Interpreting Resistance Data for HIV-1 Therapy Management – Know the Limitations

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Abstract

Antiviral drug resistance can be one of the causes for HIV-1 therapy failure. Several studies have shown some beneficial effect of antiviral resistance testing on response to the following therapy regimen. The technical performances of genotypic and phenotypic assays have been considerably improved with time. However, both are still limited in their power to fully map antiviral resistance as cause of therapy failure. Nevertheless, the results of genotypic and phenotypic assays can often deliver complementary information. The greatest challenge still remains in the accurate interpretation of in vitro resistance results, whether genotypic or phenotypic, into information that can be implemented into clinical practice. For phenotypic assays, bioinformatics analyses linking fold-resistance values and clinical response data have been performed, or are currently ongoing, to assign clinical cut-offs for all drugs. Genotypic drug-resistance interpretation systems have been developed and are continuously being updated to solve the same problem. Retrospective and prospective studies have compared systems in their performance to predict phenotype or in their ability to predict therapy outcome. All these analysis are of value, but still display some weak points. However, due to the fast evolving know-how in the field, a prospective trial in which different systems are compared head-to-head will be difficult to design. (AIDS Reviews 2006;8:37-43)

Key words


Introduction to HAART and antiviral resistance

Combinations of antiviral drugs are used for the treatment of HIV-1 infection. They are called highly active antiretroviral therapy (HAART) and generally comprise three drugs. The aim of HAART is to prevent clinical progression. The introduction of HAART in the mid-1990s resulted in a decrease of the morbidity and mortality in the HIV-1 patient population. Nevertheless, therapy failure still occurs. The markers for therapy failure, ultimately resulting in clinical progression, are decreasing CD4 cell count and rising viral load. As changes in viral load mostly precede changes in CD4 count, the determination of viral load is the most relevant laboratory procedure to monitor the short-term in vivo activity of a therapy given to an individual patient. Incomplete adherence to therapy, suboptimal therapy potency, suboptimal pharmacokinetics, and pre-existing drug resistance can be factors that result in rising viral load and therapy failure.

The evolution of resistance against HIV-1 drugs within a patient depends on the generation of genetic variation and on the selection of drug-resistant variants during antiretroviral therapy. The high HIV-1 genetic variability is caused by the error-prone nature of HIV-1 reverse transcriptase, the absence of any enzymatic proofreading activity, and the huge rate of HIV-1 replication in vivo. Some of the genetic variants created will result in alterations to the structure and function of the
molecules targeted by the antiviral drugs. These alterations could confer changes in susceptibility to one or more of these drugs. In the presence of therapy, the variants with some level of resistance will gradually overcome the wild-type variants. Under the continuous selective pressure of drugs and with the presence of residual replication due to insufficient potency of the treatment, additional mutations will accumulate.

Thus, the main goal of HAART is to reduce the viral load as much as possible to prevent further clinical progression. However, its immediate goal is to reduce the viral load to prevent the formation of variants that result in antiviral resistance to the current therapy and that, because of the phenomenon of cross-resistance within drug classes, ultimately might lead to a limitation or even lack of future treatment options.

**Resistance assays and their position within clinical practice**

Assessment of antiviral resistance can be done by phenotypic and genotypic assays. In the early days, resistance assays were performed solely to diagnose antiviral resistance upon therapy failure. Gradually the idea arose to use this information to guide subsequent therapy changes. A number of prospective studies showed a short-term, modest, beneficial effect of antiviral resistance testing on response to the subsequent regimen. These data resulted in the recommendation of resistance testing in the management of HIV-1 therapy.

However, when implementing the results of resistance assays into clinical decision making, one has to be sure that the obtained information is accurate and also be aware of the limitations of these assays.

Both phenotypic and genotypic assays rely on the amplification of the target gene by polymerase chain reaction (PCR), which makes the assays highly susceptible to contamination from its own product. However, the incorporation of good laboratory practices can overcome this problem.

