AIDS Rev 2003;5:165-171

Protease Inhibition in African Subtypes of HIV-1

Adrián Velázquez-Campoy[1], Sonia Vega[1], Erin Fleming[1], Usman Bacha[1], Yasien Sayed[2], Heini W. Dirr[2] and Ernesto Freire[1]

[1]Department of Biology, The Johns Hopkins University, Baltimore, MD, USA
[2]Protein Structure-Function Research Programme, Department of Molecular and Cell Biology, University of the Witwatersrand, Johannesburg, South Africa

Abstract

Of the 42 million people infected with HIV-1 worldwide, 30 million are in Africa. However, the HIV-1 subtypes prevalent in Africa are not the same that are prevalent in North America and Western Europe. In these developed regions, subtype B is responsible for the vast majority of HIV infections, whereas in sub-Saharan Africa subtypes A and C, and to a lesser extent subtype G, account for most of the infections. These subtypes exhibit genomic differences as large as 30% with respect to subtype B. These differences involve current drug targets, including the HIV-1 protease. Since protease inhibitors have been developed and tested against the HIV-1 B subtype, and proteases from other subtypes carry up to ten amino acid polymorphisms, it is important to assess the influence of these naturally occurring polymorphisms on the potency of existing inhibitors, as well as their synergistic interactions with mutations known to cause drug resistance. This review will examine the effects of naturally occurring polymorphisms on the efficacy of current protease inhibitors and the effects of well characterized drug-resistant mutations within the framework of non-B subtypes. At the biochemical level, non-B-subtype polymorphisms lower the binding affinities of existing clinical inhibitors, but not to the point of causing drug resistance. However, these polymorphisms amplify the effects of mutations causing drug resistance and may play a role in the long-term viability of these inhibitors.

Key words

AIDS. Africa. Drug resistance. Protease inhibitors. Non-B Subtypes. Calorimetry.

The AIDS epidemic in Africa has achieved dramatic proportions (see[1] for a recent review). Of the 42 million people infected with HIV worldwide, 30 million are in Africa. While the adult prevalence rate in North America and Western Europe is around 0.5%, the overall figure reaches 8.4% in sub-Saharan Africa. However, the HIV-1 subtypes prevalent in Africa are not the same that are prevalent in North America and Western Europe. In North America and Western Europe the B subtype is responsible for the vast majority of HIV infections, whereas in sub-Saharan Africa the A and C subtypes, and to a lesser extent the G subtype, account for most of the infections. The A subtype predominates in the northern part of sub-Saharan Africa, the C subtype in southern Africa and the G subtype in Nigeria. This situation

Correspondence to:
Ernesto Freire
Department of Biology
The Johns Hopkins University
Baltimore, MD 21218
Phone: (410) 516-7743
Fax: (410) 516-6469
Email: ef@jhu.edu

is, however, not static as population movement across different regions in the world are certain to alter the existing pattern[2-4].

While many factors contribute to the clinical effectiveness of protease inhibitors, an absolute requirement is that they exhibit high binding affinity and inhibitory potency against the intended target. Since protease inhibitors have been developed and tested against the HIV-1 B subtype, and proteases from other HIV-1 subtypes carry amino acid polymorphisms, some of which have been associated with drug resistance[5], two important questions need to be addressed: 1) are existing drugs equally effective against proteases from different HIV-1 subtypes?, and 2) How do drug-resistant mutations operate within the framework of proteases from non-B subtypes?.

HIV-1 protease variability

One of the most serious problems in the chemotherapy of HIV-1 infection arises from the high mutation rate of the virus and, consequently, its high genomic variability. This variability leads to sequence polymorphisms in the viral proteins, including those that are targets for current drugs. This problem is compounded by the existence of different viral subtypes that differ by as much as 30% in their genomes.

