

Editor's Summary

### King of the Hill

Thirty years ago, a deadly new disease burst onto the medical scene. AIDS was poorly understood, untreatable, and rapidly lethal. Today, through the herculean efforts of scientists, clinicians, and public health officers, HIV infection can be largely controlled in adherent patients with highly active antiretroviral therapy (HAART), which consists of treatment with at least three antiretroviral drugs. However, the success of HAART does not stem simply from the combined use of three drugs; indeed, some drug combinations are more successful than others. Shen *et al.* now show that the most successful drugs act cooperatively by binding multiple copies of the drug target.

Successful inhibition by a drug depends both on the drug dose and the concentration of the drug target: There must be sufficient amounts of the drug to inhibit the activity of the drug's target. Individual drugs can be more effective if they act cooperatively by binding a single target multiple times, such as one observes with a multivalent receptor, which contains more than one drug-binding site. This intramolecular cooperation can be represented by the Hill coefficient, which is a measure of how inhibition increases with drug concentration. HAART drugs have univalent targets; however, they still demonstrate cooperative inhibition in a dose-response curve (Hill coefficient > 1). Shen *et al.* propose a critical subset model that explains this unexpected cooperation: Infectivity requires multiple copies of a drug target at an individual stage of the virus's life cycle. This means that a certain number of the targeted molecules must remain unbound by the drug throughout the life cycle for infectivity to occur. Thus, the drug target actually functions as a virtual multivalent receptor, which results in the cooperative action of the HAART drugs. The authors confirm their model experimentally by modulating the number of functional drug targets per virus, verifying model predictions. These data not only explain the relative success of the current HAART therapy for HIV but also provide a framework for choosing new targets for antiviral drugs.

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## HIV

# A Critical Subset Model Provides a Conceptual Basis for the High Antiviral Activity of Major HIV Drugs

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Control of HIV-1 replication was first achieved with regimens that included a nonnucleoside reverse transcriptase inhibitor (NNRTI) or a protease inhibitor (PI); however, an explanation for the high antiviral activity of these drugs has been lacking. Indeed, conventional pharmacodynamic measures like IC<sub>50</sub> (drug concentration causing 50% inhibition) do not differentiate NNRTIs and PIs from less active nucleoside reverse transcriptase inhibitors (NRTIs). Drug inhibitory potential depends on the slope of the dose-response curve ( $m$ ), which represents how inhibition increases as a function of increasing drug concentration and is related to the Hill coefficient, a measure of intramolecular cooperativity in ligand binding to a multivalent receptor. Although NNRTIs and PIs bind univalent targets, they unexpectedly exhibit cooperative dose-response curves ( $m > 1$ ). We show that this cooperative inhibition can be explained by a model in which infectivity requires participation of multiple copies of a drug target in an individual life cycle stage. A critical subset of these target molecules must be in the unbound state. Consistent with experimental observations, this model predicts  $m > 1$  for NNRTIs and PIs and  $m = 1$  in situations where a single drug target/virus mediates a step in the life cycle, as is the case with NRTIs and integrase strand transfer inhibitors. This model was tested experimentally by modulating the number of functional drug targets per virus, and dose-response curves for modulated virus populations fit model predictions. This model explains the high antiviral activity of two drug classes important for successful HIV-1 treatment and defines a characteristic of good targets for antiviral drugs in general, namely, intermolecular cooperativity.

## INTRODUCTION

In 1997, regimens were developed that suppressed HIV-1 viremia to below the detection limit in most treated patients. These regimens combined two nucleoside reverse transcriptase inhibitors (NRTIs) with an HIV-1 protease inhibitor (PI) (1–3). Combinations of two NRTIs and a nonnucleoside reverse transcriptase inhibitor (NNRTI) also proved effective (4, 5). Collectively, these regimens, known as highly active antiretroviral therapy (HAART), transformed a previously fatal disease into a chronic condition that is well controlled in adherent patients. Despite HAART's success, a fundamental theory explaining its effectiveness is lacking.

Drug resistance, which results from both the high error rate of reverse transcriptase (RT) and the dynamic nature of HIV-1 infection, is a major cause of treatment failure (6–10). The low probability of multiple simultaneous resistance mutations on the same genome contributes to the success of triple combination therapy (11). However, suppression of HIV-1 replication is not simply the result of using three drugs; some triple NRTI combinations had suboptimal responses (5). Thus, in early treatment efforts, inclusion of a PI or a NNRTI appeared essential for control of viral replication. Although the use of drugs acting through different mechanisms also contributes to the effectiveness of combination therapy, PIs and NNRTIs appeared to have greater antiviral activity than most NRTIs. Therefore, treatment guidelines recommend inclusion of a PI or a NNRTI in most initial HAART regimens (5). Standard pharmacologic measures such as IC<sub>50</sub> and inhibitory quotient do not

distinguish PIs and NNRTIs from less active NRTIs (12). Thus, the fundamental pharmacodynamic principles underlying this successful treatment remain unclear. Instead, progress has depended on comparative clinical trials, which have recently established a role for newer drugs as well, such as integrase strand transfer inhibitors (InSTIs) and CCR5 antagonists (5).

We recently showed that the superior antiviral activity of PIs and NNRTIs relative to most NRTIs can be partially explained by the dose-response curve slope ( $m$ ) (12). This parameter is included in all fundamental pharmacodynamic equations including the Hill equation (13), the sigmoidal  $E_{\max}$  model (14), and the Chou-Talalay median-effect equation (15). The slope parameter is related to the Hill coefficient describing intramolecular cooperativity in the binding of ligands to a multivalent receptor (13, 16). Positive cooperativity ( $m > 1$ ) reflects enhanced binding of additional ligands to a receptor after the first ligand binds. However, the enzymes targeted by NNRTIs and PIs are univalent with respect to these inhibitors. NNRTIs bind to a single pocket adjacent to the deoxynucleoside triphosphate (dNTP) binding site (17), and HIV-1 protease is a homodimer with a single active site targeted by PIs, which are peptidomimetic substrate or transition-state analogs (18, 19). Therefore, noncooperative dose-response curves ( $m = 1$ ) were expected. However, in single-round infectivity assays, dose-response curves for NNRTIs and PIs have unexpectedly steep slopes ( $\sim 1.7$ ; ranging from 1.8 to 4.5, respectively), allowing these drugs to achieve extraordinarily high antiviral activity (12).

Because  $m > 1$  for NNRTIs and PIs cannot be explained by intramolecular cooperativity, a mechanistic explanation requires reconsidering the concept of cooperativity in drug action. Here, we describe a critical subset model that explains steep slopes for these drugs in terms of intermolecular cooperativity, and we provide experimental validation for this model.

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RESULTS

Critical subset model

The simplest model describing dose-response relationships is the median-effect equation (15). It is derived from the law of mass action, which governs dynamic equilibria in chemical reactions. The median-effect equation can be written as:

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

or

$$f_a = \frac{1}{1 + \left(\frac{IC_{50}}{[D]}\right)^m} \quad (2)$$

In the context of HIV-1 infection,  $f_a$  and  $f_u$  are the fractions of viruses affected and unaffected by the drug,  $[D]$  is the drug concentration,  $IC_{50}$  is the drug concentration causing 50% inhibition, and  $m$  is the slope parameter describing the steepness of the dose-response curve.