HIV-1 displays an enormous genetic diversity that is reflected at the population level by the presence of nine subtypes, 20 circulating recombinant forms (CRF) and a multitude of unique recombinant forms (URF) and at the individual level by the presence of an enormous number of quasispecies within one patient. Quasispecies differ from each other by substitutions, deletions and insertions. Both assays rely on the hybridization between primers and template for the generation of amplification product and sequences. Minor differences between primers and target sequence might lead to negative results or unrepresentative selection of particular quasispecies from the total viral population. The amplification of quasispecies with insertions and/or deletions of different length results in shifts within the fluorograms when performing population sequencing and generates non-interpretable data. In particular the envelope, the target for enfuvirtide and CCR5 antagonists, challenges researchers in their goal to develop assays that display a good performance for all patient isolates.

To understand the observed therapy failure, it is highly important to monitor the resistance pattern of the active replicating viral population in plasma samples. Results of resistance assays represent only the genotypic or phenotypic profile of the majority of quasispecies present in vivo. They have difficulty detecting minor variants that reflect less than 25% of the total viral population. If the sample is collected without the presence of selective pressure, the chances are high that wild-type will be detected instead of the mutant (Venturi, et al. 2002). Mutant viruses often display a reduced replication capacity in comparison to wild-type and therefore they are rapidly overcome by the wild-type. However, they remain as minor variants and can rapidly reemerge in a subsequent therapy causing therapy failure. When no selective pressure is present, reversal of resistance can also occur. It occurs however at a much lower frequency and much slower rate than the reemergence of wild-type. Therefore, resistance testing has to be performed before starting, stopping, or changing therapy. Resistance assays are most accurate in determining resistance to the current therapy combination. Not detecting resistance to any previously used drug does not guarantee complete drug susceptibility. If a previous isolate from a patient has been scored as resistant to a particular drug, these resistant variants can still exist as minor variants, or can be archived in latently infected cells, and the response might be limited when that drug is reused. So resistance testing is no substitute for good clinical judgment based on combined therapy and viral load histories.

**Interpretation of resistance assays**

Phenotypic assays measure the in vitro viral replication in the presence of different drug concentrations and determine the concentration of drug required to inhibit the replication of the patient’s virus by 50% (IC\textsubscript{50}). Although the IC\textsubscript{50} resembles better the requested in vivo effect, the IC\textsubscript{90} is a more reliable indicator as it is measured at the steepest part of the dose-re-
response curve. A small change in response has no dramatic impact on the IC$_{50}$ value, whereas it does when the 90% measurement is used. The final result of a phenotypic assay is a factor that compares the IC$_{50}$ of the patient's virus to the IC$_{50}$ measured for a wild-type reference strain (fold-resistance). It is a continuous variable and it is in agreement with the drug resistance continuum in vivo as resistance is not an all-or-nothing phenomenon.

However, a clinician has to decide whether or not to include a certain drug and therefore this variable is currently categorized into a more workable final output, e.g. sensitive/reduced susceptibility (PhenoSense®M, Monogram Biosciences, San Francisco, USA) or within/above the normal susceptibility range (Antivirogram®, Virco, Mechelen, Belgium). Initially, arbitrary cut-offs were used, reflecting the reproducibility range of the test. This was recognized to be inadequate and therefore biological cut-offs were determined. These cut-offs reflect the range in susceptibility to each individual drug observed in therapy-naive patients. Subsequently, clinical cut-offs have been determined for some drugs. They are based on the distinction between fold-resistance levels for which a reduced virologic response is seen and fold-resistance levels for which no or almost no virologic response is observed. The clinical cut-offs for the two commercial assays can be found at their respective websites – www.monogramhiv.com and www.vircolab.com. Bearing in mind the drug resistance continuum, it is hypothesized that a continuous resistance score might even improve the prediction of therapy outcome. A recent retrospective study showed that incremental phenotypic drug susceptibility scores (PSS) more accurately predicted the virologic and immunologic outcome in patients starting salvage therapy as compared to dichotomous PSS. Continuous PSS were also better than dichotomous PSS, but not in comparison to incremental PSS.

