Viitanen, S. (1996) *Chem. Biol. 3*, 301−314.
19. Gulnik, S. V., Suvorov, L. I., Liu, B., Yu, B., Anderson, B., Mitsuya, H., and Erickson, J. W. (1995) *Biochemistry 34*, 9282−9287.
20. Pazhanisamy, S., Stuver, C. M., Cullinan, A. B., Margolin, N., Rao, B. G., and Livingston, D. J. (1996) *J. Biol. Chem. 271*, 17979−17985.
21. Wilson, S. I., Phylip, L. H., Mills, J. S., Gulnik, S. V., Erickson, J. W., Dunn, B. M., and Kay, J. (1997) *Biochim. Biophys. Acta 1339*, 113−125.
22. Lam, P. Y. S., Jadhav, P. K., Eyermann, C. J., Hodge, C. N., Ru, Y., Bacheler, L. T., Meek, J. L., Otto, M. J., Rayner, M.

M., Wong, N., Chang, C.-H., Weber, P. C., Jackson, D. A., Sharpe, T. R., and Erickson-Viitanen, S. (1994) *Science 263*, 380−384.
23. Lam, P. Y. S., Ru, Y., Jadhav, P. K., Aldrich, P. E., Delucca, G. V., Eyermann, C. J., Chang, C.-H., Emmett, G., Holler, E. R., Daneker, W. F., Li, L., Confalone, P. N., McHugh, R. J., Han, Q., Markwalder, J. A., Seitz, S. P., Bacheler, L. T., Rayner, M. M., Klabe, R. M., Shum, L., Winslow, D. L., Kornhauser, D. M., Jackson, D. A., Erickson-Viitanen, S., Sharpe, T. R., and Hodge, C. N. (1996) *J. Med. Chem. 39*, 3514−3525.
24. Wilkerson, W. W., Hollis, A. Y., Cheatham, W. W., Lam, G. N., Erickson-Viitanen, S., Bacheler, L., Cordova, B. C., Klabe, R. M., and Meek, J. L. (1995) *Bioorg. Med. Chem. Lett. 5*, 3027−3032.
25. Rodgers, J. D., Johnson, B. L., Wang, H., Greenberg, R. A., Erickson-Viitanen, S., Klabe, R. M., Cordova, B. C., Rayner, M. M., Lam, G. N., and Chang, C.-H. (1996) *Bioorg. Med. Chem. Lett. 6*, 2919−2924.
26. Jadhav, P. K., Ala, P., Woerner, F. J., Chang, C.-H., Garber, S. S., Anton, E. D., and Bacheler, L. T. (1997) *J. Med. Chem. 40*, 181−191.
27. Cheng, Y.-S. E., Yin, F. H., Foundling, S., Blomstrom, D., and Kettner, C. A. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 9660−9664.
28. Bradford, M. M. (1976) *Anal. Biochem. 72*, 248−254.
29. Erickson-Viitanen, S., Klabe, R. M., Cawood, P. G., O′Neal, P. L., and Meek, J. L. (1994) *Antimicrob. Agents Chemother. 38*, 1628−1634.
30. Winslow, D. L., Horlick, R. A., Anton, E. A., Tritch, R. J., Zagursky, R. J., and Bacheler, L. T. (1994) *Biochem. Biophys. Res. Commun. 205*, 1651−1657.
31. Nugiel, D. A., Jacobs, K., Worley, T., Patel, M., Kaltenbach, R. F., III, Meyer, D. T., Jadhav, P. K., De Lucca, G. V., Smyser, T. E., Klabe, R. M., Bacheler, L. T., Rayner, M. M., and Seitz, S. P. (1996) *J. Med. Chem. 39*, 2156−2169.
32. Schock, H. B., Garsky, V. M., and Kuo, L. C. (1996) *J. Biol. Chem. 271*, 31957−31963.
33. Vacca, P. J., Dorsey, B. D., Schleif, W. A., Levin, R. B., McDaniel, S. L., Darke, P. L., Zugay, J., Quintero, J. C., Blahy, O. M., Roth, E., Sardana, V. V., Schlabach, A. J., Graham, P. I., Condra, J. H., Gotlib, L., Holloway, M. K., Lin, J., Chen, I.-W., Vastag, K., Ostovic, D., Anderson, P. S., Emini, E. A., and Huff, J. R. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 4096−4100.
34. Reich, S. H., Melnick, M., Davies, J. F., II, Appelt, K., Lewis, K. K., Fuhry, M. A., Pino, M., Trippe, A. J., Nguyen, D., Dawson, H., Wu, B.-W., Musick, L., Kosa, M., Kahil, D., Webber, S., Gehlhaar, D. K., Andrada, D., and Shetty, B. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 3298−3302.

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