Dose–response curve slope is a missing dimension in the analysis of HIV-1 drug resistance

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HIV-1 drug resistance is a major clinical problem. Resistance is evaluated using in vitro assays measuring the fold change in IC_{50} caused by resistance mutations. Antiretroviral drugs are used at concentrations above IC_{50}, however, and inhibition at clinical concentrations can only be predicted from IC_{50} if the shape of the dose–response curve is also known. Curve shape is influenced by cooperative interactions and is described mathematically by the slope parameter or Hill coefficient (m). Implicit in current analysis of resistance is the assumption that mutations shift dose–response curves to the right without affecting the slope. We show here that m is altered by resistance mutations. For reverse transcriptase and fusion inhibitors, single resistance mutations affect both slope and IC_{50}. For protease inhibitors, single mutations primarily affect slope. For integrase inhibitors, only IC_{50} is affected. Thus, there are fundamental pharmacodynamic differences in resistance to different drug classes. Instantaneous inhibitory potential (IIP), the log inhibition of single-round infectivity at clinical concentrations, takes into account both slope and IC_{50}, and thus provides a direct measure of the reduction in susceptibility produced by mutations and the residual activity of drugs against resistant viruses. The standard measure, fold change in IC_{50}, does not correlate well with changes in IIP when mutations alter slope. These results challenge a fundamental assumption underlying current analysis of HIV-1 drug resistance and suggest that a more complete understanding of how resistance mutations reduce antiviral activity requires consideration of a previously ignored parameter, the dose–response curve slope.

HIV/AIDS | pharmacology | virology | highly active antiretroviral therapy | evolution

With suboptimal treatment, drug-resistant HIV-1 evolves rapidly (1–7). Resistance results from mutations introduced by the error-prone HIV-1 reverse transcriptase (RT) (8–10). Treatment with inadequately suppressive regimens and/or problems with adherence allow additional cycles of replication and selection of resistant variants. For some antiretroviral drugs, single amino acid substitutions in the drug target produce high-level resistance (3, 11). Some mutations confer cross-resistance within a drug class (12–15). Many reduce enzyme or protein function, thereby decreasing viral fitness (16–19).

At the molecular level, resistance often results from mutations that interfere with drug binding to the target enzyme or protein (20–23). Additional mechanisms may also contribute. For example, resistance to zidovudine involves mutations that promote excision (24–26). Although the molecular mechanisms of resistance are well studied, the pharmacodynamics of resistance are less well understood. Resistance is typically measured as a change in IC_{50} (Table S1) relative to WT virus (27–30). Antiretroviral drugs are used at concentrations above IC_{50}, however, and inhibition at clinical concentrations can only be predicted from IC_{50} if the shape of the dose–response curve is known. The shape is influenced by cooperative interactions and is described mathematically by the slope parameter or Hill coefficient (m) (31, 32). Certain drugs, notably nonnucleoside RT inhibitors (NNRTIs) and protease inhibitors (PIs), have cooperative dose–response curves with high slopes even though they target enzymes that are univalent with respect to the inhibitor (33). These high slopes may reflect a unique form of intermolecular cooperativity operative when multiple copies of a drug target participate in a given step in the virus life cycle (33). High slopes allow for extraordinarily high-level inhibition at concentrations above IC_{50}.

In all basic pharmacodynamic models, including the Hill equation, the median effect equation (32), and the sigmoidal maximum effect (E_{max}) model (34), m has an exponential relationship to drug effect (31). Thus, slope is an important determinant of antiviral activity. Implicit in current analysis of resistance is the assumption that mutations shift dose–response curves to the right without affecting slope. The effects of resistance mutations on slope have never been described, however. If a mutation increases IC_{50} and decreases m, it may cause more resistance than is anticipated from a consideration of IC_{50} alone. Thus, the effects of mutations on slope must be understood. Here, we measure these effects and demonstrate that consideration of slope provides a unique way to understand the effects of resistance mutations.