Some anti-HIV drugs have  $m > 1$ , even though their target enzymes are univalent. To explain this observation, we developed a critical subset model based on mass action. The model envisions independent binding of multiple drug molecules to a set of identical targets, viral proteins, within each virus or virus-infected cell. Within a given virion or infected cell, the targeted proteins behave as a set of linked receptor sites that can be occupied to variable extents (Fig. 1A), depending on  $[D]$  and the affinity of individual drug-target interactions ( $K_d$ ). Drug binding involves a series of equilibrium reactions mediating conversions between all possible partially occupied states (16). Assuming that replication requires some critical threshold number ( $c$ ) of functional (unbound) enzyme molecules to complete the relevant reaction before irreversible decay processes intervene (Fig. 1B), the following expression describes  $f_a$  as a function of  $[D]$ ,  $K_d$ , the unbound virus concentration  $[V]$ , and the total number of enzyme molecules per virus  $n_T$  (Eq. 3 and Table 1):

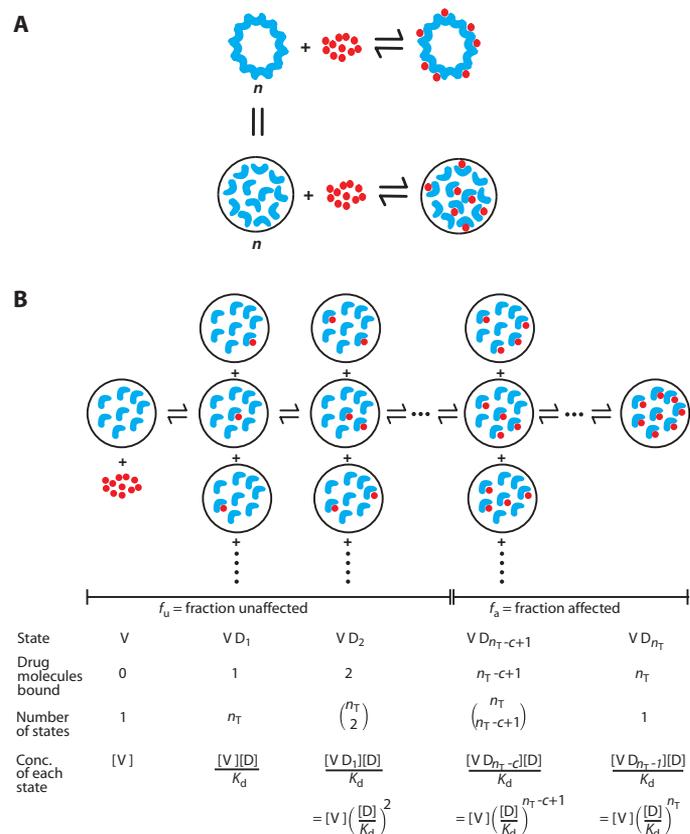
$$f_a(n_T) = \frac{\sum_{i=n_T-c+1}^{n_T} \binom{n_T}{i} [V] \left(\frac{[D]}{K_d}\right)^i}{\sum_{i=0}^{n_T} \binom{n_T}{i} [V] \left(\frac{[D]}{K_d}\right)^i} = \frac{\sum_{i=n_T-c+1}^{n_T} \binom{n_T}{i} \left(\frac{[D]}{K_d}\right)^i}{\sum_{i=0}^{n_T} \binom{n_T}{i} \left(\frac{[D]}{K_d}\right)^i} \quad (3)$$

Consider, for example, HIV-1 protease. Multiple copies of protease participate in the maturation of each virion. Although not physically linked, they are constrained spatially within the virion. Thus, the virion can be considered a multivalent receptor with  $n_T$  binding sites, where  $n_T$  is the total number of protease molecules per virus (Fig. 1A). Because simultaneous binding is unlikely, we have modeled drug binding as a series of equilibrium reactions (Fig. 1B). The derivation of Eq. 3 follows from a consideration of the concentration of each possible state along the path to saturation (16). Viruses with a critical subset of  $c$  or more unbound enzymes are infectious. To render a virus noninfectious, at least  $n_T - c + 1$  enzyme molecules must be occupied (Fig. 1B). Thus, in Eq. 3, the numerator is the sum of the concentrations of all viruses having  $\geq n_T - c + 1$  enzyme molecules bound by drug, and the denominator is the total virus concentration.

This model predicts the shapes of dose-response curves. To illustrate these predictions, we plotted hypothetical dose-response curves using

median-effect plots based on Eq. 1 (Fig. 2). The median-effect plot linearizes the dose-response curve for most drugs, with the slope of the resulting line equal to the Hill coefficient (Fig. 2, A and B). We obtained complex dose-response curves with various  $m$  depending on  $n_T$  and  $c$  (Fig. 2, C and D). When  $n_T = c = 1$ , Eq. 3 simplifies to the median-effect equation (Eq. 2) and predicts  $m = 1$ . However, for all cases with  $1 < c < n_T$ , the model predicts steeper dose-response curves with  $m \geq 1.5$  at all drug concentrations.

The model predicts complex dose-response curves with nonlinear median-effect plots. That is,  $m$  is different for different segments of the curve. As shown below, the dose-response curves of some antiretroviral drugs are similarly complex. According to the model, slope values are determined by  $n_T$  and  $c$ . As  $[D]$  increases above  $IC_{50}$ ,  $m$  approaches  $c$ . As  $[D]$  decreases below  $IC_{50}$ ,  $m$  approaches  $n_T - c + 1$  (Supplementary Note 1). Within the range of  $\log(f_a/f_u) = -2$  to 4 (1 to 99.99% inhibi-



**Fig. 1.** The critical subset model. (A) A virion (or virally infected cell) can be viewed as a multivalent receptor containing  $n$  identical copies of a given drug target such as HIV-1 protease. Although not physically linked to one another, these copies are spatially constrained within the virus particle or infected cell. (B) Sequential binding of a protease inhibitor (red circle) to the protease dimers (blue crescents) within a virion. The concentrations of the various bound states can be determined by the law of mass action, assuming a single unchanging dissociation constant,  $K_d$ . Assuming that successful completion of the relevant step in the virus life cycle requires a critical number  $c$  of functional unbound copies of the drug target,  $f_a$  can be computed as the concentration of inhibited states divided by the concentration of all possible states (Eq. 3).  $f_u$  is equal to  $1 - f_a$ . Similar arguments can be applied to RT except that the RT molecules are present within the reverse transcription complex in an infected cell.

tion), the curves inflect upward when  $c$  is close to  $n_T$  and downward when  $c$  is small relative to  $n_T$  (Fig. 2, C and D). For example, when  $n_T = 5$ , the model predicts a family of curves with slopes ranging from 1.30 to 1.88 at the  $IC_{50}$  (Fig. 2C). Slope at the  $IC_{50}$  is highest when  $c = 3$ , and lowest when  $c = 1$  or 5. However, the slope at high  $[D]$  approaches  $c$ , and thus, the maximum slope is 5. The same pattern with steeper slopes is observed for  $n_T = 30$  (Fig. 2D). Whenever simultaneous inhibition of multiple copies of a target molecule within a virion or infected cell is required to block replication, dose-response curves with  $m > 1$  are predicted by this model. This unique form of intermolecular cooperativity does not depend on changes in the binding properties ( $K_d$ ) of individual target sites.

The model also predicts the relationship between  $IC_{50}$  and  $K_d$ . When  $n_T$  is odd and  $c = (n_T + 1)/2$ ,  $IC_{50} = K_d$ . When  $c > (n_T + 1)/2$ ,  $IC_{50} < K_d$  and vice versa (Fig. 2, C and D). Similarly, for a series of curves with constant  $c$ ,  $IC_{50}$  decreases as  $n_T$  decreases (Fig. 2, E and F). This is expected because a lower  $IC_{50}$  is achieved with the same  $K_d$  when the fractional occupancy of target enzymes required for inhibition is low.

### Effect of heterogeneity on dose-response curves

A second factor affecting slope is heterogeneity in  $n_T$ . In other biological systems with all-or-none outcomes, heterogeneity in the number of drug targets flattens dose-response curves (20). For individual virions, infection is all or none, and thus, heterogeneity arguments apply. When heterogeneity is limited, dose-response curves are steeper. Intuitively, this may be understood by considering that a homogeneous virus population will be inhibited over a narrow range of drug concentrations, whereas a more heterogeneous population will contain viruses that are easier or harder to inhibit, and therefore, inhibition will be observed over a wider concentration range.

