A quantitative basis for antiretroviral therapy for HIV-1 infection

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Highly active antiretroviral therapy (HAART)1–3 has dramatically decreased mortality from HIV-1 infection4 and is a major achievement of modern medicine. However, there is no fundamental theory of HAART. Elegant models describe the dynamics of viral replication3,5–9, but a metric for the antiviral activity of drug combinations relative to a target value needed for control of replication is lacking. Treatment guidelines10,11 are based on empirical results of clinical trials in which other factors such as regimen tolerability also affect outcome. Why only certain drug combinations control viral replication remains unclear. Here we quantify the intrinsic antiviral activity of antiretroviral drug combinations. We show that most single antiretroviral drugs show previously unappreciated complex nonlinear pharmacodynamics that determine their inhibitory potential at clinical concentrations. We demonstrate that neither of the major theories for drug combinations accurately predicts the combined effects of multiple antiretrovirals. However, the combined effects can be understood with a new approach that considers the degree of independence of drug effects. This analysis allows a direct comparison of the inhibitory potential of different drug combinations under clinical concentrations, reconciles the results of clinical trials, defines a target level of inhibition associated with treatment success and provides a rational basis for treatment simplification and optimization.

To determine the antiviral activity of multidrug regimens, the inhibition produced by single drugs must be precisely quantified. Drug potency is commonly described using the concentration producing 50% inhibition in in vitro assays (IC50). However, antiretroviral drugs are used at concentrations above the IC50, and inhibition at clinical concentrations can only be determined if the shape or slope (m) of the dose-response curve is also known12. When dose-response curves are steep (m > 1), small changes in drug concentration (D) have large effects. Steep curves typically reflect cooperative binding to multivalent receptors13. However, antiretroviral drugs target enzymes that are univalent for the inhibitor. Thus, the slope parameter has been ignored even though it is a component of all fundamental pharmacodynamic equations13–15 and models describing the combined effect of multiple drugs (reviewed in refs. 16,17). We previously showed that some antiretrovirals, notably protease inhibitors, have steep slopes12. This finding suggested unexpected complexity in the relationship between drug concentration and antiviral effect. To explore this relationship, we used a single-round infectivity assay12,18, because complex factors such as growth and death of target cells distort m in multiround assays19. Infections were carried out in primary CD4+ T lymphoblasts, the main target cells for HIV-1 in vivo12. The assay accounts for differences in protein binding, for the time required for intracellular phosphorylation of nucleoside reverse transcriptase inhibitors (NRTIs) and for individual variation in cellular uptake and metabolism of drugs (see Methods).

We also used a new method of analysis. Standard dose-response curves for antiviral drugs, which plot the fraction of infection events unaffected by drug (f_u) against log of drug concentration (log(D)), obscure m, the importance of which is better illustrated in plots based on the median-effect equation20 (equations (1) and (2)):

\[
f_u = \frac{1}{1 + \left(\frac{D}{IC_{50}}\right)^{m}} \tag{1}
\]

or

\[
\log((1 - f_u) / f_u) = m \log(D) - m \log(IC_{50}) \tag{2}
\]

Equation (2) linearizes dose-response curves such that a logarithmic measure of inhibition, \(\log((1 - f_u) / f_u)\), increases linearly with log(D) (Fig. 1a). The slope of the line is m. With high inhibition (\(f_u < 0.01\)), \(\log((1 - f_u) / f_u)\) approximates log(1 / \(f_u\)), which is the instantaneous inhibitory potential (IIP), a useful and intuitive measure of antiviral activity12. IIP is the number of logs single-round infection events are reduced by a drug. Drugs with high m achieve much higher IIP than ones with the same IC50 but lower m (Fig. 1a).

The influence of m is apparent in HIV-1 infectivity assays12,18 with different drugs. For the integrase strand transfer inhibitor (InSTI) raltegravir (RAL), curves generated with cells from different donors were

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Paradigm and had slopes of ~1 (Fig. 1b). At the peak plasma concentration (Cmax), RAL produces ~2 logs inhibition (IIP = 2). Curves for the protease inhibitor atazanavir (ATV) are steeper and strikingly nonlinear (Fig. 1c). An upward inflection markedly increases inhibition for minor increases in D (Supplementary Note 1, Supplementary Fig. 1 and Supplementary Table 1). Inhibition at clinical concentrations can only be estimated by extrapolation from the upper end of the observable range (Fig. 1c, Supplementary Note 2 and Supplementary Fig. 2). At Cmax, ATV produces ~8 logs of inhibition. Thus, infection of >1 x 10^8 cells would be required to observe a single infection event.