Sequence variability within the protease molecule originates from differences between subtype, differences within subtypes and drug resistance mutations. Within any given population, sequence variability can be characterized by an entropy function, H(i), defined for each position i in the protease sequence as $H(i) = \sum_j p_{i,j} \ln(p_{i,j})$, where $p_{i,j}$ is the frequency of residue j in position i. A position that is completely conserved will have a value of H(i) = 0, whereas a position that is variable will have a high entropy value, being H(i) = 2.996 the limit value for a position completely random in which all amino acids are equally probable. Figure 1 shows the sequence entropy values for each subtype mapped into the three-dimensional structure of the protease. Values range from low variability (blue) to high variability (red). It is clear in this figure that amino acid polymorphisms do not occur at random. They are concentrated in the hinge region of the flap, in the loop connecting the ß-strands, and in the α-helix and opposite ß-strand. In the absence of inhibitor pressure, naturally occurring polymorphisms are most likely to occur in regions that do not affect the catalytic efficiency of the protease or the fitness of the virus. In general, naturally occurring polymorphisms do not occur in regions that lead to a loss of structural stability of the protein, a loss of catalytic activity or a loss in substrate binding affinity.

HIV-1 protease in African subtypes

Protease molecules from non-B subtypes of HIV-1 that predominate in Africa (A, C and G) contain naturally occurring amino acid polymorphisms that fall outside the variability range observed within subtypes. These polymorphisms involve as much as ten positions in the protease sequence. In our work, we have studied representative, recombinant versions of non-B-subtype proteases corresponding to the A, C and G subtypes. The sequence of the A-subtype protease was derived from the consensus sequence of A-subtype protease from 14 antiretroviral-naive Ugandan

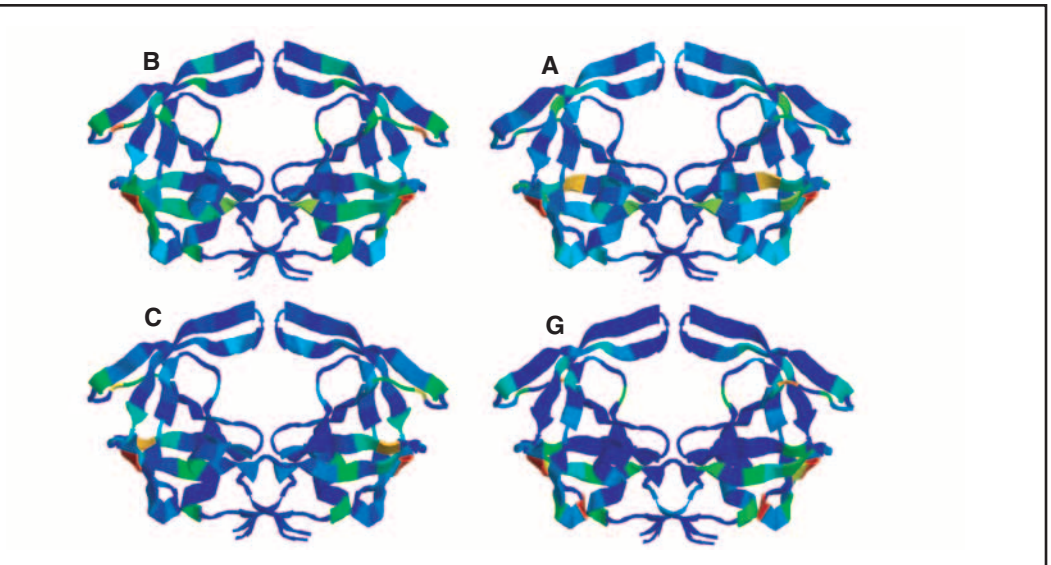

Figure 1. *Sequence polymorphisms in HIV-1 protease from different subtypes mapped into the structure of the molecule. The structures have been colored using a rainbow scale in which blue represents conserved regions (H(i) = 0) and red for regions of maximal variability. The maximal sequence entropy for each subtype ($H(i)_{max}$) is 1.3, 1.27, 1.52 and 1.1 for the B, A, C and G subtypes respectively.*