Genotypic assays determine the nucleotide sequence of the viral gene that is targeted by the drugs within the therapy combination. The nucleotide sequence is subsequently translated into an amino acid sequence and compared to a reference wild-type strain to generate a mutation list. These mutations are then compared with a list of "resistance-associated" mutations. Mutations are defined as resistance-associated (a) when they are known to be selected in vitro or in vivo in the presence of a certain drug, (b) when they have been associated with a reduction in phenotypic susceptibility, or (c) when they have been associated with a reduced clinical response. This implies that the result of a genotypic assay always depends on pre-existing knowledge.

Patients are currently treated with combination therapies that change in time and that result in complex patterns of mutations.

Some mutations that are developed under the selective pressure of a particular drug can cause cross-resistance towards other drugs from the same class without any previous experience. Already early on it was realized that mutations selected by the nonnucleoside reverse transcriptase inhibitor (NNRTI) nevirapine rendered viruses highly resistant to all NNRTI. However, viruses with a limited number of certain mutations retained some susceptibility to efavirenz. The rare multi-nucleoside resistance pathway A62V, S68G, V75I, F77L, F116Y, Q151M was first observed in 1993. This resistance pattern was observed in patients receiving sequential monotherapy or bi-therapy with nucleoside reverse transcriptase inhibitors (NRTI). It confers intermediate to high-level resistance to many NRTI. The reverse transcriptase mutations M41L, D67N, K70R, T210W, T215YF, and K219QE were first ascribed to the selective pressure of zidovudine. Afterwards it was shown that stavudine could also select for this resistance pathway and therefore this particular set of mutations was assigned as thymidine-associated mutations (TAM). TAMs, whether or not associated with an insertion at position 69 in the RT gene, are associated with a reduced phenotypic susceptibility and a reduced clinical response to many NRTI. A study based upon a subset of patients of EuroSIDA confirmed the presence of two evolutionary pathways, i.e. TAM1 and TAM2 profiles characterized by M41L, L210W, T215Y and D67N, K70R, K219QE, respectively. Stavudine appeared to retain a greater viral activity than zidovudine in patients carrying viruses displaying the TAM2 profile. In patients exposed to abacavir and part of the NARVAL trial, the strongest association between the decrease in viral load and the number of mutations was observed with a set of six mutations, of which four were at TAM positions (41, 67, 210 and 215) and two at other RT positions (74 and 184). Retrospective analysis of the trials GS-99-907 and GS-98-902 showed that response to tenofovir was reduced among patients with ≥ 3 TAM inclusive of either M41L or L210W. Slightly increased therapy responses were observed when M184V was present. The Jaguar trial was used to define a genetic score for didanosine. Eight mutations were associated with a reduced response to didanosine (M41L, D67N, T69D, L74V, V118I, L210W, T215YF and K219QE) and two mutations with...
a better response (K70R and M184VI)\textsuperscript{28}. Despite the association of particular signature mutations to each protease inhibitor (PI), a broad level of cross-resistance exists within the class of PI. In general, the level of phenotypic resistance or the level of reduced clinical response correlates to the number of PI mutations\textsuperscript{29}.

Resistance antagonism is defined as the resensitization to one drug through the selection of a particular mutation by another drug within an existing resistance pattern that originally rendered the virus resistant to the first drug. A well-known example of this resistance mechanism is the M184VI mutation that confers high-level resistance to lamivudine and emtricitabine and that, in a background of a limited number of TAM, reverses the phenotypic resistance to zidovudine, stavudine and tenofovir\textsuperscript{30}. Similar findings have been observed \textit{in vitro} for the interactions between respectively K65R, L74V, L100I or Y181C and TAMs\textsuperscript{31}. The clinical relevance of the latter re-silencing patterns is still unclear.