If  $n_T$  follows an integer-valued normal distribution with an SD of  $\sigma$ , then dose-response curves follow Eq. 4, which gives  $f_a$  for a population of viruses with variability in  $n_T$ .

$$f_a(n_T, \sigma) = \frac{\sum_{n_i=c}^{n_{\max}} [f_a(n_i)P(n_i|n_T, \sigma)]}{\sum_{n_i=c}^{n_{\max}} P(n_i|n_T, \sigma)} \quad (4)$$

where

$$P(n_i|n_T, \sigma) = \frac{1}{\sigma\sqrt{2\pi}} e^{-\frac{(n_i-n_T)^2}{2\sigma^2}} \quad (5)$$

and  $f_a(n_i)$  is calculated using Eq. 3. The derivation of Eq. 4 follows from a consideration of  $f_a$  for viruses with a given total number of enzyme molecules per virus ( $n_i$ ) and the probability of finding viruses with  $n_i$  enzyme molecules within a population of viruses with  $n_T \pm \sigma$  total enzyme molecules per virus (fig. S1A). Because viruses

**Table 1.** Glossary of abbreviations.

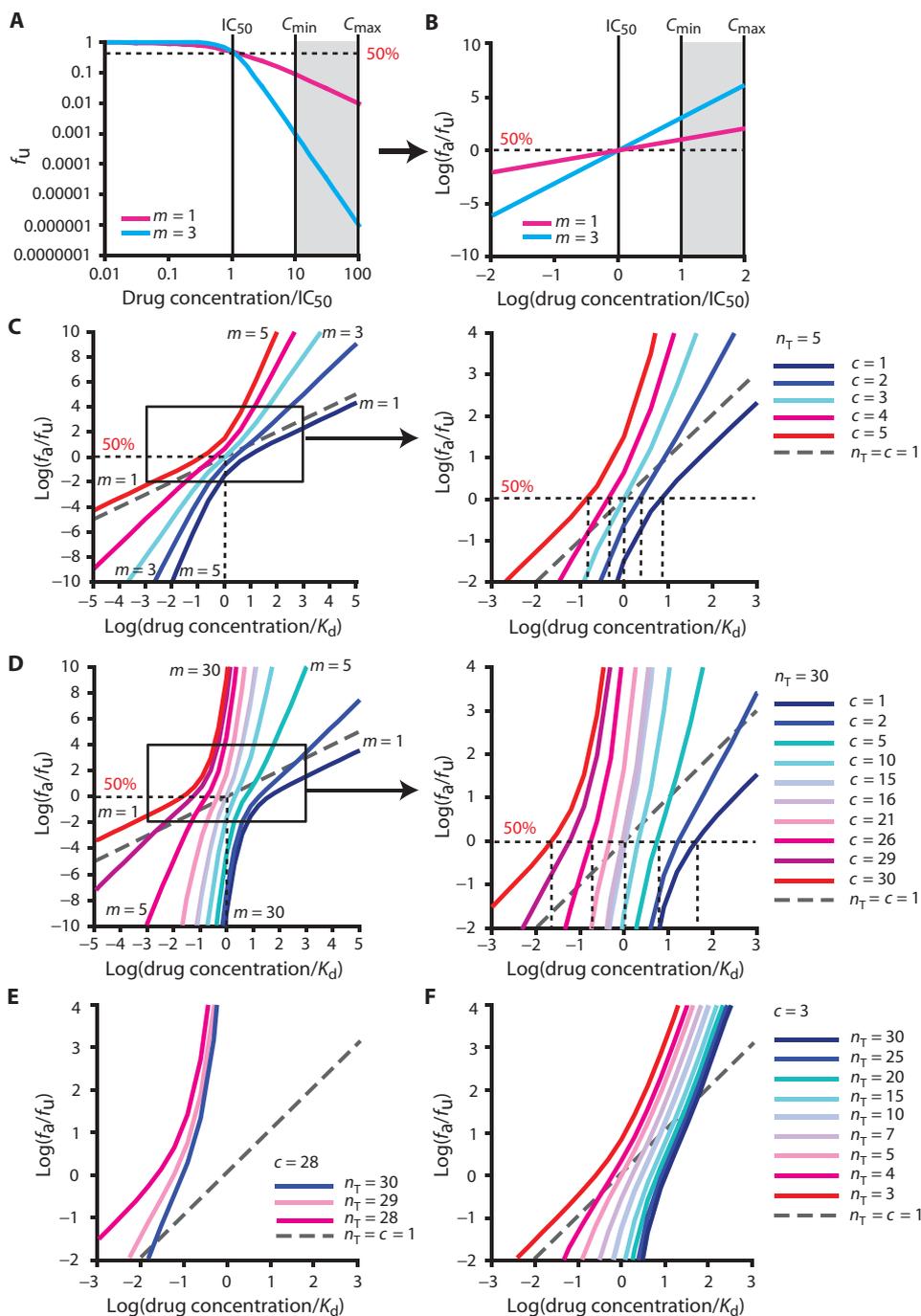
$f_a$	Fraction of viruses or infection events affected by the drug
$f_u$	Fraction of viruses or infection events unaffected by the drug
$[D]$	Drug concentration
$IC_{50}$	Drug concentration that leads to 50% inhibition
$m$	Slope parameter or Hill coefficient
$K_d$	Dissociation constant for a drug binding to an individual target enzyme molecule
$n_T$	Average of total number of enzyme molecules per virion relevant to the inhibited reaction
$c$	Critical threshold number of functional enzyme molecules required for viral replication
$[V]$	Unbound virus concentration
$\sigma$	SD for $n_T$
$n_{\max}$	Maximum total number of enzyme molecules per virion, usually set as $>n_T + 3\sigma$
$n_i$	Given total number of enzyme molecules per virion relevant to the inhibited reaction
$f_m$	Fraction of mutant plasmid transfected
$P_f$	Probability of obtaining functional enzyme multimers for a given $f_m$
$n_F$	Total number of functional target molecules per virion relevant to the inhibited reaction
RI	Relative infectivity of phenotypically mixed viruses compared to the wild-type virus

with  $n_i < c$  are noninfectious, the numerator must be divided by the probability of infectious virus for a given  $n_T$ ,  $\sigma$ , and  $c$  (fig. S1A).  $n_{\max}$  is the maximum total number of enzyme molecules per virus, which is set at  $>n_T + 3\sigma$ . Equation 4 predicts a decrease in  $m$  with increasing  $\sigma$  at drug concentrations below the  $IC_{50}$  (fig. S1B), because heterogeneity leads to a subpopulation of viruses with low  $n_T$  that are inhibited more easily at low  $[D]$ .

### Testing the critical subset model with phenotypic mixing experiments—theoretical considerations

The critical subset model makes testable predictions about how variation in  $n_T$  and  $c$  affect dose-response curves. For example, the model predicts that at constant  $c$ ,  $IC_{50}$  decreases as  $n_T$  decreases (Fig. 2, E and F). To test this model, we used a phenotypic mixing strategy (21, 22) to reduce the total number of functional target molecules per virion ( $n_F$ ) (Fig. 3A). To reduce  $n_F$ , we mixed defined fractions ( $f_m$ ) of mutant proviral constructs containing inactivating point mutations in the target enzymes with wild-type constructs and transfected them into human embryonic kidney (HEK) 293T cells to generate virions with reduced  $n_F$ . Note that  $n_F = n_T$  when no mutant enzyme is present. We then examined the relative infectivity (RI) of these virus populations compared to wild-type virus and obtained estimates of  $n_T$  and  $c$  (Fig. 3, B and C). Finally, we determined whether the dose-response curves for virus populations with lower  $n_F$  behaved as predicted by the model (Fig. 3, D and E). Note that larger decreases in  $IC_{50}$  are predicted when  $c$  is small relative to  $n_T$ .