Similar analyses showed that most antiretrovirals have complex, nonlinear median-effect plots (Fig. 1d and Supplementary Table 1). Different drugs showed highly significant differences in slope at clinical concentrations (m') (Supplementary Table 2). Grouping curves by drug class and normalizing by IC_{50} revealed that all protease inhibitors and non-nucleoside reverse transcriptase inhibitors (NNRTIs) have steep, upwardly inflected curves, with maximum slopes >3 and >2, respectively (Fig. 1e and Supplementary Table 1). These complex curves may reflect a unique form of intermolecular cooperativity (1). Complex curves were observed for several other antiretrovirals (Fig. 1e and Supplementary Note 3). These results reveal previously unappreciated complexities in dose-response relationships for antiretroviral drugs that are crucial for understanding the effects of these drugs at clinical concentrations.

To demonstrate how these curves behave in the clinical range, we normalized concentrations by C_{max} (Fig. 1f). For the protease inhibitors ATV, darunavir (DRV) and lopinavir (LPV) and the NNRTI efavirenz (EFV), curves inflect upward at concentrations below the minimum plasma concentration (C_{min}), giving extraordinarily high IIP at clinical concentrations, consistent with clinical trial results (10,11). For the protease inhibitor nelfinavir (NFV), the inflection occurs at D > C_{min} (Fig. 1f), explaining its inferior clinical performance (22). Because the protease inhibitor ritonavir (RTV) is used at low concentrations to ‘boost’ other protease inhibitors, its curve inflects at the top of the clinical range, providing little antiviral activity. Thus, understanding the complex shapes of dose-response curves at clinical concentrations is essential for assessing antiviral activity.

Pharmacokinetics are also important (12). For drugs with high m, small decreases in D greatly reduce IIP. If the half-life is also short, IIP can fall dramatically during the dosing interval (12). Therefore, we computed the average IIP over the dosing interval (IIPave) from the area under the IIP versus time curve to allow quantitative comparison of antiviral activity of different drugs at expected plasma concentrations (Fig. 1g and Supplementary Tables 3 and 4). We used equation (2) to model the dose-response relationship in the clinical concentration range, with parameters chosen to best represent the shape of the curve in that range (Supplementary Table 1). Notably, this calculation takes into account both the complex shapes of the dose-response curves revealed here and pharmacokinetic differences
between drugs. IIP_{ave} values in tissue sites that have different drug concentrations than plasma could be similarly estimated provided that the relevant pharmacokinetic data were available.

Overall, the results explain the established clinical value of NNRTIs and protease inhibitors^{10,11}. Treatment guidelines have until recently recommended that all initial HAART regimens include an NNRTI or protease inhibitor^{10,11}. Steep, upwardly inflected curves allow these drugs to achieve extremely high IIP_{ave}. We previously estimated that only NNRTIs and protease inhibitors^{10,11} immediately halt replication^{23}. Only NNRTIs and protease inhibitors approach this level. The protease inhibitor DRV has the highest IIP_{ave}. Of note, protease inhibitors are the only class for which monotherapy has been successful^{24–26}. Conventional dose-response curves obscure the marked differences in IIP observed here (Fig. 1g).

