HIV Resistance Testing: an Update

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Abstract. In recent years, resistance testing has become an important tool in optimizing the combination therapy for treating HIV infected individuals. The identification of resistance mutations has allowed physicians to select the antiviral agents with maximum therapeutic benefic and minimum toxic side effects. The current therapeutic agents approved by the Food and Drug Administration (FDA), their mechanisms of actions, and the mutations of the HIV viral genome that lead to resistance to antiviral agents are discussed. In addition, methods of resistance testing, both genotypic and phenotypic, are evaluated with consideration of their inherent advantages and disadvantages. (received 28 February 2002; accepted 5 April 2002)

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Introduction

During the early 1990s, the outcome of controlled trails using combination therapy revealed that sustained reduction of viral replication in an HIV infected patient is associated with improved clinical outcome [1-3]. The introduction in 1996 of a new class of antiviral agents, protease inhibitors provided the means to suppress HIV replication to levels that are non-detectable using the most sensitive assays (<50 particles/ml) [4-6]. Today, use of combination therapy that includes a protease inhibitor is believed to have significantly reduced HIV morbidity and mortality [7-10].

The antiviral agents interfere with various stages of the viral replication cycle and prevent additional viral expression by infected cells. Currently, there are 16 antiviral agents, including 6 nucleoside reverse transcriptase inhibitors (NRTI), 3 non-nucleoside reverse transcriptase inhibitors (NNRTI), and 6 protease inhibitors that have been approved by the FDA (Table 1). In addition, several new antiviral agents are in the late stages of clinical trials, including T20, which belongs to a new class of antiviral agents (fusion inhibitors).

Nucleoside reverse transcription inhibitors

NRTIs are analogs of the natural cellular nucleosides that lack the 3'-hydroxyl group. Once phosphorylated, they become incorporated into a growing HIV DNA molecule and form a dead-end that prevents further DNA synthesis [11]. Resistant viruses develop mutations that either discriminate against NRTI and prevent their addition to the DNA chain, or alternatively increase the hydrolytic removal of the drug (pyrophosphorolysis), enabling continued DNA synthesis [12,13]. Currently there are 7 FDA approved NRTIs: Zidovudine (AZT), Didanosine (DDI), Zalcitabine (DDC), Lamivudine (3TC), Stavudine (d4T), Abacavir (ABC), and Tenofovir (TDF), that are used to treat HIV infection. These agents are selected on the basis of their selective inhibition of HIV-1 RT with minimal inhibition of cellular polymerases.

Nonnucleoside reverse transcription inhibitors

Delavirdine (DLV), Efavirenz (EFV), and Nevirapine (NEV) are the 3 FDA approved NNRTIs. They are structurally diverse compounds that inactivate RT by irreversibly binding to a hydrophobic pocket of the enzyme. A single mutation in this pocket often results in high-level resistance to one or more NNRTIs. Although these compounds are potent
inhibitors of HIV replication, when they are used alone or in combination with a weak regimen, they rapidly select for mutants within 2 to 3 weeks. The resistant viruses have one or more mutations in the NNRTI-binding pocket. Resistance to one NNRTI is usually associated with cross-resistance to all other NNRTIs [12,13].

**Protease inhibitors**

The HIV protease enzyme is composed of 2 identical 99 amino acid monomers that are associated noncovalently. These monomers form a substrate cleft that recognizes and processes gag and gag-pol gene polyproteins. Protease inhibitors inactivate this enzyme by irreversibly binding to the enzyme substrate cleft and preventing synthesis of mature infectious virus particles [12,13]. Currently, there are 6 FDA approved HIV protease inhibitors, Amprinavir (AMV), Nelfinavir (NFV), Ritonavir (RTV), Saquinavir (SQV), Lopinavir (LPV), and Indinavir (IDV).

**HIV resistance**

HIV resistance has been reported for all classes of antiretroviral agents [14-18]. Several factors are believed to lead to the drug failure, including host immune failure, drug resistance mutations, sub-inhibitory drug levels (due to incomplete adherence, poor absorption, rapid clearance), and drug-drug interactions. These factors lead to increased frequency of viral replication, coupled with mutations that occur during each replication cycle, and a lack of RT proofreading mechanisms, resulting in the emergence of drug-resistant viruses [19].