Results

Dose–Response Curves for Antiretroviral Drugs Against Resistant Viruses. Inhibition caused by a drug can be expressed as the fraction of single-cycle infection events affected by the drug (f_a) or the fraction that remains unaffected ($f_u = 1 - f_a$) and is determined by the drug concentration D, IC_{50}, and m according to the median effect equation (32, 35):

$$f_a/f_u = (D/IC_{50})^m \quad [1]$$

or

$$log(f_a/f_u) = m log D - m log IC_{50} \quad [2]$$

We studied single mutations that confer at least partial resistance according to the International AIDS Society-USA Drug Resistance Mutations Group and the Stanford University HIV Drug Resistance Database (36–38). Importantly, we analyzed resistance using a single-round infectivity assay because multiround assays distort m (39). Primary $CD4^+$ T lymphoblasts were used as target cells because they mimic the principal target cells for HIV-1 in vivo. Assays were done in 50% (vol/vol) human serum to account for protein binding and with preincubations of target cells with nucleoside RT inhibitors (NRTIs) to allow concentrations of the active triphosphate forms of these drugs to reach steady state (*SI Methods*).

Fig. 1 shows dose–response curves for representative drugs from five classes. For each drug, Fig. 1*A* shows a standard

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semilog dose–response curve (f_u vs. log D). Resistance mutations shift curves to the right and increase the IC_{50}. For example, the K65R and M184V mutations in RT shift the curve for the NRTI lamivudine (3TC) substantially to the right (Fig. 1*A*, 3TC). Interestingly, single mutations associated with resistance to the PI atazanavir (ATV) cause only minor shifts and do not increase IC_{50} by more than 10-fold (Fig. 1*A*, ATV). A problem with semilog plots shown is that differences in antiviral activity at higher drug concentrations are obscured as f_u approaches 0. Log-log dose–response curves (Fig. 1*B*) provide a better indication of antiviral activity at high drug concentration and reveal the impact of slope. If a mutation lowers m, inhibition achieved by the drug may be dramatically reduced. This is illustrated for M184V (Fig. 1*B*, 3TC), which increases IC_{50} but also reduces m such that increases in 3TC concentration cause a much less dramatic fall in f_u than for WT. To determine IC_{50} and m, we used the median effect model (Eq. **2**) to linearize dose–response curves (Fig. 1*C*). It is possible to directly determine m from the slope of median effect plots. The effect of M184V on slope is readily evident (Fig. 1*C*, 3TC). We have previously shown that PIs have steep slopes (33), and this is clear in the plot for ATV against WT (Fig. 1*C*, ATV). Some PI mutations reduce slope. For example, I84V, I50L, and N88S caused significant decreases in m ($P = 0.0042$, $P = 0.029$, and $P = 0.016$, respectively). Interestingly, mutations conferring resistance to the integrase strand transfer inhibitor (InSTI) raltegravir (RAL) did not significantly alter m.

Inhibition of replication at a given drug concentration can be predicted using the median effect equation, given IC_{50} and m (33). Inhibition can be expressed as instantaneous inhibitory potential (*IIP*), the number of logs by which single-round infection events are reduced at a clinically relevant drug concentration:

$$IIP = log(1/f_u) = log\left[1 + (D/IC_{50})^m\right] \quad [3]$$

IIP plots for the selected resistance mutations are shown in Fig. 1*D*. As previously described (33), *IIP* values for NNRTIs and PIs are greater than the *IIP* values for drugs from other classes because of higher m values for these classes. Resistance mutations reduced *IIP* at clinical concentrations, but residual activity against resistant virus varied dramatically for different drugs and mutations. Importantly, drugs with high *IIP* values for WT [efavirenz (EFV) and ATV] retained more activity against some resistant viruses than drugs from other classes had against WT. For example, N88S in protease is considered to cause high-level ATV resistance (37), but the *IIP* of ATV against this mutant is still higher than the *IIP* of 3TC, enfuvirtide (ENF), or RAL toward WT.

With suboptimal suppression, mutants with selective advantage over WT evolve. Selective advantage is the ratio of infectivity of a preexisting mutant to infectivity of WT at a given D. It can be estimated by multiplying the ratio $f_{u\ (\text{mutant})}/f_{u\ (\text{WT})}$ at that D by the replication capacity, the fractional infectivity relative to WT in the absence of drug. Selective advantage takes