adults[5,6] and is identical to the sequences of two Ugandan isolates (#225.706 and #230.706). One C-subtype protease based upon a consensus sequence (92RW026 [Rwanda, GenBank #AF009410], C2220 [Ethiopia, GenBank #U461016], Z1226 [Zimbabwe, GenBank #AF083603], 96BW01 [Botswana, GenBank #AF110959] and C11 [Zambia, GenBank #AF107378]); another C-subtype protease corresponding to a consensus sequence from South Africa (C-SA in this paper) and a G-subtype consensus protease derived from the sequence database (http://hiv-web.lanl.gov, HIV Sequence Database, Los Alamos National Laboratory).

The amino acid polymorphisms of the studied non-B proteases relative to the B-subtype protease are shown in figure 1, mapped into the three-dimensional structure of the molecule. As shown in the figure, most of the polymorphisms are located at similar regions outside the active site. They are clustered in the hinge region of the flap (red), in the loop connecting the ß-strands (green), and in the α-helix and opposite ß-strand (yellow). Only in the G subtype, a difference present in 62% of the isolates occurs within the active site in a position associated with drug resistance in the B subtype (V82I shown in blue)[7]. Not surprisingly, differences between subtypes fall in the same regions that exhibit high variability within subtypes. In the African subtypes, the naturally occurring isoleucine at position 36 is a secondary resistant mutation in the B subtype[8] (M36I) and may act at a later stage in the viral infection, following the onset of primary resistant mutations. The polymorphisms M36I and S37N are characteristic to all non-B subtypes. Other polymorphisms, like leucine at position 93 (I93L) in the C-SA subtype and isoleucine at position 20 (K20I) in the G subtype, have been associated with drug resistance and classified as secondary resistance mutations.

Naturally occurring polymorphisms affect the behavior of the protease molecule at different levels. The structural stability of the native conformation of the A, B, C and G subtype proteases is different. Under the same experimental conditions, the A, C and G subtype proteases show higher structural stability than B-subtype protease (0.7, 1.3 and 2 kcal/mol, respectively)[9]. The C-SA-subtype protease, on the other hand, is slightly less stable than the B-subtype protease, as shown in figure 3. In this figure, the structural stability of the native conformations of the A, B, C, C-SA and G proteases is shown. These experiments were performed using the technique of differential scanning calorimetry (DSC), which directly measures the magnitude of the forces that stabilize the native structure of proteins[10]. Differences in structural stability may influence binding affinity due to the required conformational change associated with substrate and inhibitor binding. This conformational change involves the closing of the flaps and a slight rotation of each monomer around the dimer interface. A more stable unligated protease would impose a larger energetic penalty for the conformational change, thus decreasing the binding affinity. In addition, amino acid polymorphisms distal from the binding site may indirectly affect the geometry of the binding cavity by energetically favoring a slightly distorted conformation. It has been observed, before, that conformationally constrained ligands, such as the protease inhibitors in clinical use, have little capacity to adapt to chang-

