

# Drug resistance and antiretroviral drug development

Robert W. Shafer<sup>1\*</sup> and Jonathan M. Schapiro<sup>1,2</sup>

<sup>1</sup>Division of Infectious Diseases, Department of Medicine, Stanford University, Stanford, CA 94305, USA;

<sup>2</sup>National Hemophilia Center, Sheba Medical Center, Tel Aviv, Israel

**As more drugs for treating HIV have become available, drug resistance profiles within antiretroviral drug classes have become increasingly important for researchers developing new drugs and for clinicians integrating new drugs into their clinical practice. *In vitro* passage experiments and comprehensive phenotypic susceptibility testing are used for the pre-clinical evaluation of drug resistance. Clinical studies are required, however, to delineate the full spectrum of mutations responsible for resistance to a new drug and to identify the settings in which a new drug is likely to be most useful for salvage therapy.**

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## Introduction

The US Food and Drug Administration (FDA) has approved eight nucleoside reverse transcriptase (RT) inhibitors (NRTIs), seven protease inhibitors (PIs), three non-nucleoside RT inhibitors (NNRTIs), and one fusion inhibitor. Four fixed-dose NRTI combination formulations and several modified versions of existing drugs with improved bioavailability have also been approved. Additional NRTIs, NNRTIs, and PIs are in advanced clinical development and several cell entry inhibitors are in earlier stages of clinical development.

As more antiretroviral drugs are approved, resistance profiles within drug classes have become increasingly important. This importance is reflected in the recent publication by the US FDA of a draft document entitled, 'Guidance for Industry: Role of HIV Drug Resistance Testing in Antiretroviral Drug Development'.<sup>1</sup> Clinicians also look to the resistance profiles of experimental drugs when considering whether to enrol their patients in clinical trials and expanded access programmes, or to delay therapeutic changes in anticipation of new drug approvals. We have written this review to help clinicians interpret the drug resistance profiles of new compounds as they are published in pre-clinical and early clinical studies.

## Pre-clinical studies

### *In vitro* passage experiments

Experiments in which HIV-1 is cultured in the presence of increasing concentrations of a newly identified antiretroviral compound have three main purposes: (i) to identify one or more

of the genetic mechanisms of resistance to the compound (i.e. mutations) and to compare these mechanisms with those associated with resistance to other drugs of the same class, (ii) to determine the time it takes for resistance to emerge *in vitro*, and (iii) to determine whether the drug-resistant variants replicate less well than wild-type variants.

What should clinicians know to interpret the results of these experiments? First, resistance is inevitable. In fact, it is the emergence of resistance that confirms that an antiretroviral drug acts specifically by inhibiting the virus and not by poisoning the cells in which the virus replicates. Second, there are no standard protocols for performing these experiments and the time to development of resistance is highly protocol dependent. Drugs that induce resistance within 1–2 passages (about 3–7 days) will almost certainly require fewer mutations for drug resistance to develop (low genetic barrier to resistance) than will drugs that induce resistance only after many weeks of passage (high genetic barrier to resistance). But small differences in the time to developing resistance are unlikely to be reproducible.

Finally, the development of mutations that are similar to those that emerge with other drugs strongly suggests that cross-resistance will be a problem. However, the development of mutations that differ from the mutations associated with other drugs does not guarantee the absence of cross-resistance. Mutations that emerge during *in vitro* passage are sufficient to cause resistance but are rarely the only means by which resistance can develop. Indeed, the mutations that emerge *in vitro* usually represent only a subset of the mutations that emerge *in vivo*. The *in vivo* challenge is much greater than the *in vitro* challenge because of the much larger number of viruses *in vivo* and their greater heterogeneity.<sup>2</sup>

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\*Corresponding author. E-mail: rshafer@stanford.edu

*Role of drug susceptibility testing during pre-clinical drug development*

Drug susceptibility or ‘phenotypic’ testing is used to quantify the decrease in susceptibility to a new drug conferred by the mutations that emerge *in vitro* and the mutations that confer resistance to other antiretroviral drugs. These results are important to prioritize continued drug development and to determine the optimal use of this drug within the context of other approved antiretroviral drugs. If a new drug is eventually approved for clinical use, then these genotype–phenotype correlations become essential for the optimal use of genotypic drug resistance testing.

