Evaluation and Management of Virologic Failure

Introduction

Development of Antiretroviral Drug Resistance

Among persons with HIV who are not taking antiretroviral therapy, HIV replicates at an extraordinarily high rate, typically producing billions of virions daily.[1] At the reverse transcription step during the HIV replication process, mutations occur at high rate, predominantly because HIV reverse transcriptase fails to correct erroneously incorporated nucleotides ([Figure 1]).[2] These nucleotide sequence changes generate amino acid substitutions during translation that can alter the reverse transcriptase, protease, or integrase enzymes, as well as change binding proteins and envelope proteins, which can create resistance to different classes of antiretroviral medications. If a person with HIV takes antiretroviral therapy with good adherence and maintains a suppressed HIV RNA levels, there is insufficient HIV replication for these mutations to occur at a significant frequency; with inadequate adherence, however, those viral strains that develop mutations with resistance to the antiretroviral regimen will have a fitness advantage and will eventually become dominate quasispecies([Figure 2]). The emergence of dominant resistant strains of HIV can result in a suboptimal response to antiretroviral therapy and virologic failure; this type of drug resistance is referred to as acquired resistance (as opposed to transmitted resistance).[3]

Transmission and Natural History of Drug-Resistant HIV

Transmission of drug-resistant HIV occurs in an estimated 6 to 21% of new HIV infections.[4,5,6,7,8,9,10,11] In the absence of antiretroviral therapy, several outcomes may occur following such transmission: (1) back-mutation of the strains to more fit wild-type strains, (2) overgrowth of the strains by wild-type HIV, (3) persistence of the strains at low-levels if they do not impair viral fitness, and/or (4) existence of strains that contain a mixture of some persistent mutations and some mutations that have reverted to wild type. If in any of these scenarios antiretroviral therapy is administered, the drug-resistant strains may become dominant if they confer resistance to the specific antiretroviral agents that are chosen, and thus may cause virologic failure. This transmitted resistance (as opposed to acquired) has become less relevant in the integrase strand transfer inhibitor (INSTI) era, but is still important for clinicians to recognize.

Management of HIV Drug Resistance and Virologic Failure

Testing for HIV resistance to antiretroviral medications has become an important component of the clinical care of persons with HIV. Resistance assays can assist the clinician in selecting a maximally effective antiretroviral regimen. Clinicians who care for individuals with HIV should have a general understanding of the basic evaluation of HIV drug resistance and management of virologic failure. It is also important to emphasize that management of virologic failure is complex and expert advice should be obtained if the clinician
managing the virologic failure does not have this expertise.[12] In addition, it is important for clinicians to identify more complex resistance scenarios that require expert consultation. This Topic Review will address the approach to patients with detectable HIV RNA levels, the role for HIV resistance testing, the interpretation of resistance test results, and basic strategies for managing virologic failure.
**Definition of Terms Related to Virologic Responses**

**Virologic Responses**

In the Adult and Adolescent ARV Guidelines, the following definitions are used to characterize and define different virologic responses to antiretroviral therapy:[12]

- **Virologic Suppression**: A response to antiretroviral therapy with an HIV RNA level below the lower level of detection of available assays (typically less than 20 to 50 copies/mL, depending on the assay used) (Figure 3).
- **Virologic Failure**: The inability to achieve or maintain HIV RNA levels less than 200 copies/mL.
- **Incomplete Virologic Response**: Failure to suppress HIV RNA to undetectable levels after 24 weeks on an antiretroviral regimen, as documented by two consecutive HIV RNA levels 200 copies/mL or greater in a patient who has not previously achieved virologic suppression on the same antiretroviral regimen (Figure 4).
- **Virologic Rebound**: Confirmed HIV RNA level greater than or equal to 200 copies/mL after achieving virologic suppression on antiretroviral therapy (Figure 5).
- **Virologic Blip**: After achieving virologic suppression, a single detectable HIV RNA level (usually less than 200 copies/mL) that is followed by a return to virologic suppression (Figure 6).
- **Low-Level Viremia**: Persistent HIV RNA levels above the level of detection of the assay but below 200 copies/mL (Figure 7).
Causes of Virologic Failure

Causes of Virologic Failure

Multiple different factors can play a role in the development of virologic failure.[12] Cohort studies and clinical experience have shown that suboptimal adherence frequently plays a major role in the development of drug resistance and virologic failure.[12] In some instances, individuals may acquire drug-resistant HIV, which can increase the likelihood of virologic failure, depending on the specific acquired mutations and the antiretroviral regimen chosen. The Adult and Adolescent ARV Guidelines list the following three groups of factors that most often contribute to virologic failure: (1) patient/adherence-related factors, (2) HIV-related factors, and (3) antiretroviral regimen-related factors (Table 1).[12]

False Elevation in HIV RNA Levels with Plasma Preparation Tubes

Multiple studies have documented factitious transient or low-level viremia that may result from the use of plasma preparation tubes, which likely does not represent virologic failure or replication-competent viremia.[13,14,15,16,17] The false elevation in HIV RNA levels is thought to result from retained cellular material following the normal specimen processing that separates plasma from whole blood; specifically, some of the cellular material may have fragments of peripheral blood mononuclear cells that are infected with HIV and that contain proviral HIV DNA, which subsequently gets amplified during the quantitative HIV RNA assay.[17] In addition, after the initial separation process of the blood specimen, some residual platelets may remain that have HIV adherent to the platelet surface. This plasma preparation-associated false elevation in HIV RNA levels appears to occur more frequently when a freezing step is included. One group has described a solution to this problem that involves implementing a second centrifugation step.[13]
Approach to Detectable HIV RNA Levels

New detectable HIV RNA levels may or may not indicate virologic failure, and several factors must be considered when evaluating the cause of virologic failure. The following outline the recommended approach in the Adult and Adolescent ARV Guidelines for different scenarios of detectable HIV RNA levels.[12]

Intermittent Viremia (Virologic Blips)

Most available data suggest that isolated blips in HIV RNA levels do not usually correlate with HIV drug resistance or virologic failure.[18,19,20,21,22] Some studies have shown that virologic blips more often occur in patients with high baseline HIV RNA and low baseline CD4 cell count (less than 350 cells/mm$^3$).[23] One study showed an association between the magnitude of the blip and virologic failure, with blips greater than 500 copies/mL associated with increased risk of virologic rebound.[24] Studies on adherence have been conflicting, with some studies showing a correlation of viral blips with adherence whereas others have not.[20,25] Most experts do not recommend making any antiretroviral regimen changes based on an isolated virologic blip, especially with an HIV RNA level less than 200 copies/mL; the most important is to review adherence and possible drug-drug interactions and to continue monitoring of the viral load.