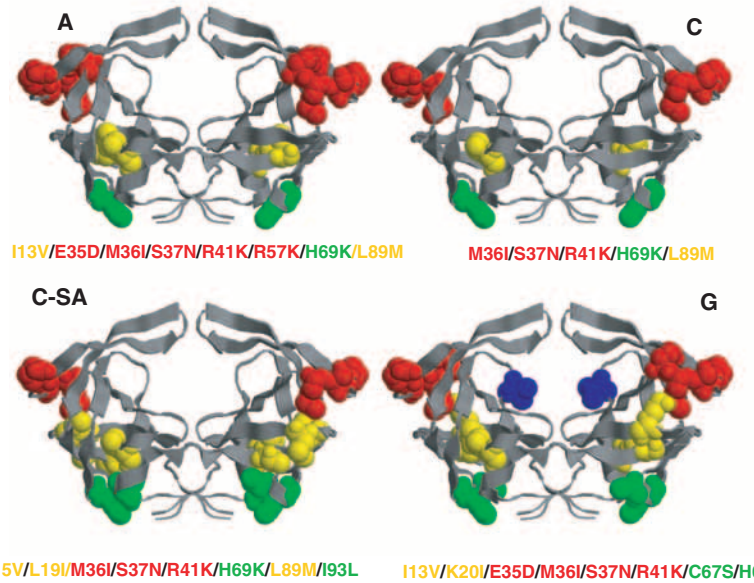

Figure 2. *Structure of HIV-1 proteases used in these studies showing the location of amino acid polymorphisms in A, C, C (South Africa) and G subtypes relative to B subtype.*

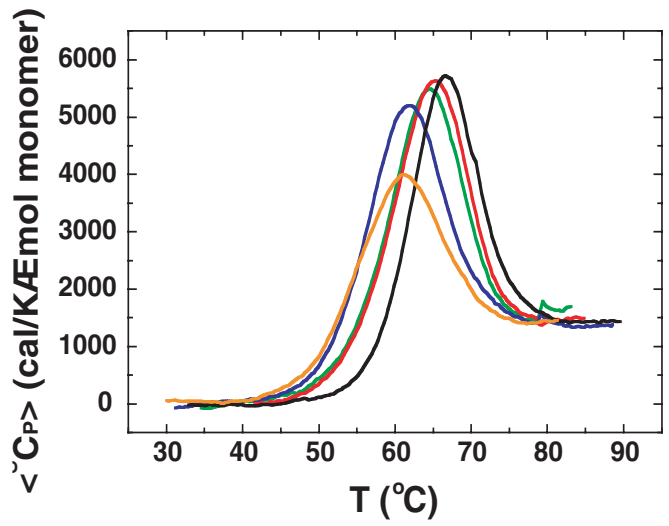

Figure 3. *The structural stability of the HIV-1 protease. The figure shows the temperature dependence of the heat capacity function for the HIV-1 protease. In this figure the blue line is for the B protease, green is for the A protease, black is for the G protease, red for the C protease and orange for the C-SA protease. All experiments were performed under the same experimental conditions (buffer glycine 10 mM, pH 3.6) at a protease concentration of 12μM (dimer).*

es in the target site and that they lose affinity significantly, even when facing very conservative mutations[11-13].

Effect of natural polymorphisms on inhibitor potency

Table 1 shows the catalytic activity of proteases from B, A, C, C-SA and G subtypes against the same substrate (a chromogenic peptide mimicking the highly conserved cleavage site ARVL/AEAM between the capsid protein and p2 in the *gag* protein precursor). From an enzymatic point of view, no major differences between the five proteases are observed, the most important difference being that the C and G proteases have catalytic efficiencies (k_{cat}/K_m) about twice that of the B subtype.

The response of the different proteases to inhibitors in clinical use is shown in table 2. All inhibitors exhibit a loss in binding affinity against the wild-type proteases from non-B-subtype HIV-1. Depending on the inhibitor and the protease, the

Table 1. *Enzymatic characteristics of wild-type proteases from different HIV-1 subtypes**

	B	A	C	C-SA	G
K_m (μM)	14	20	5.4	11.6	4.1
k_{cat} (s^{-1})	8.9	7.8	7.7	5.8	5.6
k_{cat}/K_m ($\mu M^{-1}s^{-1}$)	0.64	0.39	1.43	0.5	1.37

*Measured spectrophotometrically in sodium acetate 10 mM, pH 5.0, sodium chloride 1 M, at 25 °C against the same substrate (a peptide mimicking the highly conserved cleavage site ARVL/AEAM between the capsid protein and p2 in the *gag* protein precursor)