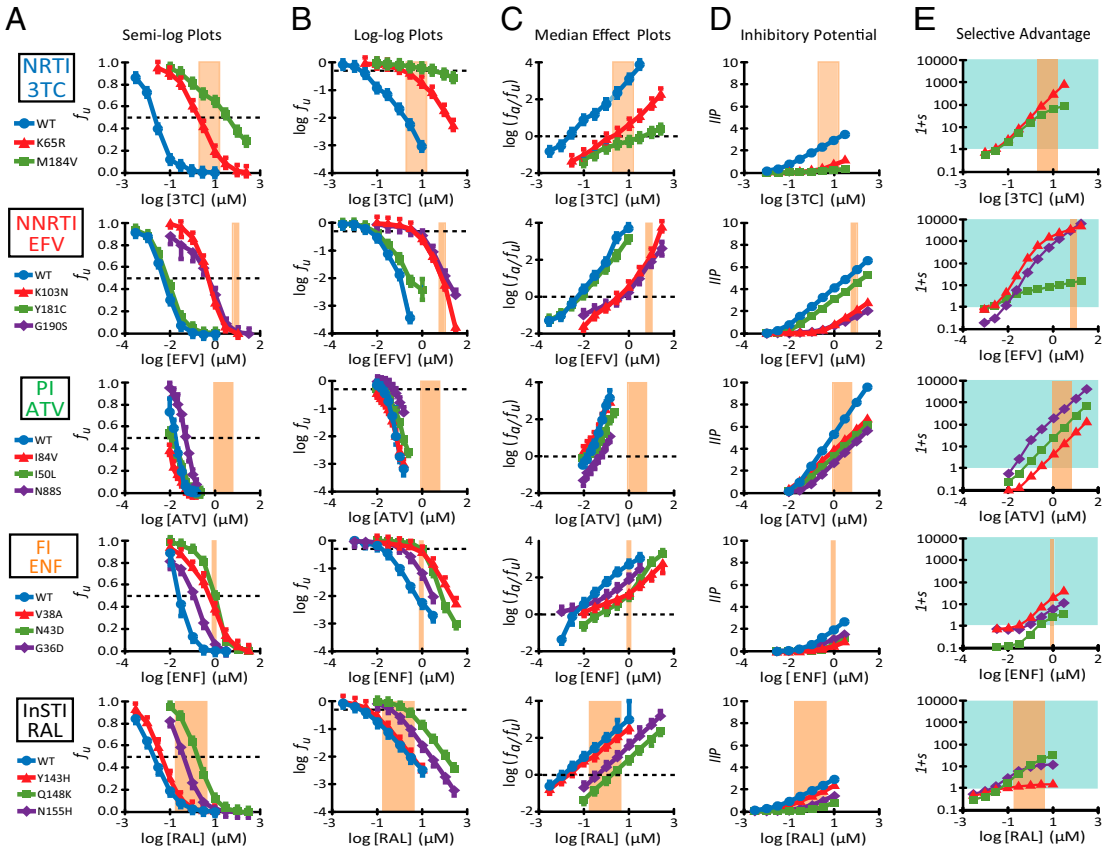

Fig. 1. Analysis of HIV-1 drug resistance mutations requires a consideration of dose–response curve slope. (*A*) Standard semilog dose–response curves for representative drugs from five different classes of antiretrovirals. Drugs were tested for inhibition of WT (blue curves) and mutant viruses bearing the indicated drug resistance mutations in a single-round infectivity assay. The fraction of infection events that remain unaffected (f_u) by the indicated D is shown. Error bars represent SE. The peach-shaded region indicates the clinical concentration range of the relevant drug. $f_u = 0.5$ (dotted line) indicates IC_{50} for each drug. Note that the D axis for 3TC and RAL is shifted by 1 log relative to the other drugs. (*B*) Log-log plots of the dose–response curves from *A*. IC_{50} for each drug can be determined from the points where the curves intersect log $f_u = -0.3$ (dotted line). Error bars represent SE. (*C*) Median effect plots of the dose–response curves from *A*. IC_{50} for each drug can be determined from the points where the curves intersect log (*fa*/*fu*) = 0 (dotted line). The m value is the actual slope of the median effect plot. Error bars represent SE. (*D*) *IIP* for WT and mutant viruses at the indicated D. *IIP* was computed using Eq. **3** and the measured IC_{50} and m. (*E*) Selective advantage (written as $1 + s$, where s is the selection coefficient) is the ratio of the infectivity of a preexisting mutant virus to the infectivity of WT at the indicated D. The blue shading indicates regions where mutant viruses have selective advantage. ENF, enfuvirtide.

into account reductions in fitness caused by the mutation. The selective advantage profiles for specific mutants are shown in Fig. 1*E*. Selective advantage >1 indicates that the mutant will prevail. In the clinical concentration range, most mutants had a selective advantage over WT, but the degree varied widely.