For FDA-approved drugs and for drugs nearing FDA approval, most phenotypic testing is done using either the PhenoSense (ViroLogic, South San Francisco, CA, USA) or Antivirogram (Virco, Mechelin, Belgium) assays. Both assays are intricate, high-throughput, commercially available tests performed in specialized facilities within each company. Each assay uses PCR to amplify from patient plasma the entire protease, much of RT and the 5′ part of *gag*.<sup>3,4</sup> The amplified material is incorporated into a *pol*-deleted recombinant virus by ligation (PhenoSense) or homologous recombination (Antivirogram). A standardized virus inoculum is then used to infect a cell line and virus replication is measured in a range of drug concentrations. In the PhenoSense assay, virus replication is monitored by a luciferase gene cassette that emits light in proportion to the number of virions present after one round of HIV-1 replication. The Antivirogram assay measures the intensity of a tetrazolium dye that produces colour when reduced by cellular mitochondrial enzymes in proportion to the number of viable cells present after several cycles of HIV-1 replication.

Drug susceptibility assays are not designed to determine the exact amount of drug required to inhibit virus replication *in vivo*, but rather to identify differences in the drug concentration required to inhibit a fixed inoculum of a virus relative to wild-type. Indeed, marked differences in the rates of NRTI triphosphorylation rates between the cells used for susceptibility testing and the wider variety of cells infected by HIV-1 *in vivo* appear to explain the poor relationship between *in vitro* and *in vivo* potency. For example, there are significant discordances between the *in vitro* and *in vivo* activities of the NRTIs. Zidovudine appears to be the most potent NRTI *in vitro* because the IC<sub>50</sub> is 10- to 100-fold lower than that of the other NRTIs. Yet, in patients, lamivudine, emtricitabine, abacavir, tenofovir, and didanosine are much more potent than zidovudine at lowering plasma HIV-1 RNA levels.

Because the absolute value of the IC<sub>50</sub> is highly assay dependent, this value is usually divided by the IC<sub>50</sub> of a wild-type control and the ratio is reported as the fold decrease in susceptibility. However, the significance of even this partially normalized result depends on four additional factors: (i) the technical variability of an assay; (ii) the range in susceptibilities observed for wild-type isolates (‘biological variability’); (iii) the range in susceptibilities to a drug observed in mutant HIV-1 isolates with decreased *in vitro* susceptibility (‘dynamic range’); and (iv) the levels of resistance which are clinically meaningful. The technical and biological variability of the PhenoSense and Antivirogram assays have been described,<sup>5,6</sup> but similar data are not available for most other assays, complicating their interpretation.

The dynamic range in susceptibility for zidovudine, lamivudine, emtricitabine, and the NNRTIs is about 1000-fold; for

the PIs about 100-fold; and for the NRTIs stavudine, didanosine, tenofovir, and abacavir about 10-fold. Small changes in susceptibility may be difficult to detect in drugs with narrow ranges if they overlap with the technical and biological variability of an assay. For example, we have recently reported that the PhenoSense assay is more precise than the Antivirogram assay and as a consequence is more sensitive at detecting decreased susceptibility to stavudine, didanosine and abacavir.<sup>7</sup>

Because drug resistance is not an all-or-nothing phenomenon, the significance of particular changes in susceptibility must be correlated with clinical data. However, it is possible to gain some insight during pre-clinical studies. Small changes in susceptibility are also more likely to be significant if they are associated with mutations that are associated with virus escape *in vitro*. For example K65R is selected *in vitro* by tenofovir.<sup>8</sup> This mutation confers slightly less than 2-fold decreased tenofovir susceptibility, yet it clearly interferes with tenofovir activity in patients.<sup>9,10</sup>

*Non-B subtypes*

During its spread among humans, the group M (Main) virus responsible for the HIV-1 pandemic has evolved into multiple subtypes that differ from one another by 10–12% of their nucleotides in protease and RT genes<sup>11</sup> and up to 30% in their envelope genes.<sup>12</sup> Currently approved antiretroviral drugs were developed using biophysical and biochemical studies targeting subtype B isolates—the predominant subtype in North America and Western Europe—and the vast majority of data on the genetic mechanisms of HIV-1 drug resistance have been generated from observations on subtype B viruses. However, HIV-1 subtype B viruses account for only ~10% of the global HIV pandemic.