The introduction of mutant enzyme subunits reduces infectivity, because some viruses will no longer contain a critical number of functional enzymes (Fig. 3A). The enzyme molecules targeted by RT and protease inhibitors are heterodimers and homodimers, respectively. If formation of mixed dimers from wild-type and mutant monomers follows random mixing, then the virions produced should contain reduced  $n_F$  following a binomial distribution. The RI of mixed virions compared to wild-type virions is a function of  $n_T$ ,  $c$ , and  $P_f$ , where  $P_f$  is



**Fig. 2.** Steep dose-response curves predicted by the critical subset model. **(A)** Hypothetical log-log plot of  $f_u$  versus drug concentration based on the median-effect model for drugs with different  $m$  values. The strong impact of slope on the suppression of infectivity is apparent at high drug concentrations. Drug concentrations are normalized by  $IC_{50}$ . The dotted line represents 50% inhibition. **(B)** Hypothetical median-effect plot [ $\log(f_a/f_u)$  versus  $\log$  drug concentration] (Eq. 1) of the curves shown in (A). The  $m$  value is the slope of the line. **(C and D)** Hypothetical median-effect plots of dose-response curves generated using the critical subset model assuming  $n_T = 5$  (C) or 30 (D) and values of  $c$  ranging from 1 to  $n_T$ . Concentrations are normalized by  $K_d$ . The right panel is an expansion of the indicated area in the left panel. As  $c$  increases,  $IC_{50}$  decreases, and slope increases at concentrations above the  $IC_{50}$  but decreases at concentrations below the  $IC_{50}$ . A standard noncooperative dose-response curve with  $m = 1$  ( $n_T = c = 1$ ) is also shown (gray dashed line). **(E and F)** Hypothetical median-effect plots of dose-response curves generated using the critical subset model with values of  $n_T$  ranging from  $c$  to 30, and  $c = 28$  (E) or 3 (F). As  $n_T$  decreases, the dose-response curves shift to the left.

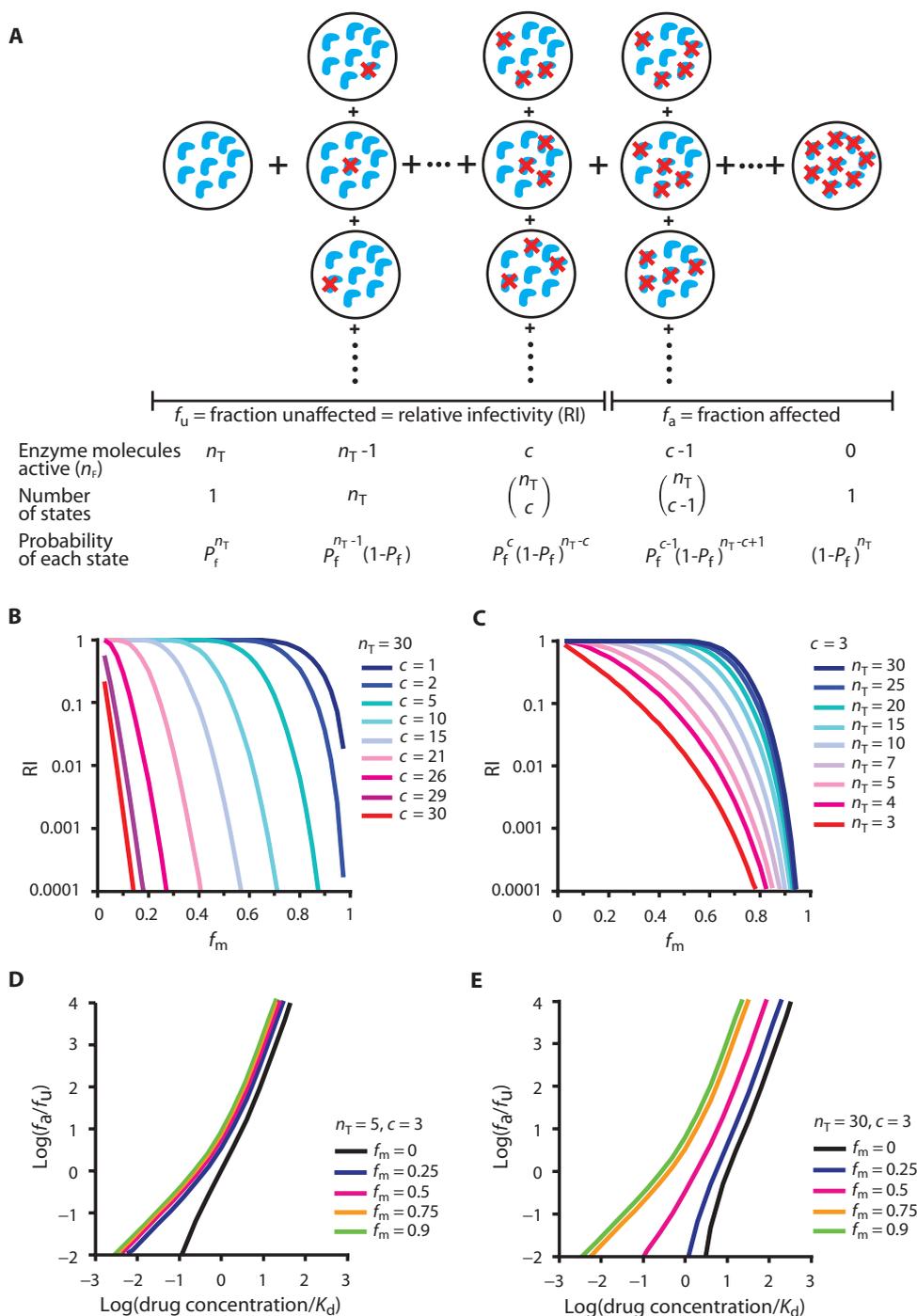
the probability of obtaining a functional enzyme multimer for a given  $f_m$  (Eq. 6).

$$RI(n_T) = \sum_{n_F=c}^{n_T} P(n_F) = \sum_{n_F=c}^{n_T} \binom{n_T}{n_F} P_F^{n_F} (1 - P_F)^{n_T - n_F} \quad (6)$$

$P(n_F)$  is the probability of getting  $n_F$  copies of functional enzyme molecules with a given  $n_T$ ,  $c$ , and  $P_F$ . The calculation of  $P_F$  from  $f_m$  is based on the subunit stoichiometry of the target enzyme and is different for RT and protease, as described in Materials and Methods. Based on Eq. 6, the RI of phenotypically mixed viruses is the sum of the probabilities of obtaining viruses with  $n_F \geq c$ . Plotting RI as a function of  $f_m$ , we obtain RI curves with various patterns depending on  $n_T$  and  $c$  (Fig. 3, B and C). When most copies of the enzyme are required to complete the relevant step in the life cycle, decreases in  $n_F$  have a major effect—a small increase in  $f_m$  results in loss of infectivity. In contrast, when  $c$  is small relative to  $n_T$  and thus available enzyme exceeds that which is necessary, infectivity is not affected until  $f_m$  is large (Fig. 3, B and C). As demonstrated below, the former situation applies to RT, whereas the latter applies to protease. Note that the analysis of RI is based on the same assumptions that underlie the critical subset model, namely, that a critical threshold number ( $c$ ) of functional copies of a viral enzyme out of  $n_T$  copies are required for infectivity (Figs. 1B and 3A).

To test the critical subset model, phenotypically mixed viruses can be assayed for susceptibility to drug inhibition. Dose-response curves for inhibition of viruses with various  $f_m$  can be predicted by Eq. 4, where the calculation of  $f_a(n_i)$  follows Eq. 7 (Fig. 3, D and E). The derivation of Eq. 7 follows from a consideration of  $f_a$  for each  $n_F$  (Eq. 3) and the probability of viruses with  $n_F$  functional enzyme molecules within a population of viruses with  $n_i$  total enzyme molecules and an average probability  $P_F$  of functional ones (Eq. 6 and Fig. 3A).

$$f_a(n_i) = \frac{\sum_{n_F=c}^{n_i} [f_a(n_F)P(n_F)]}{RI(n_i)} \quad (7)$$



**Fig. 3.** Effect of reductions in  $n_F$  on the dose-response curves for antiviral drugs. **(A)** Relative infectivity (RI) of phenotypically mixed virions. Consider a population of virions with the same  $n_T$  but various numbers of active ( $n_F$ , blue crescents) and inactive protease molecules (crescents with red cross).  $P_f$  is the probability of obtaining a functional protease molecule for a given  $f_m$ . The probability of getting  $n_F$  copies of functional enzyme molecules [ $P(n_F)$ ] as a function of  $n_T$ ,  $c$ , and  $P_f$  can be calculated using Eq. 6. The RI of a population of phenotypically mixed virions is the sum of the probabilities of all the viruses with  $n_F \geq c$ . **(B and C)** Hypothetical RI plots (RI versus  $f_m$ ) for protease with  $n_T = 30$  and various values of  $c$  (B), or  $c = 3$  and various values of  $n_T$  (C). **(D and E)** Hypothetical median-effect plots of dose-response curves for PIs against viruses with  $c = 3$ ,  $n_T = 5$  (D) or  $c = 3$ ,  $n_T = 30$  (E), and various  $f_m$  values. Concentrations are normalized by  $K_d$ . The change in  $IC_{50}$  is larger when  $c$  is proportionally small relative to  $n_T$  (E).