### Methods of HIV resistance testing

**Phenotypic resistance assays.** Traditional phenotypic resistance assays relied on isolation and resistance testing of the HIV virus harbored by an infected individual. The assay measured the extent to which a drug inhibited HIV p24 antigen production by peripheral blood mononuclear cells (PBMC) that are acutely infected with the patient’s isolate. The test is performed in a 96-well plate; HIV p24 antigen is quantified and the 50% inhibitory concentration (IC50) of the drug is determined. This assay is laborious and time consuming. Moreover, the isolation of infectious virus from a patient with low viral load is virtually impossible.

Two companies (ViroLogic, San Francisco, CA, and Virco, Mechelen, Belgium) have recently developed recombinant DNA technology-based phenotypic assays that are relatively rapid and sensitive [20,21]. These assays employ PCR amplification of viral protease and RT gene sequences in lieu of viral isolation. The ViroLogic PhenoSense test relies on cloning and expressing the amplified viral RNA in an HIV–1 vector that contains a luciferase reporter gene in place of the viral envelope gene. Replication of the recombinant virus in the presence of antiviral agent is monitored by the amount of expressed luciferase. The data are analyzed by plotting the % inhibition of replication of the patient’s virus and a drug-sensitive reference virus, measured by luciferase activity, versus the log10 concentration of drug. The drug susceptibility curve is used to calculate the concentration of drug required to inhibit viral replication by 50% (IC50).

<p>| Table 1. List of FDA approved antiviral agents |</p>
<table>
<thead>
<tr>
<th>Class</th>
<th>Antiviral Agents</th>
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<tbody>
<tr>
<td>Nucleoside reverse transcriptase inhibitors</td>
<td>AZT, DDI, DDC, 3TC, D4T, ABC, and TDF</td>
</tr>
<tr>
<td>Non-nucleoside reverse transcriptase inhibitors</td>
<td>DLV, EFV, NEV</td>
</tr>
<tr>
<td>Protease inhibitors</td>
<td>AMV, NFV, RTV, SQV, LPV, IDV</td>
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<p>| Table 2. Methods of HIV resistance testing |</p>
<table>
<thead>
<tr>
<th>Phenotypic resistance assays</th>
<th>Genotypic resistance assays</th>
</tr>
</thead>
<tbody>
<tr>
<td>p24 antigen quantitation</td>
<td>TrueGene (Visible Genetics)</td>
</tr>
<tr>
<td>PhenoSense (ViroLogic)</td>
<td>ViroSeq (Applied Biosystems)</td>
</tr>
<tr>
<td>Antivirogram (Virco)</td>
<td>GeneChip (Affimetrix)</td>
</tr>
<tr>
<td>LiPA (Innogenetics)</td>
<td>user developed</td>
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A shift in the patient’s viral inhibition curve toward a higher drug concentration, compared to the curve of the drug-sensitive reference virus, is interpreted as reduced drug susceptibility [39].

Similarly, the Virco Antivirogram test relies on amplified viral protease and RT genes. However, a mixture of PCR products and protease- and RT-deleted HIV-1 proviral clone is transfected into a CD4+ reporter cell line. The cells produce recombinant virus containing the patient’s viral PR-RT sequence. Susceptibility of the resulting chimeric virus is tested in the reporter cell line using real time monitoring of HIV replication by high-resolution optics [20]. Both of these phenotypic assays provide readily interpreted results (ie, resistant, susceptible, or intermediate) for each tested antiviral agent.

**Genotypic resistance assays.** The genotypic resistance assays detect specific mutations in the viral genome that are associated with resistance to various antiretroviral agents. Currently there are 4 commercial genotypic resistance assays: TruGene (Visible Genetics Inc., Toronto, Canada), ViroSeq HIV-1 genotyping (Applied Biosystems, Foster City, CA), GeneChip (Affymetrix Inc., Santa Clara, CA), and LiPA (Innogenetics, Alpharetta, GA). The initial step in these assays is PCR amplification of viral protease and a 250 to 350 codon segment of the HIV RT gene. This is followed by direct sequencing of the amplified products (TruGene, ViroSeq HIV-1 genotyping, GeneChip) or by hybridization based detection of selected RT codons (41, 69, 70, 74, 184, 214, 215) and protease codons (30, 46, 48, 50, 54, 82, 84, 90) that are coated as discrete lines on a nitrocellulose membrane in a strip format [22]. Amplified biotinylated DNA material, if present, hybridizes with the specific probes. After hybridization, streptavidin labeled with alkaline phosphatase is added. The streptavidin becomes bound to any biotinylated hybrid previously formed, which allows for detection by the phosphatase reaction following incubation with a chromogen [22].