An increasing number of observational studies, both *in vitro* and *in vivo*, suggest that the approved protease and RT inhibitors are as active against wild-type non-B viruses as against wild-type subtype B viruses.<sup>13</sup> Although it may not be possible to fully characterize the mutations associated with resistance to each of the most common HIV-1 subtypes during pre-clinical drug development, it is essential to confirm that a drug brought into clinical trials is active against all subtypes.

*Cross-resistance*

Pre-clinical studies of cross-resistance are designed to determine whether viruses resistant to currently approved drugs are also less susceptible to a new drug and, conversely, whether viruses that emerge during selection with a new drug are also resistant to approved drugs. All currently approved and experimental drugs belonging to the same class share some degree of overlapping resistance. However, if cross-resistance is extensive and bi-directional, then continued development is not justified unless the drug has another major advantage over other drugs of the same class.

Several drugs select for mutations *in vitro* that do not cause cross-resistance to other drugs. These drugs are often touted as excellent choices for initial therapy on the presumption that patients who fail therapy with such a drug will remain susceptible to other drugs of the same class. This situation is certainly better than full bi-directional cross-resistance, but because drug resistance is rarely completely non-overlapping and because

treatment failure often increases the risk of resistance to more than one component of a treatment regimen, this strategy can only be recommended if the new drug is at least as effective as other drugs of the same class.

New compounds that demonstrate *in vitro* antiretroviral activity are usually tested on a range of drug-resistant clinical isolates. However, because no standard sets of drug-resistant isolates are used, it is usually not possible to precisely determine the activity of a new drug relative to other experimental and approved drugs. We have created a panel of recombinant infectious molecular clones containing combinations of mutations that confer resistance to multiple NRTIs and are developing a similar panel of multidrug-resistant protease clones.<sup>14</sup> Testing the activity of new compounds against these panels will allow researchers from different laboratories to standardize their results against a set of reference viruses and will help drug developers prioritize experimental compounds on the basis of their activity against the most highly drug-resistant viruses.

#### *In vitro* assessment of resistance to antiretroviral drug combinations

Most *in vitro* drug combination studies use a range of subinhibitory concentrations of two drugs to determine during one or two rounds of replication whether the drugs are synergic, additive, or antagonistic. These experiments are essential for identifying antagonistic drug combinations which for the NRTIs is often particularly important because nucleoside analogues are pro-drugs that may compete with one another for triphosphorylation. However, clinically significant synergy must be identified *in vivo* because antiretroviral synergy results primarily from the prevention of drug resistance. Drugs that act on different viral targets are usually synergic because each drug suppresses variants resistant to the other drug. Drugs that act on the same viral target, however, may be synergic if one drug selects for mutations that interferes with resistance to the second drug.

### Clinical development

It is not possible to accurately predict solely from pre-clinical data all the mutations that cause resistance to a new drug, let alone their clinical consequences. First, *in vitro* passage experiments may not identify the most relevant drug-resistance mutations because the size and genetic heterogeneity of the HIV-1 population in a person far surpass those of a laboratory isolate. Second, *in vitro* susceptibility testing is performed using cells that do not accurately reflect antiviral activity *in vivo*. Third, many drug-resistance mutations reduce virus replication and possibly virulence; clinical benefit is often maintained by continuing therapy based on the drug-resistance mutations that are present. Finally, it is not possible to measure *in vitro* the effect of synergic drug combinations that prevent the emergence of drug resistance.

Clinical studies are therefore needed to identify mutations emerging in patients who develop virological failure while receiving a new drug and to assess the consequences of different mutations on the virological response to a new drug. Plasma HIV-1 RNA levels are the mainstay for defining virological success and failure in such studies. It has only rarely been possible to correlate drug resistance with immunological decline or clinical morbidity because these downstream consequences of

virological failure are usually confounded by changes made in response to the earlier plasma HIV-1 RNA changes.