Some drugs with low IIP_{ave} such as RAL and the chemokine receptor antagonist maraviroc (MVC) are effective in combination therapy^{27,28}. We hypothesized that these drugs might have favorable or synergistic interactions in combinations that compensate for low intrinsic IIP_{ave}. However, there is no metric for the antiviral activity of antiretroviral drug combinations. Two fundamental models describe the combined effects of multiple drugs^{16,17} (Fig. 2a). Bliss independence^{29} assumes independent action such that the combined effect (f_{combined}) is the product of the fractions unaffected by each drug (equation (3)):

\[
f_{u1+2} = f_{u1} \times f_{u2} = \frac{1}{1 + \frac{D_1}{IC_{501}}} \times \frac{1}{1 + \frac{D_2}{IC_{502}}} \tag{3}
\]

Loewe additivity^{30} is based on isobolograms and assumes similar mechanisms or competition for the same binding site. For positive inhibitory slopes, Loewe additivity is described by equation (4):

\[
1 = \frac{D_1}{IC_{501} \left( \frac{f_{u1+2}}{1 - f_{u1+2}} \right)^{-1/m_1}} + \frac{D_2}{IC_{502} \left( \frac{f_{u1+2}}{1 - f_{u1+2}} \right)^{-1/m_2}} \tag{4}
\]
Bliss independence predicts higher inhibition than Loewe additivity, with upwardly inflected curves (Fig. 2b). Synergy and antagonism can only be defined relative to the combined effect predicted by a given model. Loewe additivity is generally chosen\(^{16,17}\) despite the fact that, by simple binding-site competition criterion, Bliss independence should apply to many antiretroviral drug combinations, particularly those involving drugs acting at different steps in the life cycle. However, there has been no systematic evaluation of which model applies to antiretroviral drugs. Therefore, we analyzed 166 of 171 possible pairwise combinations of 19 commonly used antiretrovirals. Of note, we used conditions maximizing the difference in the levels of inhibition predicted by the two models (see Methods).

Experimental inhibition unambiguously fit one of the standard models for <60% of combinations (Supplementary Fig. 3). Intermediate inhibition was common and can be understood by considering NRTI-NNRTI interactions. Although these classes bind different sites on reverse transcriptase, they inhibit the same process and are not fully independent. Therefore, these combinations show inhibition below the Bliss prediction. Notably, the inhibition produced by a given combination showed a consistent relationship to predictions of the two models as drug concentrations increased (Fig. 2c). This suggests that combinations can be characterized on a spectrum defined by two states, independent inhibition (Bliss) and competitive binding (Loewe), with synergistic and antagonistic interactions at either extreme. To quantify degree of independence (DI), we developed a new index with combinations following Loewe additivity assigned $DI=0$ and combinations following Bliss independence assigned $DI=1$. The index is computed as:

$$DI = \frac{F_P - F_L}{F_B}$$ (5)

where $F_P$, $F_L$ and $F_B$ are logarithmic measures of inhibition, log$[1 - f_{obs}] / f_{obs}$, for the experimental data and Loewe and Bliss predictions, respectively (Fig. 2c). This approach incorporates both classic models and maps inhibition on a median-effect plot in relation to the models.

Representative combination experiments are shown in Figure 2d. Expected results based on the competitive binding criterion are in Figure 2e. Experimental results, categorized by DI index, are in Figure 2f and Supplementary Table 5.

Some combinations targeting different steps in the life cycle followed Bliss independence and showed higher combined effects. This is illustrated by the InSTI-NNRTI combination RAL-emtricitabine (FTC) (Fig. 2d, $R^2 = 0.99$ for Bliss) and was observed for combinations of InSTIs with drugs from all other classes (Fig. 2f). The excellent clinical performance of InSTI-based regimens\(^ {22}\) may reflect these favorable interactions. The chemokine receptor antagonist MVC also showed favorable interactions (Fig. 2f).

Drugs binding to the same site, such as the hydrophobic NNRTI pocket\(^ {31}\), should follow Loewe additivity. This is illustrated by the NNRTI-NNRTI combination etravirine (ETR)-nevirapine (NVP) (Fig. 2d, $R^2 = 0.99$ and 0.64 for Loewe and Bliss, respectively). Loewe additivity was observed for combinations of two NNRTIs, two InSTIs and two nucleoside analogs of the same base (zidovudine-stavudine (AZT-d4T), lamivudine (3TC)-FTC and didanosine-tenofovir (ddI-TDF)) (Fig. 2f). The AZT-d4T combination is considered antagonistic owing to effects at the level of phosphorylation\(^ {22}\) and suboptimal clinical responses\(^ {33}\). However, in infectivity assays, this combination fits the Loewe prediction perfectly ($R^2 = 0.99$).