According to this model, the change in  $IC_{50}$  depends on the fractional occupancy of enzyme molecules required for inhibiting replication. Two contrasting situations with the same value of  $c$  but different values of  $n_T$  are illustrated in Fig. 3, D and E. When  $c$  is close to  $n_T$  (Fig. 3D,  $n_T = 5$ ), the fractional occupancy required for inhibition is low, and the model predicts that  $IC_{50}$  should only decrease slightly with increasing  $f_m$ . This is because the number of enzyme molecules is already close to the limiting value of  $n_T = c$ . However, when  $c$  is small relative to  $n_T$  (Fig. 3E,  $n_T = 30$ ), the fractional occupancy required for inhibition is high, and the model predicts that  $IC_{50}$  can decrease over a wider range with increasing  $f_m$  until the limiting condition is reached.

### Experimental validation of the critical subset model—generation of phenotypically mixed virions

Before testing the critical subset model, we validated several assumptions. One is that wild-type and mutant enzymes can be expressed in cells and incorporated into virions with similar efficiency. We chose well-characterized point mutations in the active sites of RT (D185N), protease (D25N), and integrase (D64E) that abolish enzyme activity without effects on multimer formation or virus assembly (18, 23, 24). Transfection efficiency was similar for the wild-type plasmid and mutant plasmids by flow cytometry (fig. S2A). Wild-type and mutant RT and integrase were incorporated into the virions with similar efficiency (fig. S2, B and C). We also tested whether formation of mixed dimers from wild-type and mutant monomers follows random mixing and hence the binomial distribution. To obtain direct biochemical evidence for mixed dimer formation in cotransfected cells, we performed co-immunoprecipitations (fig. S3). We introduced either a hemagglutinin (HA) or a Flag tag into the N terminus of the wild-type and mutant enzymes. Viral proteins immunoprecipitated by either anti-HA or anti-Flag antibodies from cell lysates were immunoblotted with anti-Flag or anti-HA antibodies, respectively. Our results suggest that for both RT and protease, dimers with all of the four possible combinations (wild-type–wild-type, wild-type–mutant,

mutant-wild-type, and mutant-mutant) can be formed with about equal probability (fig. S3, A and B).

### Analysis of dose-response curves for NNRTIs and PIs against phenotypically mixed viruses

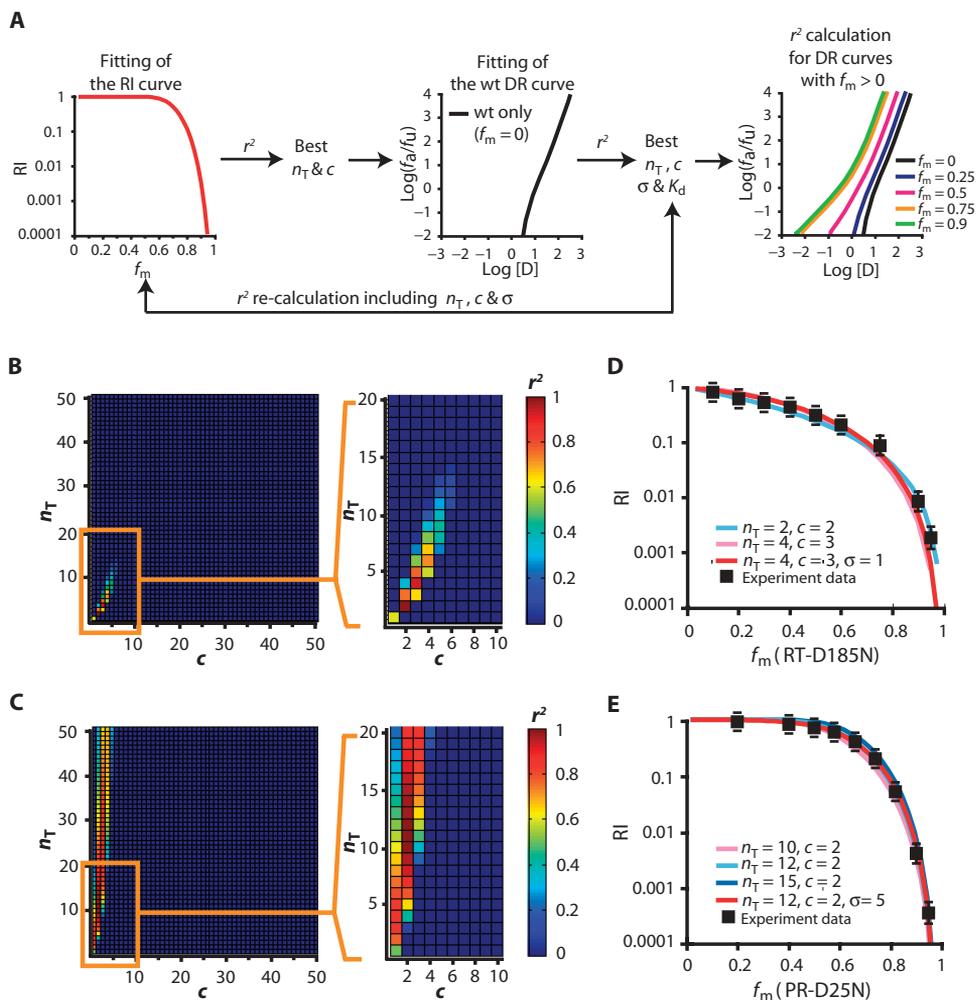
According to the model, drugs targeting steps involving multiple copies of the drug target should show steeper dose-response curves and markedly better inhibition at drug concentrations above the  $IC_{50}$ . NNRTIs can interact with all RT molecules in the pre-integration complex, and PIs can interact with all protease molecules in the maturing virion. These drug classes exhibit slopes  $>1.7$  and high inhibitory potential (12). For NNRTIs and PIs, the model predicts that  $IC_{50}$  should decrease as  $n_T$  is reduced and that the higher the fractional occupancy required for inhibiting replication, the greater the change in  $IC_{50}$  (Fig. 3, D and E).

We tested these predictions using the algorithm shown in Fig. 4A. We first determined the apparent  $n_T$  and  $c$  by measuring in the absence of drug the RI of virions with mixtures of catalytically active and inactive enzymes. The RI of phenotypically mixed virions can be calculated with Eq. 6 for any given  $n_T$  and  $c$  (Fig. 3A). We fitted experimental RI data to theoretical RI curves for RT and protease and calculated  $r^2$  values for all combinations of  $n_T$  between 1 and 100 and  $c$  between 1 and  $n_T$  (Fig. 4, B and C). The  $r^2$  values shown in Fig. 4, B and C, indicate poor fitting for  $n_T > 50$ . The experimental RI curves fit the theoretical curves best with either  $n_T = 2, c = 2$  or  $n_T = 4, c = 3$  for RT (Fig. 4, B and D), and  $n_T = 9$  to 15 and  $c = 2$  for protease (Fig. 4, C and E). With these  $n_T$  and  $c$  values, the model predicts  $m = 1.4$  to 2.0 for NNRTIs and  $m > 1.8$  for PIs, consistent with experimental observations.

To refine values for  $n_T$  and  $c$  and find the approximate  $K_d$ , we used estimates from RI curves as a starting point to find  $\sigma$  and  $K_d$  values that allowed optimal fitting of dose-response curves for NNRTIs and PIs against wild-type viruses (Fig. 4A, Eqs. 4 to 7, and Supplementary Note 2). For NNRTIs,  $n_T = 4, \sigma = 1, c = 3$  gave the best average fit (Fig. 5A), with  $K_d = 0.018, 0.33,$  and  $0.018 \mu M$  for efavirenz (EFV), nevirapine (NVP), and etravirine (ETR), respectively. The inclusion of  $\sigma$  for RT improved the fitting of the RI curve (Fig. 4D and table S1). The values developed using RI data in the absence of drugs allowed good fitting of experimental dose-response curves, including the upward in-

flexion that is apparent in the experimental data and that is predicted by the model for these  $n_T$  and  $c$  values (Fig. 5A).