Hyper-susceptibility to NNRTI has been observed for samples displaying multiple NRTI mutations and isolated from patients naive for NNRTI. The hyper-susceptibility was associated with an improved clinical outcome to efavirenz-based therapy\textsuperscript{32}. A similar phenomenon was observed with the L50I mutation within protease. Viruses displaying this mutation were selected in naive patients starting atazanavir-containing therapy and they had increased susceptibility to other PI\textsuperscript{33}.

Genotypic resistance assays are more widely used than phenotypic assays. They can be more easily implemented in regular molecular biology facilities, which is not the case for phenotypic assays. Therefore, genotyping is mostly performed locally, whereas phenotyping is commonly put out to commercial companies (Monogram Biosciences and Virco). Nevertheless, these and other companies can also supply genotypic results and interpretations. The virologists that are responsible for the testing should convert the generated mutation list into advice for the clinician. However, due to the complex nature of resistance patterns, it is not always possible to be kept informed of the latest discoveries. Therefore, most of them rely on genotypic drug-resistance interpretation systems that are updated at regular intervals\textsuperscript{34}. The commercial genotypic assays are concerted with an interpretation system (TRUGENE\textsuperscript{®} HIV-1 Genotyping System, Bayer HealthCare LLC Diagnostic Division, NY, USA; and ViroSeq\textsuperscript{™} HIV-1 Genotyping System, Celera Diagnostics, CA, USA). However, sequences can also be submitted to commercial interpretation systems (virco\textsuperscript{®}TYPE, Virco; and ViroScore, ABL, Luxembourg) or publicly available systems (geno2pheno\textsuperscript{35}; ANRS, HIVdb\textsuperscript{36}; Rega\textsuperscript{37}). Their goal is to translate complex patterns of resistance-associated mutations into a definite (e.g. susceptible and resistant) or continuous (fold-change) variable that can be more easily implemented in clinical practice. These systems can be based on Bayesian rules (e.g. ANRS and Rega), on mutation tables with additive and/or subtracting scoring (e.g. HIVdb), on bio-informatics' analysis (e.g. geno2pheno), or combinations thereof (virco\textsuperscript{®}TYPE HIV-1, GeneSeqHIV™ and AntiRetroScan)\textsuperscript{38}.

### The complementary nature of genotypic and phenotypic resistance assays

Phenotypic resistance assays give a direct measurement of susceptibility towards the tested inhibitors that includes the effect of all mutations and their interactions. In this manner, they are ideal assays for testing the susceptibility towards new antiviral drugs for which hardly any genotypic data is yet available (e.g. tipranavir and TMC114), for testing samples from patients who failed multiple therapies, and for testing samples with atypical (e.g. V106M) or less prevalent (e.g. K103S/T/H) mutations\textsuperscript{39,40}. Before the publication of these manuscripts, these mutations were not considered as resistance-associated in genotypic assays.

Before the mechanism clarifying the antagonistic effect of M184V/I within the background of TAMs was known, it was already measured by phenotypic assays\textsuperscript{41}. This reversal in phenotypic resistance is reflected by a partial virologic response\textsuperscript{42}. The interaction between TAMs and K103N results in the reversal of phenotypic resistance to efavirenz. However, it is not reflected by a change in virologic response. Patients displaying this pattern of mutations still fail efavirenz-containing therapy\textsuperscript{32}. Genotypic assays can make a distinction based upon the detection of the mutations whereas phenotypic assays cannot.