Another common pattern was a combined effect between the Bliss and Loewe predictions, reflecting some lack of independence. This is illustrated by the NRTI-NNRTI combination abacavir (ABC)-NVP (Fig. 2d). Intermediate effects were observed for many RTI–protease inhibitor combinations and some RTI-RTI and protease inhibitor–protease inhibitor combinations (Fig. 2f, Supplementary Table 5 and Supplementary Fig. 4). As discussed above, lack of independence is expected for combinations of RTIs and, for protease inhibitor–RTI combinations and protease inhibitor–protease inhibitor combinations, may represent complex effects of incomplete maturation on downstream events in the life cycle\(^ {34-36}\). Combined effects significantly greater than the Bliss prediction represent synergy and were observed for combinations of deoxythymidine analogs AZT or d4T with other RTIs (Fig. 2d, AZT-NVP and Fig. 2f) and for certain other combinations (Fig. 2f). Together, these results suggest that degree of independence of two drugs has a major impact on the combined effect.

IIP for triple combinations ($IIP_{1+2+3}$) can be estimated using three drug versions of the Bliss and Loewe models, with Bliss independence predicting higher combined effects (Fig. 3). Antagonism, defined as inhibition less than the Loewe prediction, was uncommon in pairwise analysis (Fig. 2f). Therefore, we assumed that the lower limit of $IIP_{1+2+3}$ is the Loewe prediction. Similarly, since most two drug combinations showed inhibition less than or equal to the Bliss prediction, the upper limit of $IIP_{1+2+3}$ is the Bliss prediction except for cases in which strong synergistic interactions dominate. Using these bounds, we determined the range of $IIP_{ave1+2+3}$ for all three
drug combinations of 19 commonly used antiretrovirals (Fig. 4a and Supplementary Table 6). Because the Bliss and Loewe predictions diverge with increasing D (Figs. 2b and 3b), the ranges are broad. Predicted IIPave values for different regimens vary substantially. At the high end are regimens with two drugs whose dose-response curves inflect sharply upward at $D < C_{\text{min}}$ (for example, EFV plus DRV). At the low end are dual-NRTI regimens. To examine the relationship between IIPave values and clinical outcome (Supplementary Fig. 6), correlation coefficient $= 0.686, P < 0.001)$. Only 1 of 31 evaluated regimens with IIPave < 8 had >70% of subjects with a viral load <50 copies per ml at 48 weeks. Among evaluated regimens meeting this criterion, the one with the lowest IIPave was TDF plus FTC plus RAL37. Using DI values, we estimate an IIPave of 5.05 for this regimen. This is at the high end of the Loewe-Bliss range owing to favorable RAL interactions (Fig. 4b). These results suggest that a minimum of 5–8 logs of inhibition are required for successful HAART. The triple NRTI regimen TDF plus FTC plus ABC had a similar Loewe-Bliss range but inferior efficacy38, possibly because the actual IIPave value is closer to the low end of the Loewe-Bliss range (because of low DI for ABC pairs) and below the 3-log threshold (Fig. 4b). Three of the other regimens currently recommended for initial treatment had IIPave >8, primarily owing to the IIP of the base drug (EFV, ATV/r, DRV). Above IIPave = 7, there was little correlation with outcome (correlation coefficient $= 0.125, P = 0.29$) because above this level, replication is essentially halted, and outcome depends mainly on adherence.

This approach provides a quantitative basis for HAART. HAART controls viral replication because of steep, upwardly inflected dose-response curves for some drugs and synergies reflecting the independent action for other drugs. Despite the extremely high IIPave of some regimens, HAART is not curative because of stable reservoirs of nonreplicating virus39,40. Our results demarcate a minimum threshold level of antiviral activity (IIPave = 5–8 logs) necessary for successful treatment. Above this range, additional inhibitory potential is not necessarily beneficial, particularly if it involves the use of poorly tolerated drugs. Evaluating new regimens in relation to this threshold may reduce the need for costly clinical trials for regimens lacking sufficient IIPave. This approach also permits a comprehensive search for regimens with suprathreshold antiviral activity, maximum tolerability and minimum cost. Simpler two- or three-drug regimens with adequate IIPave but lower cost and toxicity may be important for extending therapy in resource-limited settings. Finally, this approach may allow more rational selection of salvage regimens.