Effect of Resistance Mutations on IC_{50} and *m*. The above analysis was extended to major resistance mutations affecting licensed drugs from five classes. Fig. 2*A* shows the fold change in IC_{50} for each single mutant. Fig. 2*B* shows the fractional change in *m* relative to WT. Most NRTI, NNRTI, and fusion inhibitor mutations decreased *m* but increased IC_{50}. In contrast, single PI mutations caused only relatively minor shifts in IC_{50}, whereas *m* decreased. Interestingly, InSTI mutations resulted in an inverse pattern in which *m* values were preserved but IC_{50} increased (Fig. 2 *A* and *B*). Thus, depending on drug class, there were fundamental differences in the way resistance mutations affected the dose–response curves.

To illustrate the effects of slope, we looked for mutations that altered *m* but not IC_{50}. The V82F mutation in protease does not affect IC_{50} for indinavir (IDV) but does decrease *m* (Fig. 2 *A* and *B*). As shown in Fig. S1, this change results in a marked decrease in IDV susceptibility concentrations above IC_{50}. This is most evident in a median effect plot. Extrapolation into the-clinical concentration range reveals that the change in *m* alone can give a 5-log decrease in inhibition at minimum plasma concentration.

To extend the generality of these observations, we examined clinical isolates bearing the common resistance mutation M184V or K103N (Fig. S2). Isogenic viruses lacking the relevant mutation were generated by reverting the relevant codon to WT. As expected, M184V caused a large decrease in the slope of the 3TC dose–response curve, whereas K103N caused a moderate decrease in the slope of the EFV dose–response curve. Thus, the principles described here are not unique to laboratory strains.

Effect of Resistance Mutations on *IIP*. Because changes in *m* can have a major effect on antiviral activity, we compared *IIP* values at the peak plasma concentration (C_{max}) for each drug between WT and mutant viruses (Fig. 2*C*). Most resistance mutations decreased *IIP* compared with WT, albeit to varying extents. Interestingly, PI mutations caused substantial reductions in *IIP* even though they had only minor effects on IC_{50}. This reflects the fact that *IIP* takes *m*, as well as IC_{50}, into consideration.

The effect of the common M184V mutation on susceptibility to tenofovir disoproxil fumarate (TDF) illustrates the potential clinical significance of these concepts. M184V is thought to cause minimal resistance or even hypersusceptibility to TDF (40–43). As shown in Fig. S3*A*, there is little difference in IC_{50} between WT and M184V; however, the log-log plot (Fig. S3*B*) reveals a shallower slope for the M184V mutant, indicating substantial resistance to TDF at the high end of the clinical concentration range. This resistance would not be evident in assays that report only changes in IC_{50}. At high concentrations, both WT and mutant viruses are inhibited. As is discussed below, the results of several clinical trials are consistent with the notion that M184V causes TDF resistance. Similar observations were made with clinical isolates carrying M184V (Fig. S3*C*).

Fold Change in IC_{50} Correlates Poorly with Fractional Change in *IIP* When Mutations Affect *m*. Our results indicate that the analysis of drug resistance is compromised by failure to include slope when this parameter is altered by a mutation. For integrase mutants, there were no significant fractional changes in *m* compared with WT (+0.064 to −0.058; $P > 0.05$). In contrast, PI mutants show up to a 0.578 fractional decrease in *m* ($P < 0.05$) (Fig. 2). Thus, PI resistance cannot be accurately assessed by considering IC_{50} alone. Fig. 3 demonstrates a lack of correlation between fold change in IC_{50} and fractional change in *IIP* when a mutation significantly changes *m*. For InSTI mutations, fractional change in *IIP* correlates well with fold change in IC_{50} because *m* is not altered (correlation coefficient = 0.99) (Fig. 3*A*). On the other hand, a poor correlation coefficient (0.35) was obtained for PI mutants (Fig. 3*B*). Hence, using fractional change in *IIP* ensures that the effects of a change in *m* are accounted for.