Table 2. *Loss of inhibitor potency against proteases from different HIV-1 subtypes**

	B $K_{d,B}$ nM	A $K_{d,A}/K_{d,B}$	C $K_{d,C}/K_{d,B}$	C-SA $K_{d,C\text{-}SA}/K_{d,B}$	G $K_{d,G}/K_{d,B}$
Indinavir	0.48	6.7	2.9	5.8	6.0
Saquinavir	0.40	2.8	1.9	5.8	2.0
Nelfinavir	0.26	2.7	2.3	3.9	1.8
Ritonavir	0.03	7.6	5.5	24.2	15.4
Amprenavir	0.015	2.5	2.2	3.1	2.3
Lopinavir	0.008	5.8	4.7	7.8	7.3

*Measured calorimetrically in sodium acetate 10 mM, pH 5.0 at 25 °C as described before[12,21]

Table 3. *Effect of drug-resistant mutation V82F/I84V on B, A and C subtype proteases and I84V on G subtype protease*

	B $K_{d,B\text{-}mut}/K_{d,B}$	A $K_{d'A\text{-}mut}/K_{d,B}$	C $K_{d'C\text{-}mut}/K_{d,B}$	G $K_{d'G\text{-}mut}/K_{d,B}$
Indinavir	67	400	208	26
Saquinavir	21	60	40	7
Nelfinavir	20	62	46	9
Ritonavir	382	2,833	2,116	72
Amprenavir	160	433	347	37
Lopinavir	123	558	533	64

drop in affinity ranges from 2 to 25. It is noteworthy that all inhibitors lose affinity against non-B-subtype proteases, reflecting the fact that they were optimized against the B subtype. This is not the case for the substrate, judging from the variation in K_m which may increase or decrease within a narrow range, reflecting a more permissive binding interaction. When the catalytic efficiency data (Table 1) and the affinity data are combined, it is observed that the African subtypes have a higher biochemical fitness in the presence of existing inhibitors[14]. By themselves, however, the observed effects on inhibitor binding affinity are not large enough to cause drug resistance, and current inhibitors can be expected to be effective against the wild-type forms of these subtypes.

Effect of drug resistance mutations

Only recently, reports describing different patterns of resistance mutations to protease inhibitors in non-B subtypes have appeared in the literature. For example, L90M, a primary mutation for nelfinavir and saquinavir, appears to be a key resistance mutation in the C subtype. In two recent reports dealing with genotypic differences in treated and untreated C-subtype patients[15,16] L90M appeared as the most significant mutation after the initiation of protease inhibition therapy. Other studies suggest certain biases in the appearance of secondary and compensatory mutations in B and non-B subtypes[4,17,18]. For example, B and non-B subtypes seem to differ in preference for mutations at positions 10, 20, 36, 63, 71 and 77. Although these positions have been associated with resistance and considered as secondary or compensatory mutations, they could be important in advanced stages of the infection, or lead to different routes or patterns for resistance development.

At the biochemical level, the effects of a common resistance mutation (the active-site double mutation V82F/I84V) have been studied in B- and non-B-subtype proteases[9]. V82 is located in subsites P2 and P2', whereas I84 is located in sub-site P1 and P1'. By studying the same set of mutations within the framework of different subtypes, it is possible to evaluate the influence of baseline polymorphisms on the effects of resistance mutations.

The dissociation constants for the six inhibitors in clinical use against the wild-type B-subtype protease are: saquinavir = 0.4 nM; nelfinavir = 0.26 nM; indinavir = 0.48 nM; ritonavir = 0.03 nM; amprenavir = 0.015 nM; and lopinavir = 0.008 nM, measured under identical conditions in this laboratory (Table 3). In the B subtype, the double mutation V82F/I84V lowers the binding affinity for saquinavir and nelfinavir by a factor ~20, indinavir by 70, ritonavir by 380, amprenavir by 160 and lopinavir by 120. In the non-B subtypes, the loss in binding potency is significantly amplified by the pre-existing lower binding affinity observed with these proteases as summarized in table 3. In the non-B subtypes, affinity losses can be as high as 2800, depending on the protease and inhibitor. For the G-subtype protease, only the I84V mutation was introduced, since this protease already carries the V82I polymorphism. According to the biochemical data, the addition of the single I84V mutation within this context is not equivalent to the effects of the V82F/I84V double mutation within the other frameworks.