Because  $c = 2$  for protease predicts a slope approaching 2 at high  $[D]$ , the model does not explain  $m > 2$  for some PIs or the sharp upward inflection at high  $[D]$ . Therefore, we tested the model using PIs with  $m \sim 2$ . For the wild-type dose-response curves, we considered only the initial linear part as described previously (12) and excluded the two data points at high  $[D]$ .  $n_T = 9$  to 15,  $\sigma = 4$  to 6,  $c = 2$  all fit the wild-type



**Fig. 4.** Experimental validation of the critical subset model. **(A)** Algorithm for model validation from experimental data. Curves describing the dependence of the relative infectivity (RI) of phenotypically mixed virions on  $f_m$  in the absence of drug were first obtained. Experimental RI curves were fitted with theoretical curves (Eq. 6) using various  $n_T$  and  $c$  values, and  $r^2$  values were calculated. The  $n_T$  and  $c$  values that gave the best five  $r^2$  values were used in the next step. These pairs of  $n_T$  and  $c$  values were used as a starting point in a systematic search for values of  $\sigma$  and  $K_d$  that allowed the best fit with dose-response curves for NNRTIs or PIs against wild-type (wt) viruses. The  $r^2$  values for the fitting of the RI curves were recalculated with the resulting sets of  $n_T, c,$  and  $\sigma$  values. Once an optimal set of  $n_T, c, \sigma,$  and  $K_d$  values was obtained, these values were used to predict dose-response curves for phenotypically mixed virions. The  $r^2$  values representing the fitting of predicted dose-response curves to the experimental curves were then calculated. **(B and C)** Estimates of  $n_T$  and  $c$  for RT (B) and protease (C). The  $r^2$  values for fitting of RT and protease RI curves to each pair of  $n_T$  and  $c$  values are shown. Red squares indicate  $r^2$  close to 1, and blue squares indicate  $r^2 < 0.2$ . **(D and E)** RI curves for RT (D) and protease (E). Theoretical RI curves predicted by the critical subset model are plotted as a function of  $f_m$  (solid lines). The actual experimental data are shown in black squares. The RI of a given viral stock was calculated by normalization to the infectivity of the viral stock carrying only wild-type enzyme. Each data point represents the mean  $\pm$  SEM from more than three experiments.

curves. With  $n_T = 12$ ,  $\sigma = 5$ ,  $c = 2$ , we obtained  $K_d = 0.0068$ ,  $0.039$ , and  $0.021 \mu\text{M}$  for lopinavir (LPV), nelfinavir (NFV), and amprenavir (APV), respectively (Fig. 5B). The introduction of  $\sigma$  for protease improved the fitting of the RI curve slightly (Fig. 4E and table S1).

Having established values of  $n_T$  and  $c$  that allow fitting of both RI data and dose-response curves for wild-type virus, we next examined the effect of decreasing  $n_F$  on the dose-response curves. As predicted, the dose-response curves for NNRTIs shifted only slightly to the left with increasing  $f_m$ . The experimental data fit the model predictions with  $r^2 > 0.94$  for each of the curves obtained for EFV and ETR (Fig. 5A and Supplementary Note 2). For NVP, the  $r^2$  values were  $> 0.90$  (Fig. 5A). For PIs, the model predicts a substantial left shift of the dose-response curves with increasing  $f_m$ . Again, the experimental data fit the model predictions well with  $r^2 > 0.87$  for all drug curves with various  $f_m$  (Fig. 5B and Supplementary Note 2). These results are consistent with the fractional occupancy of RT or protease required for inhibiting replication. The finding that the  $\text{IC}_{50}$  decreased with decreasing  $n_F$  for PIs can be understood by considering that virus particles with lower  $n_F$  are more easily inhibited, because fewer pro-

tease molecules must be bound by the drug to reduce the number of unbound protease molecules to below  $c$ . This result is also consistent with reports that the threshold activity of protease necessary for infectivity is small relative to the total protease activity per virion (25). In contrast, most of the RT molecules present are required for viral replication. Therefore, decreasing  $n_F$  had minimal effect on the  $\text{IC}_{50}$  of the NNRTIs.

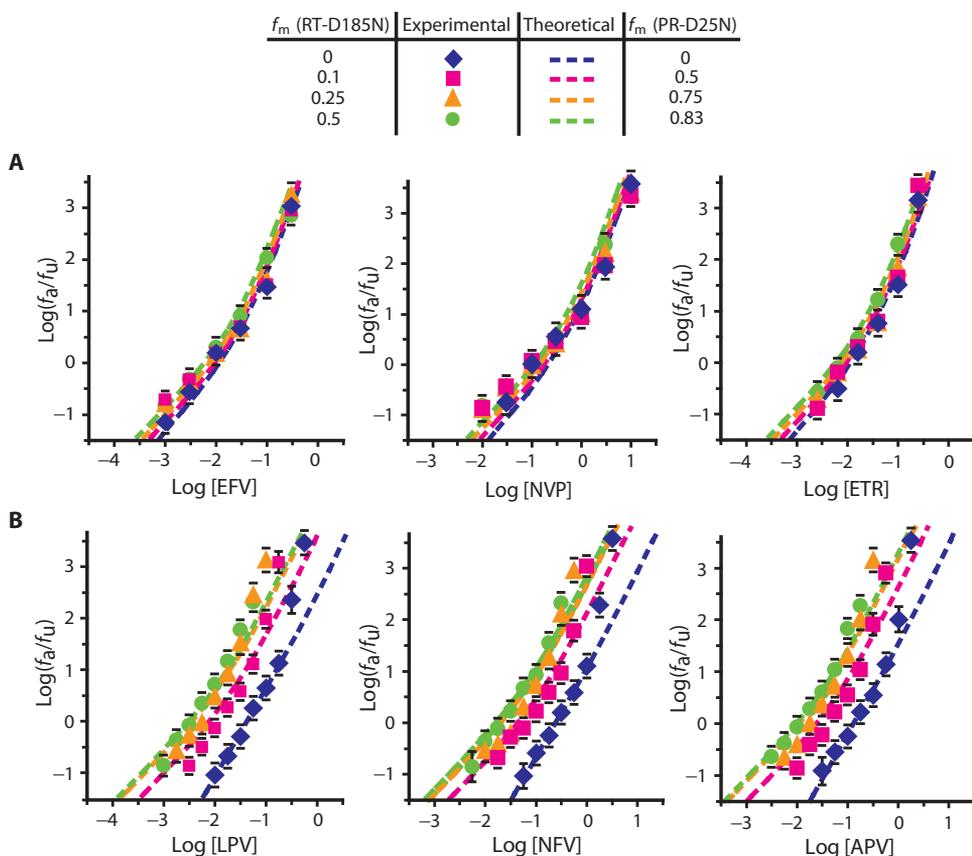
Together, these results suggest that the participation of multiple copies of RT and protease in the processes of reverse transcription and virion maturation, respectively, results in a form of intermolecular cooperativity that is manifested as steeper dose-response curves for the NNRTIs and PIs. The cooperative curves can be fit using the critical subset model without the need to postulate interactions between the active sites of individual enzyme molecules.