HIV RNA Detectable but Below the Limit of Quantitation

In some instances, HIV can be detected in a sample, but the amount of virus is so low that an accurate count of the HIV level cannot be determined; this situation is typically referred to as detectable below the limit of quantitation, and is sometimes called “very low-level viremia” (Figure 8). Individuals in this situation are generally considered to have well-controlled HIV and do not require any change in antiretroviral therapy based on this very low-level viremia (most commercial assays have a cutoff of 20 or 40 copies/mL for reporting quantitative HIV RNA levels).[26,27] Most experts do not alter lab monitoring intervals or make any other changes based on HIV RNA levels detectable in this range.

Low-Level Viremia (HIV RNA Detectable but Less than 200 copies/mL)

Most experts define low-level viremia as a detectable HIV RNA level above the lower limit of detection of the assay (usually 20 to 50 copies/mL) but less than 200 copies/mL. Confirmed low-level viremia may be due to suboptimal adherence, drug interactions, drug-food interactions, or early virologic failure. If an individual with HIV has persistent low-level viremia in the setting of excellent adherence with a potent antiretroviral regimen, then the cause is likely detection of proviral DNA or replication incompetent HIV by the RNA assay, and these low HIV RNA levels may not be clinically important. Most, but not all, studies have shown a low risk of virologic resistance in patients with persistently detectable HIV RNA levels that remain below 200 copies/mL. When HIV RNA levels are less than 200 copies/mL, standard genotypic drug resistance testing usually cannot be performed. The Adult and Adolescent ARV Guidelines recommend that patients with low-level viremia continue the same antiretroviral regimen, but undergo monitoring with HIV RNA levels every 3 months.[12,27] Some experts, however, would take a more aggressive approach to patients with persistently detectable HIV RNA at level between 50 and 200 copies/mL, including evaluating with a DNA genotype and switching the antiretroviral therapy to a regimen with a high barrier to resistance in order to reduce the risk of virologic failure.

HIV RNA Level between 200 and 1,000 copies/mL

In persons on antiretroviral therapy, persistent HIV RNA levels between 200 and 1,000 copies/mL while on antiretroviral therapy is often associated with the emergence and evolution of drug resistance; this level of viremia is considered as virologic failure.[28,29] The emergence of mutations typically occurs more rapidly in antiretroviral medications that have a lower genetic barrier to resistance. It is also important to note that virologic failure may occur in the absence of new resistance mutations. For example, if an individual with HIV is taking a regimen that includes an anchor drug with a high barrier to resistance (e.g. dolutegravir,
bictegravir, or a boosted protease inhibitor) and they miss multiple doses of the antiretroviral medications, they may experience virologic rebound to above 200 copies/mL without the emergence of new mutations. Although this scenario is defined as virologic failure, virologic suppression can be achieved on the same antiretroviral regimen with excellent subsequent adherence. If the HIV RNA level is 500 or greater copies/mL, genotypic drug resistance testing should be performed; if the HIV RNA level is between 200 and 500 copies/mL, resistance testing should be considered, although the ability of laboratories to successful perform resistance testing at this level of viremia is not reliable. Management of these individuals is complex and should be based on the drug resistance genotype, if applicable, and by expert consultation.

**HIV RNA Level Greater than 1,000 copies/mL and Drug Resistance Identified**

In persons with HIV RNA levels greater than 1,000 copies/mL, a drug resistance genotype can reliably be performed. If new or old drug resistance mutations are identified that are relevant to the regimen the individual is taking, then a prompt change in the antiretroviral regimen is warranted and the choice of the new regimen should incorporate expert consultation. Some experts would also immediately change the regimen once the elevated HIV RNA is identified. The decision to immediately change the regimen or wait until the genotype results return is complex and should be informed by expert consultation.

**HIV RNA Level Greater than 1,000 copies/mL and Drug Resistance Not Identified**

In persons with HIV RNA levels greater than 1,000 copies/mL, a drug resistance genotype can reliably be performed. Individuals with an HIV RNA level greater than 1,000 copies/mL and no drug resistance mutations identified likely have suboptimal adherence as the major factor for virologic failure. Further, persons with dramatic increases in HIV RNA levels to well above 1,000 copies/mL who do not show any drug resistance mutations typically have completely stopped taking antiretroviral medications, or are rarely taking their medications. It is only in the setting of lack of substantial drug pressure that concomitant development of major increase in HIV RNA level and absence of drug resistance mutations.
HIV Drug Resistance Assays

Types of Drug Resistance Assays

Two basic types of drug resistance assays are currently available to assess HIV resistance to antiretroviral agents: genotypic assays and phenotypic assays.[30] The sensitivity of either the genotype or phenotype for detecting minority HIV populations is limited. More recently, some investigators have employed newer techniques, such as allele-specific PCR, single-genome, and ultra-deep sequencing, to assess the role of minority HIV variants that harbor drug resistance but are not detectable by current standard genotypic or phenotypic assays; these tests are not currently commercially available but have contributed to our understanding of HIV resistance.[31] A new HIV genotypic drug resistance assay (GenoSure Archive) that analyzes archived proviral HIV DNA is commercially available for use in patients with undetectable or very low HIV RNA levels.

Conventional Genotype Testing

The most commonly used HIV drug resistance test in clinical practice is the conventional HIV RNA genotype assay, which uses population (Sanger) sequencing. The conventional HIV drug resistance genotype test involves multiple steps: (1) polymerase chain reaction (PCR)-amplification of HIV RNA circulating in the patient's plasma; (2) direct sequencing of these amplified regions, which averages the sequence, obscuring minor genotypes; (3) comparison of the patient's gene sequences to known HIV "wild-type" gene sequences; and (4) determining the corresponding amino acid alterations that would result from the specific identified DNA mutations (Figure 9).[30] The assays used by commercial laboratories to perform conventional drug resistance genotypic testing typically sequence the polymerase gene (encodes HIV reverse transcriptase) and the protease gene.[30] In addition, many laboratories now have the capacity to analyze regions of the HIV integrase gene and some can perform genotypic analysis of the HIV envelope gene, including genotypic analysis of the V3 region to determine HIV tropism.[32,33] Most commercial genotypic assays detect the majority HIV quasispecies (present in at least 80% of the HIV in the sample).