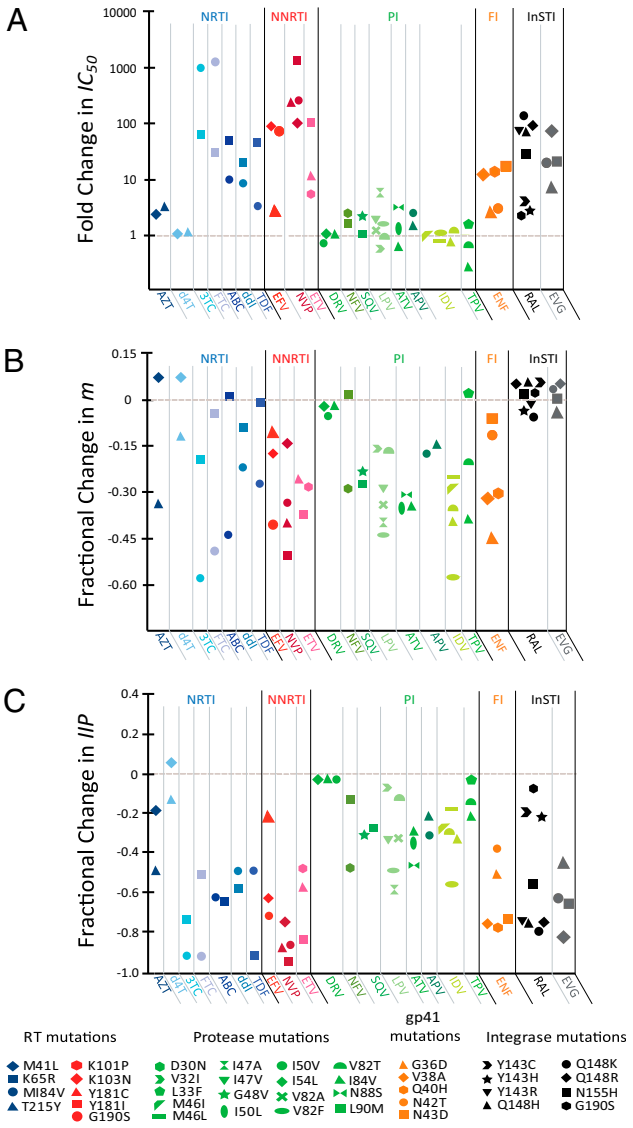

Fig. 2. Effect of single drug resistance mutations on IC_{50} (*A*), *m* (*B*), and *IIP* (*C*). IC_{50} and *m* were determined for each mutant using median effect plots (Eq. **2**) as previously described (33). The fractional change in *IIP* was computed as: $1 - (IIP_{mutant}/IIP_{WT})$. *IIP* values for WT and mutant viruses were computed using Eq. **3** and the reported *D* at C_{max} (33). Drugs are grouped by class: NRTIs (blue shades), NNRTIs (red shades), PIs (green shades), fusion inhibitor (orange), and InSTIs (black and gray). Within each class, mutations are indicated by the shape of the symbol. ABC, abacavir; APV, amprenavir; AZT, zidovudine; d4T, stavudine; DRV, darunavir; ENF, enfuvirtide; ETV, etravirine; EVG, elvitegravir; FI, fusion inhibitor; FTC, emtricitabine; LPV, lopinavir; NFV, nelfinavir; NVP, nevirapine; SQV, saquinavir; TPV, tipranavir.

Residual *IIP* Against Drug-Resistant Viruses. Because *IIP* is simply the number of logs by which a drug reduces in single-round infectivity, it can be used to compare the expected antiviral activity of different drugs against different viruses under clinical conditions. Fig. 4 displays the predicted *IIP* of each drug against WT and mutant viruses at C_{max} based on *m* and IC_{50} measured in primary cells. The reduction in *IIP* produced by single mutations varies dramatically. Certain drugs, notably the PIs, retain substantial *IIP* against single mutants, consistent with the clinical observation that PI resistance generally requires multiple muta-

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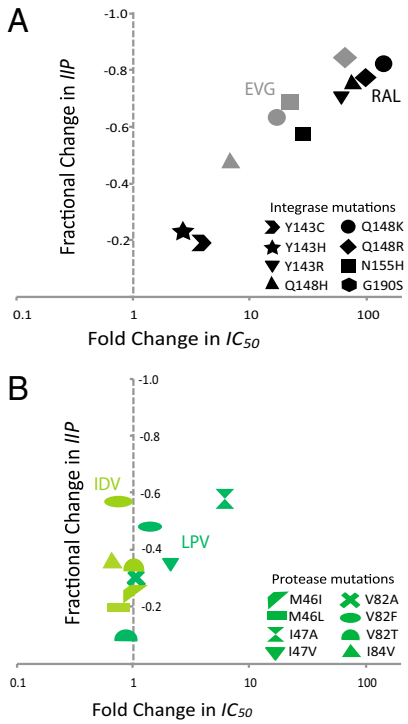

Fig. 3. Correlation of fractional change in *IIP* with fold change in IC_{50}. (*A*) Relationship between fractional change in *IIP* C_{max} and fold change in IC_{50} for integrase mutants with respect to RAL and elvitegravir (EVG). (*B*) Relationship between fractional change in *IIP* C_{max} and fold change in IC_{50} for protease mutants with respect to LPV and IDV. LPV, lopinavir.

tions. To some extent, differences in residual *IIP* reflect the tremendous differences in antiviral activity of different drug classes caused by differences in *m*.