A quantitative analysis of the data in table 3 indicates that the amplification elicited by non-B-subtype polymorphisms is generally additive; i.e. the effect of the drug resistance mutation on the potency of the inhibitors is amplified by a factor that roughly corresponds to the pre-existing loss in binding affinity due to the background polymorphisms. This conclusion is illustrated in figure 4. If the loss of binding affinity due to the background polymorphisms is multiplied by the loss in binding affinity due to the V82F/I84V resistance mutation in the B subtype, the resulting value is very close to the experimentally measured loss in binding affinity for the non-B-subtype mutants. In addition to the losses in binding affinity, it must be noted that the mutant non-B proteases maintain a catalytic efficiency similar to that observed for the B subtype with the same mutations (k_{cat}/K_m = 0.23, 0.2, 0.4 and 0.94 $mM^{-1}s^{-1}$ for the B, A, C and G mutant proteases) i.e. the effect of the mutations is more pervasive in the non-B proteases, an observation that may anticipate a faster progression to drug resistance. The more pronounced effects of drug resistant mutations within the framework of non-B-subtype proteases may lead to a more rapid failure of protease inhibition therapies.

While the effects of existing background polymorphisms appear to be additive to that of other

mutations, this is not the case for combinations of mutations associated with drug resistance, especially those associated with multi-drug resistance. Recently Ohtaka, et al.[19] measured the effects of a set of mutations (L10I/M46I/I54V/V82A/I84V/L90M) that lead to multi-drug resistance in the B subtype. The set of mutations contains one pair of mutations within the active site (V82A/I84V), one pair of mutations in the flaps (M46I/I54V) and one pair of mutations at the dimerization interface (L10I/L90M). In this case, the effects of the individual pairs of mutations are not additive and the resulting loss in binding affinity cannot be accounted for by the product of the individual losses, indicating the presence of cooperative potentiation effects. In this case, the mutations at the dimerization interface play a major role in the cooperative enhancement of drug resistance. Incidentally, L90M appears to be a critical resistance mutation in the C-subtype protease[6,15].

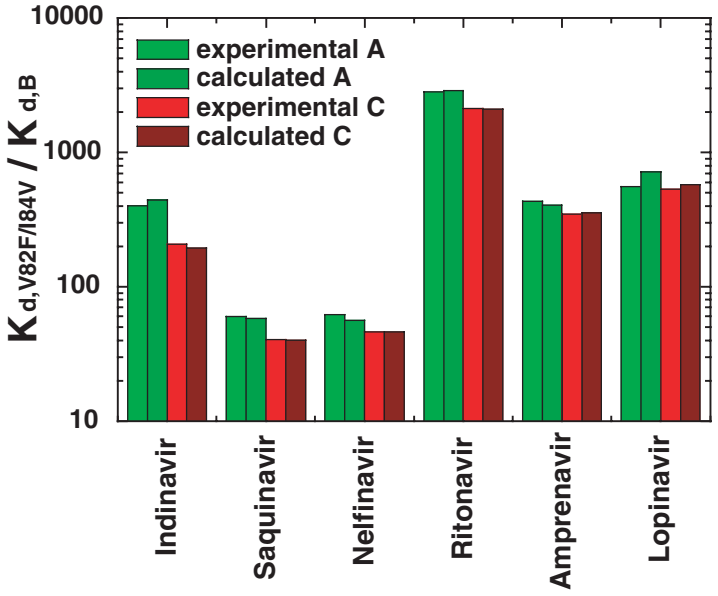

Figure 4. *The effect of naturally occurring background polymorphisms is mostly additive with the drug resistance mutation V82F/I84V. The effect of this mutation can be accurately predicted by multiplying the loss of affinity in the B-subtype protease by the loss of affinity of the wild-type forms of each subtype relative to the B subtype.*