DNA Genotyping

The HIV DNA drug resistance genotype test, often referred to as a proviral (or archive) DNA genotype, evaluates cell-associated proviral HIV DNA, either as integrated proviral DNA or unintegrated cytoplasmic HIV DNA (Figure 10).[34,35] The HIV DNA drug resistance genotype can be performed with very low or even undetectable HIV RNA levels, but the assay requires use of whole blood (not plasma) samples and immediate freezing of the whole blood sample without performing centrifugation. One study reported a high level of concordance of HIV RNA and DNA genotype in viremic patients,[36] whereas others found incomplete information from the DNA genotype when compared with cumulative drug resistance mutation data from previous drug resistance genotypes.[37,38,39] Therefore, use of standard HIV RNA drug genotyping (taking into account cumulative data from past RNA resistance tests when available) remains the preferred test for decision making in persons with virologic failure. Nevertheless, in some individuals with low or fully suppressed HIV RNA levels, historical HIV drug resistance data may not be available and proviral DNA genotyping may inform decisions about switching antiretroviral therapy.[38,40] The overall clinical utility and optimal use for this test in clinical practice remains unclear.

HIV Drug Resistance Phenotype Assay

The phenotype assay requires a patient blood sample and is performed on a blood sample using PCR amplification of reverse transcriptase, protease, and possibly the envelope genes; the process relies on the patient's dominant circulating strain of HIV. The amplified HIV genes are then inserted into a laboratory HIV strain from which these genes have been deleted, generating large numbers of recombinant HIV clones. These clones are then tested for drug susceptibility to antiretroviral agents using automated assays.
**Indications for HIV Drug-Resistance Testing**

Based on existing evidence and expert opinion, the Adult and Adolescent ARV Guidelines recommend drug resistance testing in the situations as summarized in the following discussion (Figure 11).

### Virologic Failure

For persons with HIV who develop virologic failure and have an HIV RNA greater than 1,000 copies/mL, genotypic HIV drug resistance testing should be performed. If virologic failure occurs and the HIV RNA level is between 500 and 1,000 copies/mL, HIV drug resistance testing should be considered, but some laboratories may not be able to successfully perform the test due to inadequate viral amplification. With recent advances in the sensitivity of genotypic drug resistance testing, many laboratories can now reliably perform resistance testing on samples from persons with a plasma HIV RNA level between 500 to 1,000 copies/mL and some can successfully perform testing with HIV RNA in the range of 200 and 500 copies/mL. If a person develops virologic failure while taking an INSTI-based regimen, it is important to include (or add on) integrase resistance testing in the evaluation (note that standard HIV drug resistance testing does not include integrase resistance testing). Ideally, HIV drug resistance testing should be performed while the person with HIV is still taking the failing regimen, or within 4 weeks of discontinuation of antiretroviral therapy. Nevertheless, in some situations, performing drug resistance testing within 6 weeks of stopping antiretroviral therapy is not possible; in these situations, resistance testing should still be performed since some mutations may still be detected.

### Acute HIV Infection

In the setting of acute or early HIV, obtaining genotypic drug resistance testing is recommended, regardless of whether the decision is made to immediately initiate antiretroviral therapy. Obtaining a resistance test in the setting of acute or early HIV provides the greatest likelihood of detecting transmitted drug-resistant HIV. If therapy is deferred, the guidelines suggest considering repeat resistance testing prior to initiating antiretroviral therapy, since the person may possibly acquire drug-resistant HIV in the interim.

### Antiretroviral-Naïve with Chronic HIV

Because most individuals with HIV are diagnosed with HIV long after HIV acquisition, the first opportunity for HIV drug resistance testing typically occurs years after initial HIV transmission. Drug resistance testing should be performed in the setting of chronic HIV infection at the time a person with HIV enters into HIV medical care. Multiple studies, when taken together, estimate that 10 to 17% of antiretroviral-naïve persons with HIV have evidence of resistance to at least one antiretroviral medication. Several studies performed prior to the widespread use of INSTIs as the preferred anchor drug have shown that baseline drug-resistant HIV (in persons with chronic HIV infection) can negatively impact response to antiretroviral therapy (with regimens that did not include an INSTI). In the setting where drug resistance testing first occurs after a significant gap from initial HIV infection, acquired HIV drug-resistant HIV may fail to be detected because of overgrowth by wild type HIV strains. In the current era, given the infrequent transmission of integrase-resistant HIV, most experts would promptly initiate antiretroviral therapy with an INSTI-based regimen, with the plan to subsequently modify therapy, if needed, based on the results of the drug-resistance genotype assay.

### Pregnancy

Drug resistance testing is recommended for all pregnant women with HIV infection who are antiretroviral-naïve and have an HIV RNA level above the threshold for resistance testing (500 to 1000 copies/mL) prior to starting an antiretroviral therapy regimen in pregnancy. Resistance testing is also indicated before modifying antiretroviral therapy regimens for women with HIV infection who have detectable HIV RNA levels (above 500 to 1000 copies/mL) upon entry to prenatal care while taking antiretroviral therapy or who have
suboptimal virologic suppression to a new regimen started in pregnancy.[47] In this situation, note that some laboratories have improved the sensitivity of their drug resistance assays and can successfully perform drug resistance testing with HIV RNA levels in the range of 200 to 500 copies/mL.

**After Discontinuation of Antiretroviral Therapy**

Drug resistance assays are usually not recommended for patients who have taken suppressive antiretroviral agents in the past but have been off antiretroviral therapy for more than 4 weeks.[41] When HIV drug resistance develops in response to antiretroviral therapy, populations of wild-type and resistant strains of HIV may co-exist, but upon discontinuation of antiretroviral therapy, replication of wild-type strains outpace the growth of most drug-resistant strains, due to greater virologic fitness; in this scenario, without selective antiviral pressure, resistant strains will likely become a minority species in the overall viral population (Figure 12). Because currently available resistance assays do not reliably identify strains of HIV that constitute less than 20% of the overall viral population, the "minority resistant" strains often evade detection by resistance assays in chronically infected persons who discontinue therapy.[48,49,50] Although drug-resistant strains may not be evident on resistance testing in this situation, they can quickly become dominant if the patient reinitiated antiretroviral therapy that included medications to which the patient had previously developed resistance.[48]
Interpretation of Drug Resistance Assays