METHODS
Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemedicine/

Note: Supplementary information is available on the Nature Medicine website.

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AUTHOR CONTRIBUTIONS
B.L.J., M.Z., M.E.S., C.K.B. and J.L. conducted the experiments. B.L.J., M.Z., S.A.R., L.S. and R.F.S. carried out the computational analysis. R.F.S. supervised the project and wrote the manuscript.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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ONLINE METHODS

We measured drug inhibition of HIV-1 infection using a single-round infectivity assay as previously described\textsuperscript{12,18}. We chose a single-round assay because complex factors such as growth and death of target cells distort \( m \) in multiround assays\textsuperscript{19}. To accurately mimic infection events in vivo, we carried out infections in primary CD4\(^+\) T lymphoblasts obtained by phytohemagglutinin (Remel, Leneva) activation of peripheral blood mononuclear cells from healthy donors as previously described\textsuperscript{18}. All blood donors provided informed consent according to a protocol approved by the Johns Hopkins Institutional Review Board. We infected CD4\(^+\) T lymphoblasts with recombinant HIV-1 pseudoviruses generated by transfection of HEK293 T cells (American Type Culture Collection) with a previously described\textsuperscript{12} HIV-1 vector (pNL43-ΔE-EGFP) containing a GFP-tagged defective envelope. The envelope was provided in trans by co-transfection with an envelope expression vector. In most cases, we used a CXCR4-tropic envelope (HXB2; NIH AIDS Research and Reference Reagent Program) because it gave higher-level infection of primary CD4\(^+\) T lymphoblasts, thus affording a wider dynamic range. However, IC\(_{50}\) and \( m' \) values obtained in infections with CCR5-tropic pseudoviruses were not significantly different from those obtained with CXCR4-tropic viruses (Supplementary Table 7), and modes of drug interaction were also similar (Supplementary Table 7). To account for individual variation in cellular uptake and metabolism of drugs, we performed replicate assays in lymphoblasts from 10–60 healthy donors for each drug and computed average levels of inhibition. We used high serum concentrations (55%) to account for protein binding. For protein-bound drugs, titrations of drug effect versus serum concentration demonstrated that inclusion of 50% human serum and 5% FCS provided a close approximation for in vivo drug binding. We pre-tested lots of human serum for low toxicity to CD4\(^+\) T lymphoblasts. Drugs were added to lymphoblast targets, and cells were incubated at 37 °C for 16 h before infection to allow sufficient time for triphosphorylation of NRTIs. Longer preincubations did not enhance antiviral activity. Protease inhibitors were also added at the stage of virus production in 293T cells\textsuperscript{18}. After spin infection in 96 well plates, targets were incubated for 3 days at 37 °C. Cells were then fixed in 2% formaldehyde, and GFP expression was analyzed by flow cytometry. After gating for viable cells, the fraction of infection events unaffected by drug (\( f_u \)) was determined as the % GFP\(^+\) cells in the presence of drug divided by the % GFP\(^+\) cells in control wells without drug. Calculation of IIP from \( f_u \) is described in the Supplementary Methods. All drugs were obtained from the NIH AIDS Research and Reference Reagent Program.

To determine whether the combined effect of two antiretroviral drugs followed the predictions of the Bliss or Loewe models, we tested pairs of drugs at constant molar ratios chosen to maximize the difference between the levels of inhibition predicted by the two models. Drug combinations are often evaluated in surface experiments in which all possible combinations of several different concentrations of each drug are tested\textsuperscript{26,27}. However, the difference between the inhibition predicted by the two models increases with drug concentration (Fig. 2b) and is maximal when both drugs contribute equally to the observed suppression (R.F.S., unpublished observation). Therefore, we chose drug concentrations such that at the highest concentrations used, the total inhibition predicted by the Bliss model would be within the dynamic range of the assay (IIP < 3), with each drug contributing equally. Drugs were mixed at these concentrations and serially diluted. Experimental analysis was carried out in cells from 5–15 donors per combination as described above. Protease inhibitors were added at the virus production stage. Analysis of combination experiments is described in the Supplementary Methods.

Additional methods. Detailed methodology is described in the Supplementary Methods.