The current phenotypic assays have difficulty in detecting resistance to some NRTI, especially when associated with TAMs. In a comparative study, PhenoSense\textsuperscript{™} was more precise than Antivirogram\textsuperscript{®} and was more likely to detect resistance to abacavir, didanosine and stavudine in patient strains displaying M41L, M184V and T215Y (± L210W)\textsuperscript{43}. NRTI compete with the natural deoxynucleotide-triphosphates (dNTP) for binding to the reverse transcriptase and incorporation into the growing DNA chain. The phenotypic assays use activated cells with high intracellular dNTP pools, which leads to the generation of dead-end complexes at low NRTI concent-
The predictions of phenotypic susceptibilities by genotypic drug-resistance interpretation systems have been investigated in a few studies. The linear model developed by Wang, et al. in 2004 outperformed existing genotypic drug resistance interpretation systems (HIVdb, TRUGENE, ANRS, Rega and geno2pheno) in their prediction of the phenotype. Ross, et al. investigated the agreement between phenotypic results and the interpretation according to seven interpretation systems (CHL, HIVdb, ANRS, Rega, DMC, GAV and TRUGENE) for two subsets of patient populations. In the first analysis, 206 samples from PI-experienced patients were analyzed. Good or excellent agreement ($κ ≥ 0.40$) was observed for ritonavir, indinavir, saquinavir and nelfinavir. Poor agreement ($κ < 0.40$) was obtained with four of the seven systems for amprenavir and with four or three of the seven for lopinavir, when applying the biological cut-off of 2.5-fold or the clinical cut-off of 10-fold, respectively. In the second analysis, 70 therapy experienced patients were investigated. The overall percentage concordance was above 80% for all of the interpretation systems, except for GAV. When evaluating individual drugs, concordance was very good (above 80%) for lamivudine, zidovudine, stavudine, nelfinavir, ritonavir, amprenavir, indinavir, saquinavir, efavirenz and nevirapine. There was less concordance for abacavir, didanosine and zalcitabine.

The variability in prediction of therapy outcome by different systems was investigated in 261 therapy-failing patients who subsequently were changed to a new regimen. The association of the genotypic susceptibility score (GSS) of the new regimen, calculated according to 11 interpretation systems, with the virologic outcome for three and six months was examined. Only three systems (ANRS, Rega and TRUGENE) showed significant prediction of the three-month response. The six-month response was predicted by four systems (HIVdb, TRUGENE, Retrogram and HIVResistanceWeb). The same investigators performed a similar analysis in 415 recently infected, therapy-naive patients. Of the patients, 10% had at least one major resistance-associated mutation due to transmitted drug resistance. In multivariate analysis, the GSS from two systems significantly predicted the time to virologic success (Rega and HIVResistanceWeb) and three systems showed a trend towards a significant prediction of success (Retrogram, Menéndez and HIVdb).

The RADAR study, in which two interpretation systems were prospectively compared, could not demonstrate major differences in the virologic outcome between patients for whom there was access to the rule-based in-
interpretation system TRUGENE or to VirtualPhenotype\textsuperscript{23}. However, in this study, the final interpretation was not performed by the treating physicians, but by experts, and the authors hypothesized that possible differences in drug prescription based upon both systems might have been minimized due to the expert advice as resistance mutations were also available on the Virtual-Phenotype report.

Conclusions

A number of studies have shown a short-term, modest, beneficial effect of antiviral resistance testing on the response to a subsequent regimen. The contribution of resistance testing to HIV-1 therapy management could be improved by increasing the assay’s sensitivity for the detection of minor resistant variants. However, the major challenge in gaining predictive power remains the correct interpretation of resistance. Retrospective comparisons may guide in defining the most relevant cut-offs and the most predictive mutational patterns for each drug. However, it will be difficult to draw any final conclusions from these studies as the original outputs of the interpretation systems were often re-interpreted into new variables for comparison purposes and different end-points and statistical analyses were used. Additionally, due to differences in prevalence of certain mutations and the complexity of mutational patterns and subscribed drug combinations, it will not be possible to validate all drug rules. Current retrospective comparisons will always suffer from the fact that genotypic resistance guided the choice of therapy change, possibly even by one of the systems analyzed. Prospective studies would theoretically not suffer from this bias. However, as clinicians get more and more acquainted with resistance mutations, it might be difficult to measure the independent value of interpretation systems. Additionally, as HIV research is a fast evolving field where interpretation systems constantly are upgraded as soon as new information and new drugs become available, it will be virtually impossible to design a prospective randomized trial in which different systems will be compared head-to-head.

References