Interpreting Drug Resistance Genotype

The HIV genotype assay is performed by sequencing HIV DNA genes relevant to drug resistance based on those sequences the corresponding amino acids can be automatically inferred. The actual genotype report does not provide DNA sequence data, but instead it provides information on the HIV amino acid sequences that have been inferred from the HIV DNA sequences (Figure 13). In the genotype report, specific information is provided regarding the amino acids that deviate from those found in "wild-type" strains of HIV (Figure 14). The shorthand convention used in genotype reports provides the wild-type amino acid, followed by the position of the amino acid in the protein, followed by the substituted amino acid that confers resistance; for example, the K103N mutation occurs as a result of a mutation in the DNA nucleotide which results in the replacement of the amino acid lysine (K) by asparagine (N) at amino acid position 103 in the reverse transcriptase protein (Figure 15). In addition, most drug resistance genotype reports provide an interpretation of the impact of the drug resistance mutations on antiretroviral medications. The genotype interpretation typically classifies drug susceptibility in one of three categories: no evidence of resistance, low-level resistance, or high-level resistance. In addition, some mutations can result in viral hypersusceptibility to medications, as is the case with the M184V reverse transcriptase mutation that enhances virologic response to tenofovir DF, tenofovir alafenamide, zidovudine, and stavudine.[51,52,53,54]

Interpreting Drug-Resistant Phenotype

A phenotypic resistance assay evaluates the susceptibility of HIV to antiretroviral agents by directly measuring the viability of the predominant strain of HIV in the presence of antiretroviral medications.[30] For each antiretroviral agent tested, the phenotype report provides an IC\textsubscript{50} or IC\textsubscript{90} value—this value represents the drug concentration required to inhibit the replication of HIV by 50\% (for IC\textsubscript{50}) or 90\% (for IC\textsubscript{90}). The IC\textsubscript{50} (or IC\textsubscript{90}) of the patient's sample is divided by a reference IC\textsubscript{50} (or IC\textsubscript{90}) value from wild-type virus to generate a "fold change" value; the "fold change" represents relative resistance of the patient's HIV to the antiretroviral medication (Figure 16). Based on the "fold change" observed when testing the patient's HIV against an antiretroviral medication, the HIV can be considered either susceptible, resistant, or hypersusceptible to the medication tested (Figure 17). The final report shows the fold change for each of the antiretroviral medications tested and provides an interpretation for the clinician. Phenotypic assays are now infrequently used, but may have some value in patients with extensive HIV drug resistance, particularly with the protease inhibitor class of drugs.

Limitations of Resistance Testing

Resistance testing has some significant limitations. For example, both genotypic and phenotypic resistance assays analyze the dominant circulating HIV strains and thus may fail to detect mutant strains that constitute a minority of the patient's HIV population. The failure to detect minority populations can occur in several scenarios: (1) as mutant populations begin to emerge, they may exist in low numbers and not be evident on the resistant test; (2) if a patient discontinues antiretroviral therapy, wild-type virus tends to outgrow and obscure resistant virus; (3) if a patient switches antiretroviral therapy, the selective drug pressure changes and resistant populations may diminish to a level below the threshold for detection on the resistance assay; and (4) in the setting of absent or altered drug pressure, previously generated resistance mutations may exist archived at low levels but will re-emerge to confer resistance if the previously used antiretroviral agent is reintroduced.[48,55] Thus, resistance tests provide the most accurate information if they are performed while the patient is on antiretroviral therapy; in addition, HIV drug resistance tests most accurately reflect resistance to medications the patient is currently taking. The tests less reliably detect resistance to drugs taken in the past.

Relevance of Past Resistance Tests
Clinicians should regard the patient's antiretroviral resistance as the cumulative resistance that has developed throughout the entire time the patient has been living with HIV infection, recognizing that a recent standard resistance assay may not detect all of these resistance mutations. Resistance results must be well documented for future consideration. If resistance tests were not performed at the time of failure of previous regimens, then it becomes especially important for the clinician to consider the patient's complete antiretroviral history, including virologic responses to past regimens, when designing a salvage regimen. If drug resistance is identified, consultation with an HIV expert is recommended to design a new antiretroviral regimen that will effectively suppress the HIV RNA level. If no HIV drug resistance is identified, the most explanation is very poor adherence taking antiretroviral medication, since there would not be ample selective pressure to stimulate formation of the mutations. In addition, if the drug resistance assay was performed after more than 4 weeks of not taking antiretroviral medication, wild-type virus can overgrow the resistant minority variants, obscuring the ability of the ability of the resistance assay to detect the mutations. In persons with no resistance identified, expert consultation may help in deciding how to further evaluate the situation and whether to rechallenge with the prior antiretroviral regimen or to change to a new regimen.

**Resources for Interpreting Drug Resistance Testing**

Data on the use and interpretation of HIV drug resistance testing are constantly evolving, and neither phenotypic nor genotypic HIV drug resistance testing has been standardized. Mutation resistance guides, algorithms, and sensitivity cutoffs need to be updated frequently to keep pace with the latest research findings. Given the complexity and uncertainties associated with the interpretation of resistance assays, expert clinical consultation is advised for clinicians who do not have significant experience in interpreting HIV drug resistance tests. The following are excellent resources for help with resistance issues:

- **Stanford University HIV Drug Resistance Database**: This free website provides comprehensive information on HIV drug resistance. Most importantly, the site features a Genotypic Resistance Interpretation Algorithm that allows the user to input specific genotypic mutations from a patient sample in a drop-down menu and then view the analysis of the genotype. Options for input include mutations involving reverse transcriptase, protease, and integrase.
- **National HIV Clinician Consultation Center: HIV/AIDS Management**: This free service offers clinicians expert consultation service for any aspect of HIV clinical care, including evaluation and management of virologic failure and interpretation of HIV drug resistance genotype tests. This service is available Monday through Friday 9:00 a.m. to 8:00 p.m. EST. The phone number is 800-933-3413.
- **International AIDS Society-USA (IAS-USA): Drug Resistance Mutation Figures**: The IAS-USA regularly publishes a concise and updated compendium of HIV drug resistance mutations and the impact of each of these mutations on antiretroviral medications. The HIV drug resistance mutation figures and the accompanying notes provide an excellent visual overview of the current state of knowledge for relevant HIV drug resistance mutations.
Nucleoside Reverse Transcriptase Inhibitor Resistance