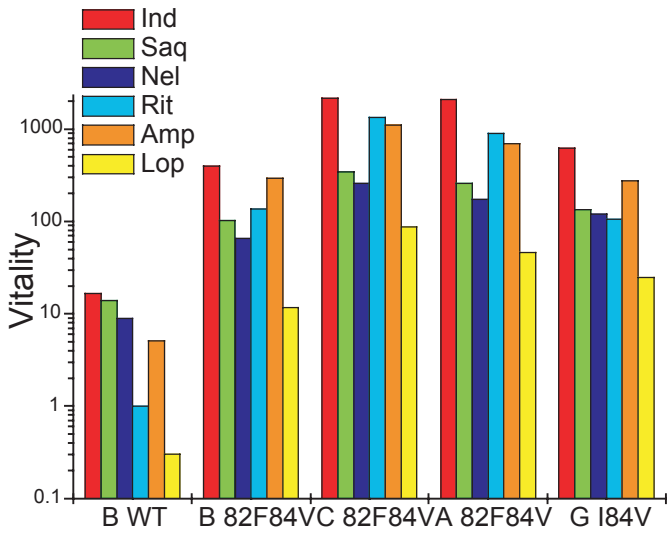

Figure 5. *The biochemical fitness of different proteases in the presence of inhibitors. The data is normalized with respect to the B wild-type and ritonavir which has a value of one (see text for details).*

Biochemical fitness

In order to compare the selective advantage of different protease mutations in the presence of specific inhibitors, Gulnik, et al.[8,20] introduced an empirical parameter, called vitality, which provides a measurement of the biochemical fitness of a specific mutation in the presence of a given inhibitor. Since different inhibitors have different binding affinities, a similar drop in binding affinity does not have the same effect on the vitality of the virus. For example, a drop of 10 in the affinity of a picomolar inhibitor is not the same as the same drop in a nanomolar affinity. From the point of view of arresting viral maturation, the first inhibitor is still effective, whereas the second one is not. A better descriptor of the biochemical fitness of the proteases is given by a modified vitality function, normalized to a reference inhibitor:

$$\text{relative vitality} = \frac{(K_d \times k_{cat}/K_m)}{(K_{d,ref} \times k_{cat,Bwt}/K_{m,Bwt})}$$

We chose the widely prescribed protease inhibitor ritonavir as the reference inhibitor and the wild-type B-subtype protease as the reference protease. Figure 5 shows the relative vitalities for the B, C and A subtype V82F/I84V resistant mutants. In this graph the wild-type B-subtype protease has a normalized vitality of one in the presence of ritonavir. The double mutation V82F/I84V improves the relative vitality of the protease up to ~400 in the presence of clinical inhibitors. The effect is more pronounced for the C and A subtype, in which the relative vitality might approach and even exceed 1000.

The biochemical data discussed in this paper indicates that background polymorphisms might affect the inhibitory potency of inhibitors, but not to the point of causing drug resistance. The main effects of these polymorphisms are most likely to manifest themselves after the onset of drug resistance mutations associated with antiretroviral therapy. If this is the case, the amplification of drug resistance effects might have serious consequences on the long term viability of protease inhibition therapy in non-B-subtype patients relative to B-subtype patients.

Acknowledgments

This work was supported in part by the National Institutes of Health grant GM 57144. Y. S. and H. W. D. supported by the South African National Research Foundation grant 2062139. We would like to thank Dr. Lynn Morris, from the National Institute for Communicable Diseases, for the South African HIV-1 subtype-C protease sequence data.

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