In addition to the commercial kits, many laboratories have developed “home brew” assays that use PCR methodology to amplify HIV protease and varying segments of RT gene, followed by dideoxyterminator sequencing of the amplification products. Several investigators have evaluated these assays and compared the genotypic findings to the clinical outcomes [22-30]. A “home brew” assay, if performed properly, is the most cost-effective means of HIV genotyping. The LiPA assay requires a minimum initial investment but is limited by the number of capture probes on the mutation detection strip. DNA chip technology, while promising, lacks the flexibility to detect all HIV subtypes and genotypes. Baring mutation on the primer binding site, or loss of viral particles during collection, transport, or storage, the apparent sensitivity of most genotypic assays range from 500 to 1000 viral particles/ml [19]. In addition, while the relationship between phenotypic and genotypic analyses of resistance is often direct, this relationship can be altered in some cases, since emergence of resistance is a dynamic process, and multiple strains of virus with various susceptibilities frequently coexist in a patient. In general, these assays detect the mutations possessed by the most prevalent genotype in a patient’s sample. Therefore, the absence of resistance by genotypic or phenotypic assays does not rule out the presence of a reservoir of resistant viruses that cannot be detected by these assays [19].

While technical expertise is the most challenging aspect of phenotypic resistance testing, correctly interpreting the identified mutations and assessing their impact on drug resistance are the most challenging aspects of the genotypic assays. In addition, only small proportions of the HIV-1 RT and protease sequences are publicly available and there is no standard way to relate these sequences to other data (eg, drug treatment history, in vitro drug susceptibility, and clinical outcome). Several drug manufacturers and investigators (notably Robert Shaffer of Stanford University) have developed internet-based websites with large databases that have partially alleviated this problem [12,13]. These frequently updated websites include the most recently published data.

**Virtual phenotyping**

The VirtualPhenotype genotypic assay, marketed by Virco Laboratory, uses a database to predict phenotypic resistance from the genotype. Viral protease
and a 400 kbase segment of RT gene from the patient are sequenced and the sequences are compared to all the sequences in Virco’s database. Previous samples with similar patterns of resistance mutations are identified. Phenotypes for these matching samples are then retrieved from the database and an mean resistance figure (change of IC50) is calculated for each drug. As yet, there has been no independent study to show that the VirtualPhenotype assay provides any advantages over properly performed and interpreted genotypic assays.

Mutations with resistance to NRT inhibitors

The most common mutations detected in clinical HIV-1 samples from patients receiving AZT are various combinations of the following mutations that occur at codons M41L, D67N, 70, L210W, T215Y/F, and K 219Q [31-33]. These mutations may also confer resistance to other nucleoside RT inhibitors, including d4T, ddI, and ABC.

M184V is the most common mutation detected in patients treated with 3TC. This mutation causes high-level resistance to 3TC [34,35]. It may also cause low level resistance to ABC [35,36], ddI, and ddC [21,37-39]. M184V combined with multiple AZT resistance or combined with mutations at positions K65R, L74V, or Y115F leads to ABC resistance [40,41]. Several studies have shown that M184V mutation partially reverses T215Y-mediated AZT resistance [34,42], which may explain the slow evolution of AZT resistance in patients receiving AZT plus 3TC [43,44]. However, it can be overcome by the presence of ≥4 AZT resistance mutations [35,42].

T69D causes low-level resistance to Zalcitibine [33]. However, the presence of any inserted codon at this point will cause low-level resistance to each of the NRTIs. In addition isolates containing insertions together with T215Y/F and other AZT-resistance mutations have high-level resistance to each of the NRTIs [47].

Mutation at codon 74 (L74V) confers low-level resistance to ddI and ABC; similarly, M184V may reverse AZT resistance [14,36,40].