Principles of Nucleoside Reverse Transcriptase inhibitor Resistance

The nucleoside reverse transcriptase inhibitor (NRTI) medications block HIV reverse transcriptase, the process where HIV RNA is converted into HIV DNA.[56] Specifically, the inhibition occurs when HIV reverse transcriptase incorporates the NRTI into the elongating HIV DNA strand, whereby the NRTI act as chain terminators due to absence of a 3' hydroxyl group.[57] The development of NRTI resistance by HIV involves one of two biochemical mechanisms that take place in the reverse transcriptase process: (1) discrimination (decreased incorporation) of the antiretroviral medication into the elongating HIV DNA strand (Figure 18) and (2) excision (primer unblocking) of the antiretroviral medication from the HIV DNA strand (Figure 19).[58,59,60,61,62] The discriminatory mutations allow the reverse transcriptase enzyme to preferentially select the naturally occurring deoxynucleotides present in the cell, thereby creating a relative decrease in the incorporation of the NRTI-triphosphate into the elongating HIV DNA strand. Excision mutations enhance the phosphorolytic excision of the NRTI-triphosphate already added to the elongation HIV RNA-DNA complex, resulting in unblocking of the primer by the NRTI. Examples of mutations that cause discrimination (decreased NRTI incorporation) include K65R, K70E, L74V, M184I/V, and the Q151M complex (Q151M followed by the accessory mutations A62V, V75I, F77L, and F116Y).[59,60,63] Characteristic mutations that occur via the “primer unblocking” pathway include the M41L, D67N, K70R, L210W, T215Y/F, and K219Q/E.[59,60,63]

Specific NRTI Mutations

M184V

The M184I/V mutations are the signature mutations that develop with resistance to the medications lamivudine and emtricitabine. The M184I mutation typically develops prior to the M184V, but usually is rapidly replaced by the M184V, primarily because the M184I mutation causes a greater impairment in viral fitness than does the M184V.[65] The M184I and M184V mutations develop via the discriminatory resistance pathway.[59,61] The M184V mutation causes high-level resistance to emtricitabine and lamivudine, low-level resistance to abacavir and didanosine, and enhanced susceptibility to stavudine, tenofovir DF, tenofovir alafenamide, and zidovudine.[66]

Thymidine Analog Mutations

The thymidine analog mutations (TAMs), which include M41L, D67N, K70R, L210W, T215Y/F, and K219Q/E, develop in the setting of virologic failure with a regimen that includes either of the thymidine analogs, stavudine or zidovudine (Figure 20).[51,63,66,67,68,69] The TAM mutations tend to accumulate in one of two characteristic, but overlapping patterns: (1) the type-I pattern that has M41L, L210W, and T215Y or (2) type-II pattern consisting of D67N, K70R, T215F, and K219Q/E (Figure 21).[63,70,71,72,73] In general, type-I TAM mutations result in higher levels of resistance to stavudine and zidovudine, as well as greater cross-resistance to abacavir, didanosine, and tenofovir DF, and tenofovir alafenamide.[63,74] For patients on a thymidine analog plus either lamivudine or emtricitabine, the development of TAMs is typically preceded by the development of an M184V mutation. Some patients develop the D67N mutation with the type-I cluster.

K65R Mutation

In clinical trials, the development of the K65R mutation has primarily occurred in patients taking a non-suppressive antiretroviral regimen that did not include a thymidine analog (stavudine or zidovudine).[75,76] In early trials of abacavir monotherapy, approximately 10% of patients developed the K65R mutation.[77] Even higher rates (greater than 50%) of K65R mutation were observed in patients treated with the triple NRTI regimen of abacavir plus lamivudine plus tenofovir DF.[78] Addition of a drug from a class other than NRTI and use of a fully suppressive regimen markedly reduce the likelihood of developing the K65R mutation. The
K65R mutation alone causes high-level resistance to didanosine, tenofovir DF, and tenofovir alafenamide, intermediate-level resistance to abacavir, emtricitabine, lamivudine, and stavudine, and hypersusceptibility to zidovudine.\[63\] The K65R mutation results in decreased incorporation of NRTI drugs and shows bilateral antagonism with the primer unblocking (excision) activity of the reverse transcriptase enzyme that contains TAMs.\[75,76,79\] If an M184V mutation occurs in conjunction with the K65R, the level of resistance to abacavir, emtricitabine, lamivudine changes to high-level resistance. Alternatively, the K65R mutation reverses the hypersusceptibility effect of the M184V on stavudine, tenofovir DF, tenofovir alafenamide, and zidovudine.\[75,80\] With abacavir resistance, the M184V typically precedes the K65R.\[81\] In clinical trials and clinical practice, it is very uncommon to observe the K65R mutation in conjunction with multiple TAMs.\[75,76\] The K65E/N variants have been reported in patients failing tenofovir DF.\[51\]

**L74V Mutation**

The L74V mutation was first identified with didanosine and abacavir monotherapy; this mutation alone causes high-level resistance to didanosine and intermediate-level resistance to abacavir. Similar to the M184V mutation, the L74V mutation causes in vitro hypersusceptibility to tenofovir, zidovudine, and possibly stavudine. The L74V in combination with M184V has been seen in patients treated with an abacavir plus lamivudine, or didanosine plus lamivudine, NRTI backbone.\[63\] Overall, the L74V mutation is an uncommon NRTI mutation, but is identified in approximately 25% of samples that contain HIV with K101E plus G190S mutations and in approximately 50% of samples that contain L100I plus K103N mutations.\[82\]

**Multi-NRTI Resistance Mutations: T69 and Q151M Insertion Complexes**

The multi-nucleoside resistance mutations occur relatively infrequently but may have a major impact on the NRTIs. The T69-insertion mutation consists of double amino acid (dizerine) insertion between codons 69 and 70 in the reverse transcriptase enzyme.\[83,84\] The T69 occurs only in the setting of existing TAM-1 mutations and together the T69-insertion and TAM-1 generate high-level resistance to all of the NRTI medications, except for lamivudine and emtricitabine, which have intermediate resistance.\[63,83,85\] The Q151M mutation complex usually occurs with several accessory mutations (A62V, V75I, F77L, and F116Y) and these mutations in tandem cause high-level resistance to abacavir, didanosine, and zidovudine, as well as intermediate resistance to emtricitabine, lamivudine, and tenofovir.\[59\] The Q151M mutation complex develops only in the setting of prolonged viremia while on therapy.\[59\]