### Analysis of the dose-response curves for InSTIs and NRTIs against phenotypically mixed viruses

The InSTIs and the NRTIs exhibit slopes close to 1 (12). Unlike NNRTIs and PIs, these drugs target reactions in which a single molecular complex

of one enzyme with viral nucleic acid mediates a critical step. For the InSTIs, the inhibited process is performed by precisely one molecular complex per infected cell, consisting of an integrase tetramer bound to the ends of the viral genome (26, 27). There is no possibility for intermolecular cooperativity because only a single drug target per virus mediates the targeted reaction. Other integrase molecules present in the pre-integration complex are not targeted by InSTIs and are irrelevant. Similar reasoning can be applied to RT by breaking reverse transcription into individual steps, each consisting of attachment of one nucleotide. The NRTIs target a single enzyme–nucleic acid complex per infected cell, namely, the RT molecule that is adding the next nucleotide to the viral complementary DNA (cDNA) (17). Other RT molecules in the reverse transcription complex are irrelevant and are not targeted. When a single target mediates the critical reaction ( $n_T = c = 1$ ), Eq. 3 reduces to the median-effect equation (Eq. 2) with  $m = 1$  (Fig. 2, C and D), consistent with the experimental observations (12). The model predicts that phenotypic mixing should not affect  $m$  or  $\text{IC}_{50}$  because nonfunctional complexes are irrelevant for drug inhibition and functional complexes are inhibited by drug in the same manner for both wild-type and phenotypically mixed viruses.

To test these predictions, we generated phenotypically mixed virions in which a fraction of the integrase or RT molecules had the inactivating catalytic site mutations D64E or D185N, respectively (23, 24).

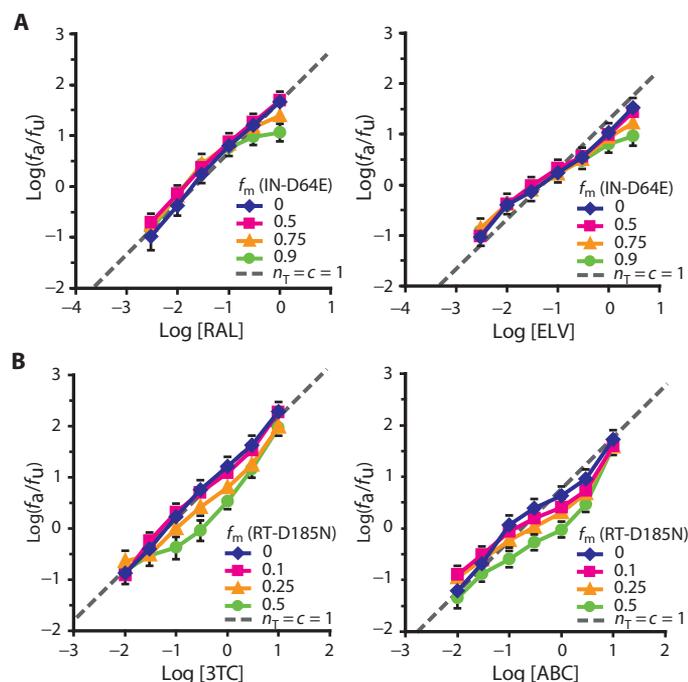


**Fig. 5.** Effect of phenotypic mixing on dose-response curves for NNRTIs and PIs. **(A)** Median-effect plots of theoretical (dashed lines) and experimental (solid markers) dose-response curves for the NNRTIs EFV, NVP, and ETR with wild-type and phenotypically mixed virions. The dose-response curves for phenotypically mixed virion preparations exhibited the expected slight decrease in  $\text{IC}_{50}$  compared to wild-type virions. **(B)** Median-effect plots of theoretical (dashed lines) and experimental (solid markers) dose-response curves for the PIs LPV, NFV, and APV with wild-type and phenotypically mixed virions. The two data points at high drug concentrations for the wild-type dose-response curves were excluded from the fitting. Dose-response curves for phenotypically mixed virion preparations showed the expected left shift with increasing  $f_m$ . Each data point represents the mean  $\pm$  SEM from more than three experiments.

Drug susceptibility was measured using the single-round infectivity assay. The slopes were close to 1 and exhibited minimal change against modulated viruses as predicted by the model (Fig. 6 and fig. S4). The  $IC_{50}$  values for the InSTIs raltegravir (RAL) and elvitegravir (ELV) against phenotypically mixed virions were unchanged (Fig. 6A). For all NRTIs except for zidovudine, there was no decrease in  $IC_{50}$ , consistent with the model.  $IC_{50}$  values for most NRTIs such as lamivudine (3TC) and abacavir (ABC) increased slightly relative to wild type at  $f_m = 0.5$  (Fig. 6B and fig. S4). The dose-response curves for zidovudine represented an exception, because the  $IC_{50}$  was markedly decreased as  $f_m$  increased (fig. S4). This increased sensitivity may reflect reduced excision of zidovudine when there are fewer active RT molecules (21). With this exception, no InSTI and NRTIs showed decreased  $IC_{50}$  when some copies of the relevant enzymes carried inactivating mutations (Fig. 6 and fig. S4). This behavior is in sharp contrast to the NNRTIs and PIs, drugs for which phenotypically mixed virions invariably showed decreased  $IC_{50}$ . Both sets of observations are consistent with the critical subset model.

## DISCUSSION

Steep dose-response curves generally reflect positive cooperativity. In the classical Hill model, a slope  $>1$  reflects cooperative binding



**Fig. 6.** Effect of phenotypic mixing on dose-response curves for InSTIs and NRTIs. **(A)** Dose-response curves for the InSTIs RAL and ELV against wild-type virus and viruses generated with increasing fractions of the D64E mutant plasmid. The  $IC_{50}$  and slope did not change with increasing  $f_m$ . As previously described (12), the flattening of the dose-response curves at high concentrations of InSTIs and high  $f_m$  may reflect low-level GFP expression from unintegrated proviruses. **(B)** Dose-response curves for the NRTIs 3TC and ABC against wild-type virus and viruses generated with increasing fractions of the D185N mutant plasmid. There was a two- to fourfold increase in  $IC_{50}$  and minimal change in slope with increasing  $f_m$ . Each data point represents the mean  $\pm$  SEM from more than three experiments.

of multiple ligands to a multivalent receptor. However, RT and protease have only one binding site for NNRTIs and PIs, respectively, and extensive kinetics studies with purified RT and protease have not provided evidence for cooperativity. Rather, the high slopes of NNRTIs and PIs in infectivity assays reflect unique aspects of the inhibition of viral replication that are not apparent in the behavior of the isolated enzymes.

Here, we present a critical subset model that provides a molecular explanation for these steep dose-response curves. We hypothesized that within a given infected cell or virus particle, the viral proteins targeted by a drug behave as a set of linked receptor sites whose fractional occupancy determines whether the relevant step in the virus life cycle is completed before irreversible decay processes intervene. When multiple copies of a target are involved in the inhibited reaction, there exists a form of intermolecular cooperativity that does not depend on alterations in the binding properties of individual sites. This model predicts slopes  $>1$  for NNRTIs and PIs because multiple copies of RT and protease are involved in the processes inhibited by these drugs, and a critical subset of these molecules is required for viral replication. The model successfully predicted shifts in the dose-response curves against viruses with decreased  $n_T$ . For NRTIs and InSTIs, only one copy of RT or integrase is relevant to the inhibited reaction. In this situation,  $m = \sim 1$  is predicted, as is observed experimentally.

According to the model, slope is determined by  $n_T$  and  $c$ . There are  $\sim 5000$  copies of Gag per virion, and the ratio of Gag-Pol to Gag is 1:10 to 1:20 (28, 29). Thus, if the Gag-Pol polypeptides are converted with 100% efficiency into functional dimeric RT and protease enzymes, there would be a maximum of 125 to 250 copies of these enzymes per virion. The  $n_T$  values for RT and protease obtained here are smaller, perhaps reflecting inefficiencies in the assembly of functional enzymes and additional complexities in the relevant reactions. The  $n_T$  and  $c$  parameters in this model are apparent numbers that allow description of the relevant reactions in terms of a simple model that does not capture the full complexity of the processes of reverse transcription and virion maturation. In addition,  $n_T$  and  $c$  refer only to enzyme molecules located in the microenvironment where the reaction occurs. For these reasons,  $n_T$  and  $c$  may differ from the actual physical number of RT or protease molecules present.