Effect of Resistance Mutations on Replication Capacity and Selective Advantage. We also measured the replication capacity of each viral clone in the absence of drug by comparing the infectivity of standardized amounts of WT and mutant viruses in primary cells (Fig. 5*A*). As expected, most single mutations reduced replication capacity. The effects observed were consistent with but of lower magnitude than effects observed in multiround assays (44, 45). Single mutations in protease produced varied effects on replication capacity. The L90M, I47V, and M46I mutants exhibited higher replication capacity than WT, consistent with a previous study (46). The protease mutants I50V and I47A had highly impaired replication capacity (<0.1) relative to WT. Overall, these results suggest that common resistance mutations impair replication capacity by up to 10-fold in a single cycle.

Changes in replication capacity must be considered in assessing the selective advantage conferred by a mutation and are incorporated in the calculation of selective advantage described above. Fig. 5*B* shows the selective advantage of all mutants studied relative to WT at C_{max}. Most single mutants had a 10- to 10,000-fold selective advantage over WT. For the thymidine analog stavudine and the PI darunavir, single mutants had little selective advantage, consistent with the observation that resistance to these drugs involves accumulation of multiple mutations (47, 48).

Analysis of Resistance Mutations Using Primary Cells vs. Transformed Cell Lines. The above results were obtained using primary $CD4^+$ T cells, which mimic the principal target cells for HIV-1 in vivo. Clinical methods for determining drug resistance phenotype rely on transformed cell lines, some of which are nonlymphoid (29). The use of cell lines facilitates high-throughput analysis and eliminates the donor-to-donor variability that can be seen in primary cell assays. Although IC_{50} may vary slightly from donor to donor in our primary $CD4^+$ T-cell assay, the effect of resistance mutations on slope is relatively constant (Fig. S4). Nevertheless, primary cells are not optimal for high-throughput analysis; therefore, we compared dose–response curves in primary $CD4^+$ T lymphoblasts, a transformed human T-cell line (Jurkat), and the human embryonic kidney cell line (293T) used in current clinical phenotypic assays (29) (Figs. S5 and S6). In all three cell types, M184V decreased the slope of the 3TC dose–response but there were differences in the extent of inhibition and the degree of slope change, particularly with the nonlymphoid 293T cells. Interestingly, the TDF effect described in Fig. S3 was not evident in either transformed line. Analysis of resistance in primary cells may reveal effects not evident in cell lines, but whether this advantage outweighs the difficulty, cost, and variability inherent in primary cell assays requires further study.

Discussion

IC_{50} is used as a measure of the potency of antiretroviral drugs. Resistance is typically expressed as the fold change in IC_{50} relative to WT. Clinical concentrations of drugs used are typically well above IC_{50}, however. Therefore, determining inhibition at clinical concentrations requires a method for extrapolating from IC_{50} to higher concentrations. IC_{90} is sometimes used (46, 49); however, like IC_{50}, it represents only a single point on the dose–response curve. However, *m* describes how inhibition changes with drug concentration, and is thus critical for understanding drug effects at clinical concentrations. Previous analyses of resistance have ignored *m* or assumed that mutations do not alter it. Here, we show that some single resistance mutations cause changes in slope that dramatically affect the amount of inhibition produced by drugs.