**Lamivudine and Emtricitabine**

In clinical trials involving combination antiretroviral therapy, the M184V mutation is characteristically one of the most common mutation to develop with initial virologic failure in regimens that include lamivudine or emtricitabine.\[86\] One early study showed that patients treated with lamivudine monotherapy develop the M184V mutation and virologic failure within 4 weeks of starting lamivudine; even after the development of the M184V lamivudine continues to generate approximately 0.4 to 0.5 log10 decrease in HIV levels, probably due to impact on viral fitness.\[87\] Several later studies suggested continuing treatment with lamivudine in the presence of an M184V mutation may confer benefit, potentially through decreased viral fitness, hypersensitivity to several other NRTIs, and perhaps delayed development of mutations in other NRTIs.\[88,89\] In the absence of drug pressure from either emtricitabine or lamivudine, the M184V mutation rapidly disappears, reflecting the overall negative impact of the M184V on viral fitness.\[90\] Once the M184V mutation develops, there are no further cascading mutations that develop that would negatively impact other antiretroviral medications. The M184V mutation is not known to impact medications outside of the NRTI class, but the M184I mutation augments resistance to rilpivirine when present in conjunction with the E138K mutation.\[91\]

**Stavudine and Zidovudine**

The thymidine analog mutations (TAMs), which include M41L, D67N, K70R, L210W, T215Y/F, and K219Q/E,
develop in the setting of virologic failure with a regimen that includes either of the thymidine analogs, stavudine or zidovudine (Figure 20). [51, 63, 66, 67, 68, 69] Although these medications are infrequently used in current clinical practice, patients with long-standing HIV may have acquired TAMs in the past. In addition, patients from resource-limited regions who have immigrated recently to the United States may have received stavudine or zidovudine in recent years, or may be currently taking these medications. In the United States, TAMs infrequently develop in patients on modern antiretroviral regimens that do not include zidovudine or stavudine. The presence of an M184V mutation reduces the impact of the TAM mutations to some degree, but the favorable impact of the M184V is negligible with high numbers of TAM mutations.[92]
Non-Nucleoside Reverse Transcriptase Inhibitor Resistance

Principles of Non-Nucleoside Reverse Transcriptase Inhibitor Resistance

The NNRTIs block HIV replication via non-competitive inhibition of the HIV reverse transcriptase enzyme. The NNRTIs binding to a hydrophobic pocket of the HIV reverse transcriptase, which cause functional changes in the enzyme (Figure 22).[56,57] The NNRTI hydrophobic binding pocket region is predominantly lined by amino acid codons 98 to 108 and 179 to 190; the key amino acids that line the binding pocket are L100, K101, K103, V106, T107, V108, V179, Y181, Y188, V189, G190, F227, W229, L234, Y318 (of p66), and E138 (of p51).[48,60] Resistance to NNRTIs typically occurs as a result of one or more mutation(s) involving amino acids that line the NNRTI binding pocket or are adjacent to the pocket; these mutations can prevent NNRTI binding either by altering the NNRTI binding site (Figure 23) or by preventing adequate access of the NNRTI to the binding pocket (Figure 24).[57,60] Although all six NNRTI drugs—delavirdine, doravirine, efavirenz, etravirine, nevirapine, and rilpivirine—bind to the same general region in the binding pocket, subtle differences exist in the interaction between the specific drug and the hydrophobic pocket, and as a result drug-specific mutations can develop. Unfortunately, the emergence of characteristic NNRTI-associated mutations can occur with rapid virologic rebound and high-level phenotypic resistance. These characteristic mutations presumably exist at low levels in all antiretroviral therapy-naïve patients. When an NNRTI is used in a sub-optimal regimen, or when patients do not adequately adhere with therapy, NNRTI-resistant mutants can be selected within 1 to 4 weeks.[48,93]

Delavirdine

With early delavirdine failure, the K103N or Y181C is generally observed as the initial mutation; failure with delavirdine is only of historic interest since this NNRTI is no longer used in clinical practice in the United States.[94]

Doravirine

Limited data exist regarding emergence of drug resistance in persons taking doravirine, but data from clinical trials that included 11 patients with virologic failure showed doravirine resistance-associated substitutions in reverse transcriptase that included one or more of the following mutations: A98G, V106I, V106A, V106M/T, V108I, E138G/K, Y188L, H221Y, P225H, F227C, F227C/R, and Y318Y/F.[95,96] Two mutation pathways of resistance have been identified with doravirine: (1) a major pathway with selection of the V106A mutation followed by the F227L or L234I mutation or (2) a minor pathway that involves selection of V108I and L234I mutations.[97,98] Clinical experience with cross-resistance for doravirine and other NNRTIs is limited but emergence of resistance to doravirine may not impact all NNRTIs.[98] Doravirine appears to maintain good activity in the presence of some of the most common NNRTI mutations, including K103N, Y181C, and G190A.[97,99] In vitro data showed marked reduced susceptibility to doravirine with (1) Y188L substitution alone or in combination with K103N or (2) V106I, V106A in combination with G190A and F227L, or (3) E138K in combination with Y181C and M230L.

Efavirenz

Among patients who fail an efavirenz-based regimen, the K103N mutation is the most common NNRTI mutation observed; three other mutations can cause primary resistance: Y188L, G190S, and G190A.[100] Although early virologic failure with efavirenz characteristically involves a single mutation, such as the K103N mutation (or dual mutations), prolonged virologic failure leads to the accumulation of multiple mutations that may include L100I, V108I, Y181C/I, and P225H; the development of these multiple mutations compounds the level of NNRTI resistance.[48,101]

Etravirine
Etravirine is a second generation NNRTI that has a higher barrier to resistance than the first generation NNRTIs and retains activity against HIV with resistance to efavirenz and nevirapine. Because the resistance profile is more complex than the first generation NNRTIs, where a single mutation can affect virologic response, a scoring rubric has been developed to help determine the impact of specific resistance mutations on susceptibility to etravirine and thus, predict virologic response.[102] According to the etravirine-weighted genotypic score (“etravirine score”), a score of 0 to 2 indicates susceptibility, 2.5 to 3.5 indicates intermediate resistance, and a score of 4 or greater indicates resistance; for example, the Y181C mutation yields a resistance weight factor of 2.5 (intermediate) whereas the G190A mutation yields a resistance weight factor of 1 (low).[102] Etravirine resistance has mostly been studied in the context of coadministration with ritonavir-boosted darunavir in the DUET studies, and the performance of the weighted scoring system has not been validated with other antiretroviral agents.[51]