Because  $n_T$  and  $c$  for a given drug target should be constant, the model does not explain slope differences within the PI class. The model particularly cannot explain dose-response curves for PIs with  $m > 2$ , because  $c = 2$  for protease predicts a maximum slope of 2. We hypothesize that drug-to-drug variability in  $m$  for PIs may reflect the complexity of viral maturation, which involves multiple molecular states of the enzyme acting on multiple distinct substrates (30). The protease domains of adjacent Gag-Pol precursor proteins must first dimerize to form an active protease enzyme, which then carries out intramolecular cleavages to liberate itself from the Gag-Pol precursor protein. Ultimately, free protease dimers complete the remaining cleavages of the Gag and Gag-Pol precursor proteins (31). The embedded protease is less sensitive to PIs than the mature, free enzyme (30, 31). The effective substrate concentration may also differ for intramolecular and intermolecular reactions. Different PIs may preferentially inhibit distinct molecular forms of protease for which  $n_T$  and  $c$  are different, resulting in slope differences. In addition, virions generated in the presence of PIs may be blocked at subsequent steps such as reverse transcription (32). Thus, unlike other antiretroviral drugs, PIs affect multiple steps in the life cycle, possibly with different  $n_T$  and

$c$  values. These complexities may explain the upward inflection of PI dose-response curves at high  $[D]$ .

The concepts developed here can be applied to other drug classes and other viruses. However, additional factors may complicate the analysis. For drugs affecting HIV-1 entry, the targets are trimeric, and interactions within and between trimers must be considered. One particularly unique aspect of HIV-1 RT, protease, and integrase inhibitors is that they target enzymes that are present within the virion or that are assembled from precursor polyproteins present within the virion. In this situation, constraints inherent in virion assembly limit heterogeneity ( $\sigma$ ) in  $n_T$ . As shown here, heterogeneity flattens dose-response curves. Thus, in the case of viruses for which the drug targets are expressed and act within infected cells, heterogeneity in the number of enzymes per cell may obscure the cooperativity that is expected when multiple copies of a drug target act together to complete a step in the life cycle.

A variety of elegant models describing steep dose-response curves for ion channels and other multivalent receptors have been developed (16, 33–35), but models to explain cooperative dose-response curves for antiviral drugs have not been described. It is possible that other models will be developed to explain the complex dose-effect relationships for antiretroviral drugs. For example, saturable drug binding to alternative intracellular sites could result in an upward inflection within a small concentration range above the concentration that saturates the alternative sites. However, a model such as the one described here would still be needed to explain slopes  $>1$  in other regions of the dose-response curve. Although the overall slopes for NRTIs are close to 1, there are subtle inflections in the dose-response curves for some NRTIs (Fig. 6B and fig. S4). These may in part reflect binding to alternative intracellular sites or the reactions needed to produce the active triphosphate forms of the NRTIs. Nevertheless, the fact that slope varies in a class-specific way despite large intraclass differences in structure suggests that the slope parameter is a direct reflection of the mechanism of inhibition, as in the model described here.

The critical subset model represents an oversimplification of a very complex problem. Nevertheless, it explains the steep dose-response curves of NNRTIs and PIs without assuming that the binding of drug to one site affects the  $K_d$  of binding to other sites. A steep slope means that small increases in drug concentration above the  $IC_{50}$  can produce large increases in antiviral activity, permitting extremely high levels of inhibition in the clinical concentration range. Thus, the present study provides a conceptual framework for understanding the high antiviral activity of NNRTIs and PIs, drugs that form the basis of successful HIV-1 treatment, and provides important insights to the characteristics of good targets for antiviral drugs.

## MATERIALS AND METHODS

Detailed descriptions of experimental Materials and Methods are available in the Supplementary Material.

### Analysis of RI curves

The RI values of recombinant viruses generated with different  $f_m$  were obtained by normalizing to the infectivity of the wild-type virus. Theoretical RI values were calculated for given values of  $n_T$  and  $c$  using the binomial distribution (Eq. 6). This analysis required a consideration of the subunit stoichiometry of the relevant multimeric targets. The probability of obtaining a functional multimer ( $P_f$ ) is a function of  $f_m$ , the

valence of the multimer, and the functional status of mixed multimers. For example, the enzymatic activity of protease requires the D25 residues from both subunits of the dimer (18). Thus, for protease,  $P_f = (1 - f_m)^2$ . For RT, although a D185N mutation in the p66 subunit abolishes activity, a D185N mutation in the p51 subunit has no effect on enzyme activity (23). Thus, for RT,  $P_f = (1 - f_m)^2 + f_m(1 - f_m) = 1 - f_m$ . To estimate the apparent  $n_T$  and  $c$  values required for virus replication, we fitted experimental RI curves with theoretical curves predicted by the model (Eq. 6) using various  $n_T$  and  $c$ , and we obtained  $r^2$  values for both RI and log RI (Fig. 4, B and C). Whereas fitting using the linear scale puts more weight on higher RI values, fitting using the log scale emphasizes lower RI values. Pairs of  $n_T$  and  $c$  values giving the best five average  $r^2$  values with both methods were used for further analysis of dose-response curves (Fig. 4A). To estimate the 95% confidence interval for  $n_T$  and  $c$ , we bootstrapped the experimental RI curves 1000 times by perturbing the mean by a random value centered around the mean with SD equal to the SEM of the data.

### Analysis of dose-response curves

Dose-response curves for virus stocks with various  $f_m$  were obtained by normalizing to the %GFP<sup>+</sup> (percentage of green fluorescent protein-positive) cells without drug treatment. When necessary, the  $IC_{50}$  and slope based on the median-effect equation (Eq. 1) were calculated through least-squares regression analysis as described previously (12). To refine the values for  $n_T$  and  $c$  and find the approximate  $K_d$ , we used the pairs of  $n_T$  and  $c$  values estimated from RI curves as a start point in a systematic search for values of  $\sigma$  and  $K_d$  that would fit best with the experimental dose-response curves for NNRTIs or PIs against wild-type viruses (Eq. 4). Values of  $\sigma > n_T/2$  were not allowed. Once an optimal set of  $n_T$ ,  $c$ ,  $\sigma$ , and  $K_d$  values was obtained, these values were used to predict theoretical dose-response curves for phenotypically mixed virions (Fig. 4A). The  $r^2$  values representing fitting of predicted theoretical dose-response curves to the experimental curves were then calculated. Note that it is not necessary to consider the binding of a drug to the inactive enzyme for calculation of  $f_a$  (Supplementary Note 3).

### Mathematical modeling and statistical analyses

All theoretical curve simulations, calculations, curve fitting, and statistical analyses were performed with Matlab version 7.1 (The MathWorks) or Microsoft Office Excel 2003.

## SUPPLEMENTARY MATERIAL

[www.sciencetranslationalmedicine.org/cgi/content/full/3/91/91ra63/DC1](http://www.sciencetranslationalmedicine.org/cgi/content/full/3/91/91ra63/DC1)  
Materials and Methods

Fig. S1. Effect of heterogeneity in the total number of enzyme molecules ( $n_T$ ) on dose-response curves for anti-HIV drugs.

Fig. S2. Virus production and levels of reverse transcriptase (RT) and integrase (IN) associated with virions produced by cells transfected with wild-type or mutant proviral constructs.

Fig. S3. Mixed dimer formation detected by co-immunoprecipitation (co-IP) of viral protein variants.

Fig. S4. Effect of phenotypic mixing on dose-response curves for NRTIs.

Table S1.  $r^2$  values for the fitting of wt dose-response curves with and without the inclusion of  $\sigma$ .

Table S2. Experimental and theoretical values for  $\log(f_a/f_u)$  for inhibition of wild-type virus by different concentrations of EFV.

Table S3. Predicted and experimental values for  $\log(f_a/f_u)$  for inhibition of phenotypically mixed viruses by different concentrations of EFV.

Table S4. Experimental and theoretical values for  $\log(f_a/f_u)$  for inhibition of wild-type virus by different concentrations of LPV.

Table S5. Predicted and experimental values for  $\log(f_a/f_u)$  for inhibition of phenotypically mixed viruses by different concentrations of LPV.

Note 1. Calculation of slopes for complex dose-response curves generated by the critical subset model.

Note 2. Detailed description of model predictions and experimental confirmation for EFV and LPV.

Note 3. Effect of drug binding to inactive enzymes on  $f_a$ .

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