The importance of slope is illustrated by the M184V mutation and its effect on the response to TDF. This mutation reduces the slope such that in some regions of the dose–response curve, the mutant virus is resistant to TDF despite minimal change in IC_{50}. Our results may explain the unexpected failure of a TDF, 3TC, and abacavir regimen (50). M184V was detected as the main mutation in most patients failing this regimen. It has been suggested that failure may have been attributable to viruses with K65R in addition

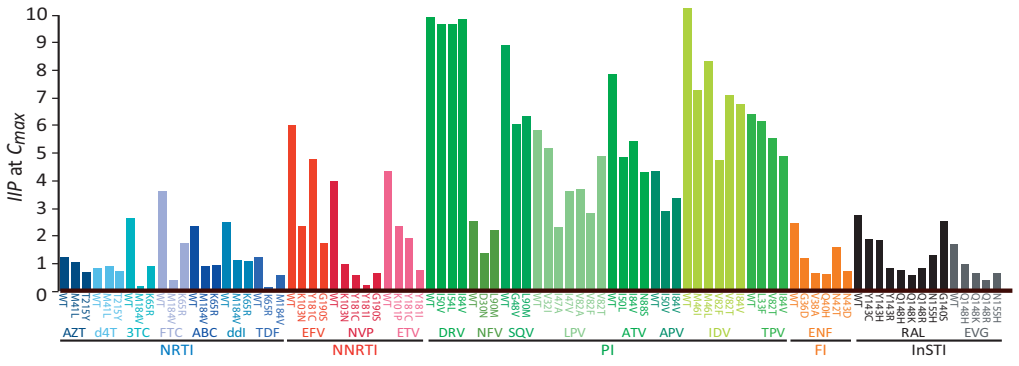

Fig. 4. Residual *IIP* against resistant viruses. Bars indicate the *IIP* against WT and viruses carrying the indicated single drug resistance mutations at C_{max}. ABC, abacavir; APV, amprenavir; AZT, zidovudine; d4T, stavudine; DRV, darunavir; ENF, enfuvirtide; ETV, etravirine; EVG, elvitegravir; FI, fusion inhibitor; FTC, emtricitabine; LPV, lopinavir; NFV, nelfinavir; NVP, nevirapine; SQV, saquinavir; TPV, tipranavir.

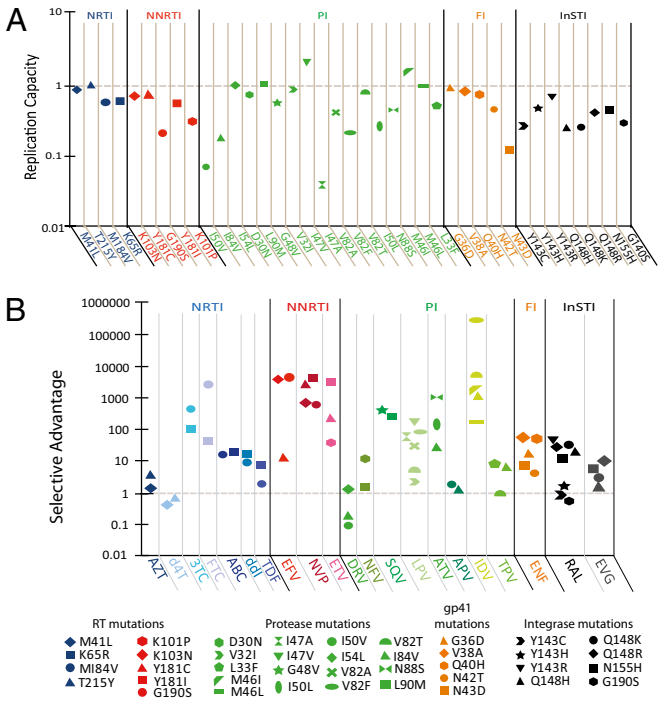

Fig. 5. Effect of mutations on replication capacity and selective advantage. (*A*) Replication capacity was determined as the ratio of the infectivity of resistant virus to the infectivity of WT. (*B*) Selective advantage of viruses bearing single resistance mutations in the presence of the indicated drugs at C_{max}. ABC, abacavir; APV, amprenavir; d4T, stavudine; DRV, darunavir; EFV, efavirenz; ENF, enfuvirtide; ETV, etravirine; EVG, elvitegravir; FTC, emtricitabine; LPV, lopinavir; NFV, nelfinavir; NVP, nevirapine; SQV, saquinavir; TPV, tipranavir.

to M184V (51, 52). In many failing patients, however, this double mutant was not detected. A simpler explanation is that M184V alone confers partial resistance to TDF. M184V also decreases slope of the didanosine (ddI) dose–response curve, which may explain the unexpected failure of TDF, 3TC, and ddI.*

Our results also show that some single resistance mutations leave substantial residual *IIP*. Because *IIP* takes into account alterations in both IC_{50} and m, it provides a unique way to quantitate how much antiviral activity a drug retains against mutant virus. We previously showed that the high slope values of the PIs and the NNRTIs result in high *IIP* (33). Not surprisingly, single mutations do not abolish all this antiviral activity. The PI class retains the highest residual *IIP* values in the presence of single mutations. Measuring the fraction of *IIP* remaining against a mutant virus provides an intuitive numerical value that is more quantitative than the current system of categorizing resistance as low, intermediate, or high level, based on fold change in IC_{50}. Fold change in IC_{50} ignores m and is potentially boundless, resulting in demarcations of resistance that are not consistent across drug classes. Because fractional decrease in *IIP* uses a finite intuitive numerical value incorporating m, we propose that it may be a more useful way to compare the extent of resistance.