Nevirapine

Patients treated with nevirapine monotherapy most commonly develop the Y181C mutation.[93] In about 20% of patients who fail a nevirapine-based regimen, the mutations V106A/M or Y181C/I initially emerge.[103] It appears the Y181C is less likely to emerge in persons taking a thymidine analog, namely zidovudine or stavudine.[48,93,103] Among patients with early virologic failure while taking a combination antiretroviral therapy regimen that includes nevirapine, approximately 80% develop the K103N or Y188C/L/H mutations.[48,103]

Rilpivirine

Individuals who have virologic failure while on rilpivirine most often develop a mutation that impacts the E138 amino acid position (E138A/G/K/Q/R/V); the E138K is the most common among these mutations.[104] A solitary E138 mutation is associated with intermediate level resistance to rilpivirine activity, but when it occurs in conjunction with the M184I mutation, high-level resistance develops, with potential virologic failure on a rilpivirine-based regimen.[91,104,105,106,107] Less often, patients taking rilpivirine develop a K101E mutation, which typically causes intermediate-level resistance.[108]

NNRTI Cross-Resistance

The K103N mutation, which often occurs in the setting of virologic failure with an efavirenz-based regimen, confers cross-resistance to nevirapine, but not to doravirine, etravirine, or rilpivirine.[37,109,110,111,112] Available data regarding cross-resistance and the Y181C mutation, which can occur with nevirapine failure, suggest intermediate resistance with efavirenz, etravirine, and rilpivirine; doravirine appears to maintain good activity against HIV strains with the Y181C mutation.[109,113,114,115,116] Similarly, the G190E mutation causes high-level resistance to nevirapine, and intermediate level resistance to efavirenz, etravirine, and rilpivirine.[117] For invidividuals who fail a rilpivirine-containing regimen with an E138K mutation, this generates potential low-level cross-resistance to efavirenz, etravirine, and nevirapine.[104] Thus, typically patients who fail an efavirenz-based regimen have a better chance of responding to etravirine in the future than those who fail a rilpivirine-based regimen. As noted previously, the use of etravirine in patients with prior NNRTI failure is complicated and scoring systems have been generated to predict the likely efficacy of etravirine in patients with multiple NNRTI-associated mutations.[102,106,118,119]

Resistance with NNRTIs and Viral Fitness

All resistance mutations have the potential to impair viral replication. Mutations in the NNRTI class appear to have markedly less impact on viral replication (fitness) than do protease inhibitor-related mutations.[112] Taken together, data from multiple studies suggest the K103N mutation has minimal impact on viral fitness and thus virus that contains the K103N mutation can exist as a highly resistant and highly fit virus. Accordingly, experts do not recommend continuing NNRTI medications in the setting of the K103N mutation. In patients who fail rilpivirine and have E138K and M184I mutations, the E138K mutation appears to nullify
any fitness benefit that might be achieved by maintaining the M184I mutation.[120]
Integrase Strand Transfer Inhibitor Resistance

Principles of INSTI Resistance

The integrase strand transfer inhibitors (INSTIs) interfere with the insertion of HIV DNA into host DNA.\[121\] The integration of HIV into host DNA is a complex process that involves multiple steps. The integrase inhibitors exert their action by blocking the HIV integrase-mediated strand transfer of the HIV DNA into the host DNA. The HIV integrase enzyme is a 288-amino acid protein comprised of the C-terminal domain, the N-terminal domain, and the catalytic core domain; most INSTI resistance mutations occur in proximity to the integrase enzyme active site in the catalytic core domain (Figure 25).\[122,123,124\] In a recent review, 15.6% of patients had viruses with at least one integrase major mutation.\[122\] Viruses can also accumulate minor, or accessory, mutations that can raise the level of overall resistance.

Bictegravir

Available data suggest that bictegravir has a high genetic barrier to resistance, similar to dolutegravir.\[125\] In addition, in vitro data has shown that bictegravir retains activity against a wide range of HIV strains with INSTI-resistance substitutions. Specifically, bictegravir retains good activity (less than twofold reduced susceptibility) with the following common single integrase mutations: 92Q, T97A, Y143C/R, Q148R, and N155H. Nevertheless, bictegravir it is not recommended for the treatment of persons with integrase-resistant HIV, primarily because enhanced dosing of bictegravir is not an option (it is available only as the fixed-dose combination tablet bictegravir-tenofovir alafenamide-emtricitabine). The combined mutations G140A/C/S plus Q148H/R/K is often associated with more than a 2.5-fold reduced susceptibility to bictegravir, especially with an additional INSTI-resistance substitution at L74M, T97A, or E138A/K.

Dolutegravir

Dolutegravir has a higher barrier to resistance than either raltegravir or elvitegravir and retains activity against all single-mutation variants.\[122\] To date, emergence of resistance to dolutegravir has not been reported in antiretroviral therapy naive patients.\[126,127,128\] The infrequent development of resistance to dolutegravir has hampered an understanding of the evolutionary pathways of resistance with primary virologic failure with dolutegravir. In treatment-experienced integrase-naïve patients, however, dolutegravir-associated mutations have developed, including the R263K and N155H.\[129\] Most patients who have initial virologic failure with raltegravir have HIV that retains susceptibility to dolutegravir.\[130\] In patients failing a raltegravir- or elvitegravir-containing regimen, the accumulation of Q148H in combination with the secondary mutations G140S/A/C, L74M, and E138K/A cause a greater than 10-fold reduced HIV susceptibility to dolutegravir;\[51,131\] the N155H mutation, followed by the A49P, L68F, T97A, E138K, and L234V can also lead to dolutegravir resistance.\[132\] In addition, mutations G118T and F121Y, which have been reported in patients who failed raltegravir, cause broad cross-resistance to all integrase inhibitors.\[133,134\]

Elvitegravir

The primary resistance mutations in HIV-1 integrase selected with elvitegravir (T66I, E92Q, and Q148R) confer reduced susceptibility elvitegravir.\[135\] The E92Q mutation is the most common initial mutation to arise with elvitegravir failure, followed in frequency by N155H and Q148H/K/R.\[122\] The E92Q mutation alone reduces elvitegravir susceptibility by more than 20-fold and reduces raltegravir susceptibility by 5-fold.\[51\] The T66I mutation causes an approximate 10-fold reduction in elvitegravir susceptibility, but it does not have significant impact on raltegravir or dolutegravir. When resistance to raltegravir develops, high-level cross-resistance with elvitegravir usually occurs.\[130,136,137\]