Mutation at codon 151 (Q151M) by itself causes intermediate levels resistance to AZT, ddI, d4T, and ABC [23,47]. The Q151M mutation combined with mutations at positions V75I, F77L, F116Y, shows high-level resistance to AZT, DDI, d4T, and ABC and low-level resistance to 3TC [12].

Mutations with resistance to NNRT inhibitors

Several mutations of the RT gene lead to resistance to NNRTIs. Mutation at codon 103 (K103N) and 230 (M230L) causes resistance to available NNRTIs [21,47-49]. Mutation at codon Y181C/I causes resistance to NVP and DVL and low level resistance to EFV [21, 49-51]. Y188C/L/H causes high-level resistance to each PI and intermediate resistance to DVL [21,49,51,52]. G190A/S causes high-level resistance to NVP and EFV but does not cause in vitro resistance to DVL [21,52].

Mutations with resistance to protease inhibitors

Several mutations lead to resistance to currently available protease inhibitors (PI). These mutations frequently lead to cross-resistance to one or more additional antiviral agents. For example, mutation at codon 82 causes resistance to IDV, RTV, and lopinavir [53].

Mutation at codon 84 (I84V) has been reported in patients receiving IDV, RTV, SQV, and APV [16,40,54-57] and causes phenotypic and clinical resistance to each PI. D30N occurs only in patients receiving NFV and confers no in vitro or clinical cross-resistance to the other PIs [5,57-59]. I50V has been reported only in patients receiving APV. In addition to causing reduced APV susceptibility, I50V mutation causes low-level RTV resistance [60].

Although these mutations may individually provide adequate information about each drug, the eventual resistance or susceptibility of a virus to a given drug is determined by the overall effect of all the accumulated mutations. For example mutation in codon 184 causes resistance to 3TC, however the same mutation may negate a mutation at codon 215 that cases AZT resistance.

Shafer et al [12,13] have developed a mutation-scoring scheme that assigns a numerical value for each mutation versus a given drug. These values are added and interpreted as follows:

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If the total value for all the mutations versus a drug is (0-9), the virus is considered susceptible to that drug.

A total value of (10-14) is indicative of potential low-level resistance; virus isolates of this type have mutations that by themselves may not cause drug resistance, yet indicate the possibility of previous drug selection.

A total value of (15-29) is reported as low-level resistance; virus isolates of this type have reduced in vitro susceptibility to the drug and patients with viruses of this genotype may have suboptimal response to treatment.

A total value of (30-59) is reported as intermediate resistance; this virus genotype suggests a degree of drug resistance greater than low-level resistance but lower than high-level resistance.

A total value of (>60) is reported as high-level resistance; this genotype is similar to isolates with the highest levels of in vitro drug resistance and patients infected with such isolates generally have little or no virologic response to the regimen [12].

Conclusions

Preventing or counteracting the emergence of drug-resistance to antiviral agents is the goal of HAART therapy. Emerging data suggest that antiretroviral resistance testing will play an important role in the clinical management of HIV disease. Recent studies by Little et al [61,62] indicate that a significant number of newly infected HIV patients harbor viruses that are resistant to at least one drug; a small yet significant proportion of these patients harbor viruses resistant to all available antiviral agents. In 2000, the International AIDS society-USA panel recommended the use of resistance testing to aid in the choice of new regimens after treatment failure and to guide therapy for pregnant women. The panel also recommended that drug resistance testing be considered prior to initiating therapy in patients with acute HIV infection [19]. Chlaix et al [63] reported that the cost of drug resistance testing in their patients was offset by reduced use of protease inhibitors. Weinstein et al [29] concluded that genotyping following antiviral failure is cost effective and that primary resistance testing may become reasonable as the prevalence of primary resistance increases. In addition, as the number of newly developed antiviral agents with potentially reduced side-effects increases, resistance testing may be helpful in identifying specific drugs in a combination regimen that is failing. The test may also be used to assess the transmission of resistant virus in an acute infection. Since treatment failure can be caused by factors other than drug resistance, resistance testing can assist in evaluating patient compliance with a therapeutic regimen and assessing other pharmacological issues that may influence HIV replication.

References


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