Our results also have implications for mechanisms of drug action. InSTI mutations affected IC_{50} but not m. Increases in IC_{50} can be attributed to decreased affinity, resulting in the need for higher drug concentrations for inhibition. Biochemical analyses of integrase mutants indicate that the off-rate is remarkably increased relative to WT (53). This finding, coupled with our observation of a constant slope close to 1, indicates that integrase inhibitors affect WT and mutant viruses by blocking a noncooperative reaction. At low multiplicity of infection, the critical reaction is mediated by a single molecular complex per cell consisting of a single integrase tetramer bound to the ends of the complete linear cDNA.

In contrast, single PI mutations decrease slope but have minor effects on IC_{50}. Protease mutations are classified by position relative to the active site: substrate cleft mutations, protease flap mutations, and those at other conserved residues (23, 54). All single mutations studied exhibited the same pattern, however, showing modest change in IC_{50} but decreased slope. The comparatively small fold change in IC_{50} caused by protease mutations has been observed previously (55). We hypothesize that the reduction in m may reflect the fact that PIs block a cooperative process in which multiple copies of protease participate in virion maturation (33). The loss in enzyme efficiency caused by mutations affects the number of protease molecules needed to complete maturation before irreversible decay processes intervene.

As we and others have pointed out (33, 56–58), *IIP* is only one of several factors that determine the magnitude and durability of viral suppression by antiretroviral drugs. Other factors include drug half-life, distribution, toxicity and tolerability, drug interactions, and genetic barriers to resistance. A study by Henrich et al. (57) shows that *IIP* and inhibitory quotient have only modest correlations with clinical trial outcomes as measured using intent-to-treat analysis after 48 wk. This is expected, because many factors in addition to intrinsic antiviral activity determine clinical outcome. In addition, it is becoming clear that many regimens have sufficient inhibitory potential to suppress viral replication completely. In this situation, clinical outcome will be dominated by other factors. These caveats also apply to the residual *IIP* against resistant viruses. Furthermore, residual *IIP* only indicates the extent of inhibition a drug exerts on mutant virus and does not indicate how well this mutant virus is able to replicate and compete with other variants, including WT. Selective advantage profiles account for changes in replication capacity and indicate the relative probability that one mutant will be selected for at various drug concentrations. The observed high residual *IIP*s of some drugs, notably the PIs, suggest that these viral variants may nevertheless be adequately suppressed despite their higher selective advantage.

Taken together, these results demonstrate that a consideration of dose–response curve slope is important for assessing resistance. Values of replication capacity, IC_{50}, m, and *IIP* obtained in our primary cell system may differ from those obtained with other isolates and with assays using cells lines. For simplicity, we studied single mutations, but the concepts discussed are grounded in fundamental laws of pharmacology and, with further development, can be applied to more complex patterns of resistance. It will be of interest to determine how m and IC_{50} are altered by complex patterns of mutations that arise in some patients, such as the thymidine analog mutations; the Q151M-complex; and those seen with PI resistance, including Gag-cleavage site mutations.

Methods

Detailed materials and methods are provided in *SI Methods*.

Dose–response curves for anti–HIV-1 drugs were obtained using an NL4-3 construct (33, 59) bearing single resistance mutations in a single-round infectivity assay. CXCR4-pseudotyped WT viruses encoding GFP in the *env* gene were used to infect primary $CD4^+$ T lymphoblasts. Patient isolates bearing the M184V and K103N mutations were cloned into the NL4-3 backbone, and viruses generated from these were used in the single-round assay.

Infectivity was quantified by flow cytometry, and f_u was calculated as $\%GFP^+$ cells in the presence of drug normalized by $\%GFP^+$ cells without drug. Using Eq. **2**, dose–response curves were linearized, and IC_{50}, m, and *IIP* were determined as previously described (33).

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