#### *Genotypic mechanisms of resistance in vivo*

It has become increasingly difficult to identify the primary mutations responsible for the virological failure of new drugs and drug combinations. Most recently approved drugs are used either for salvage therapy or as part of highly effective initial HAART regimens. Virological failure during salvage therapy results from mutations that confer cross-resistance to a new drug but these mutations are not necessarily those that would occur had the new drug been the first drug of its class administered to a patient. In contrast, most virological failures on very effective initial HAART regimens result from non-adherence or drug toxicity and are usually associated only with the emergence of resistance to those drugs with the lowest genetic barriers to resistance, such as the NNRTIs or lamivudine and emtricitabine. Therefore, data on the primary mechanisms of resistance to a new drug will accrue slowly and post-approval surveillance will be necessary to obtain a complete understanding of the mutations selected *in vivo* by a new drug or drug combination.

#### *Genotypic and phenotypic predictors of virological response*

The second goal of clinical studies is to identify the genotypic and phenotypic predictors of response to a new treatment regimen. In these studies a new drug is used as part of a salvage therapy regimen and the pre-therapy genotype is correlated with the virus loads obtained following the change in therapy. Because salvage therapy fails more commonly than initial therapy, it is possible to obtain many correlations between genotype and clinical outcome. However, it is difficult to obtain robust conclusions because of the complexity of clinical genotypic (and to a lesser extent) phenotypic data and because the virological response to a new treatment regimen is confounded by additional parameters including drugs received in the past, the drugs with which the new drug is combined, and the baseline RNA level and CD4 counts. Although adherence is also a confounder, adherence data are not usually available.

The complete treatment history must be included in the model because the baseline genotype may not contain mutations that were selected by an earlier discontinued regimen even though such mutations often interfere with the response to a new regimen. The drugs used in combination with a new drug regimen must also be included in a predictive model because a new drug is likely to be more effective when combined with other new active drugs. This has made 'intensification' studies—the addition of a single drug to a failing regimen—a popular method for limiting the complexity of models for predicting clinical outcome. However, intensification is recommended in only a few clinical situations and it is uncertain whether the data from these studies can be extrapolated to studies in which complete regimens are changed.

Different clinical studies also often examine different virological end points, such as the decrease in virus load or the proportion of persons with undetectable viraemia by week 4, 12, 24, or 48. Whereas short-term virological suppression results from the intrinsic inhibition of the most common virus variants within an individual, long-term virological suppression results from the inhibition of the whole virus population within an individual



and the regimen's ability to prevent the emergence of new resistant viruses.

More than 20 studies have been performed in which pre-therapy genotype is correlated with the virological response to a new treatment regimen. Most of these studies have been severely underpowered containing about 50–100 patients each. A summary of these studies can be found in the 'Genotype-Clinical Outcome' section of the Stanford HIV Drug Resistance Database.<sup>15</sup> Most of these studies have used multivariate analysis to develop one or more heuristic approaches or rules for interpreting genotypic resistance tests. For example, the presence of three thymidine-associated mutations and the mutation M184V have been associated with little virological response to abacavir intensification.<sup>16</sup> Or the presence of six or more mutations from a list of 11 mutations has been associated with a reduction in the proportion of patients responding to salvage with lopinavir.<sup>17</sup>

### Conclusions

Drug resistance testing is an important factor to be considered when prioritizing the development of new antiretroviral drugs. Although *in vitro* passage experiments do not lend themselves to standardization, they are a necessary step in drug development and clinicians need to be able to interpret the results of these studies in light of the caveats noted above. The choice of drug susceptibility tests for quantifying drug resistance and of isolates for measuring cross-resistance, however, can benefit from increased standardization. Pre-clinical drug resistance data are important not just for deciding what type of clinical trials to conduct; if a new drug is approved, they are essential for the optimal use of genotypic resistance testing done for patient management.

Analyses that correlate between genotype and clinical outcome are complicated but essential. These studies have often been performed during both pre- and post-drug approval. Most studies are based on small numbers of patients and yield results that require validation in larger studies. Therefore, despite the excellent guidelines for the initial treatment of HIV, guidelines for treating persons who have failed an initial regimen due to drug resistance or who have been primarily infected with a drug-resistant strain are vague and somewhat contradictory.<sup>18</sup> Increasing numbers of correlations between genotype and phenotype and response to a new treatment regimen are also required for the optimal use of new drugs following approval and widespread use.

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