Raltegravir

Three major resistance pathways have been identified with HIV resistance to raltegravir: N155, Q148, and
The most common raltegravir-resistance pathways are: (1) Q148H plus G140S, followed by (2) N155H plus E92Q, and (3) Y143R plus T97A; other secondary mutations can develop and the initial N155H mutation often crosses over to the Q148 pathway (Figure 26). Although a single major mutation reduces raltegravir susceptibility by 10-fold, the combination of Q148H plus G140S causes more than 150-fold reduced susceptibility to raltegravir and elvitegravir. Importantly, the accessory mutations G140S and T97A restore viral fitness to the mutated virus whereas E92Q does not have an impact on viral fitness. In patients taking raltegravir, emergence of integrase resistance mutations can occur, even with low-level viremia. One group analyzed patients with low-level viremia (50 to 500 copies/mL) while taking raltegravir and found 3 (7.7%) of 39 patients had developed INSTI mutations. Development of drug resistance to raltegravir generally translates to high-level cross-resistance with elvitegravir. In addition, patients taking raltegravir with incomplete virologic suppression who remain on raltegravir will gradually experience a progressive emergence of higher levels of drug resistance to raltegravir, eventually developing high-level, class-wide integrase resistance (Figure 27). The infrequently seen G118R and F121Y raltegravir-associated mutations induce broad cross-resistance to raltegravir, elvitegravir, and dolutegravir.
Protease Inhibitor Resistance

Principles of Protease Inhibitor Resistance

The HIV PIs bind to and inhibit HIV protease, an enzyme that is only 99 amino acids in length but functions to cleave the Gag and Gag-Pol precursor proteins; the inhibition of HIV protease can impact multiple enzymes that are generated during the normal cleavage process.[143] More mutations are selected by PIs than by any other class and multiple protease mutations are required to significantly impact the virologic response to a ronavir-boosted PI (Figure 28).[51,63] In general, PIs boosted with either ritonavir or cobicistat have medium or high potency and a medium or high genetic barrier to resistance.[59,144] Among the commonly used ritonavir-boosted protease inhibitors, ritonavir-boosted darunavir has the highest barrier to resistance, followed by lopinavir-ritonavir, followed by ritonavir-boosted atazanavir.[59,144] Although less studied, the barrier to resistance with cobicistat-boosted PIs is presumably similar to that seen with ritonavir-boosted PIs. The majority of virologic failures in patients taking a PI-containing regimen occur without phenotypic or genotypic protease resistance, which may reflect the relatively high genetic barrier to resistance with boosted protease inhibitors (compared with most other drugs) as well as complex and unique features of HIV protease inhibitors.[51,63,143] PI mutations are classified as major or minor; minor mutations generally do not affect drug susceptibility but may improve replicative capacity of the virus. Some PI mutations enhance susceptibility to one or more protease inhibitors. For example, I50L increases susceptibility to all PIs except atazanavir whereas L76V enhances susceptibility to atazanavir.[63]

Atazanavir

Atazanavir is predominantly used boosted with ritonavir or cobicistat, but in some circumstances it is used without boosting. The atazanavir barrier to resistance is substantially lower with unboosted atazanavir and fewer mutations are required to develop resistance than with boosted atazanavir.[51] Treatment-naïve patients who have virologic failure while taking boosted atazanavir usually do not have evidence of new protease mutations at the initial time of virologic failure.[91,145] Atazanavir can select out the I50L mutation, which has little impact on other PIs. Multiple PI mutations have been associated with reduced atazanavir susceptibility, with major mutations identified I50L, I84V, and N88S.[51] One study suggested the L76V mutation, with or without the M46I generates atazanavir hypersusceptibility if no additional PI mutations are present.[146]

Darunavir

Darunavir boosted with either ritonavir or cobicistat is considered to have a very high genetic barrier to resistance. Antiretroviral-naïve patients who develop virologic failure on a ritonavir-boosted darunavir-based regimen generally do not develop major (primary) protease-inhibitor resistance-associated mutations.[147,148,149] Investigators have identified eleven protease mutations at 10 protease positions that have been associated with reduced HIV susceptibility to darunavir: V11I; V32I, L33F, I47V, I50V, I54L, I54M, T74P, L76V, I84V, L89V.[51,150,151,152] Diminished virologic response to darunavir generally requires at least three darunavir resistance-associated mutations to emerge.[153] Major mutations include I47V, I50V, I54M, L76V, and I84V.[51] In the United States, following the approval of darunavir in 2006, the prevalence of genotypic or phenotypic resistance to darunavir has significantly decreased over time in commercially tested isolates, presumably as a result of the very high genetic barrier to resistance with boosted darunavir and the requirement for accumulation of multiple protease mutations before darunavir resistance develops.[149] Several studies have shown that treatment-experienced patients, including those with protease resistance, respond equally well to darunavir boosted with ritonavir when given once daily (800/100 mg) versus twice daily (600/100 mg), if no baseline darunavir resistance-associated mutations are present.[154,155,156]

Lopinavir-Ritonavir

Antiretroviral-naïve patients who develop virologic failure while receiving two NRTIs combined with lopinavir-
ritonavir typically do not have evidence of lopinavir drug resistance at the time of virologic failure.[157,158,159] Accordingly, lopinavir boosted with ritonavir is considered to have a high genetic barrier to resistance.[59] Major mutations associated with lopinavir resistance include V32I, I47V/A, L76V, V82A/F/T/S; there are 13 sites identified as minor mutations.[157,158,160] The combination of lopinavir-ritonavir requires accumulation of at least 3 lopinavir-associated mutations for a reduced virologic response to lopinavir-ritonavir.[59,144] Furthermore, certain mutations either alone or in combination with other mutations confer high-level resistance: I47A/V and V32I are each associated with high-level resistance, L76V in combination with 3 PI mutations also increases resistance to lopinavir-ritonavir.[51,146] Although lopinavir-ritonavir resistance infrequently develops in virologic failure in treatment-naïve patients, mutations can emerge in persons who previously failed other protease inhibitors.[161] In addition, significant cross-resistance exists with lopinavir-ritonavir and atazanavir.[162]
Entry Inhibitor Resistance

Principles of Resistance to CCR5 Antagonists

CCR5 Antagonists

The C-C chemokine receptor 5 (CCR5) antagonists exert their mechanism of action by binding to the human cell CCR5 coreceptor, causing a conformational change in the coreceptor that prevents the gp120 region of R5-tropic HIV from effectively binding with the CCR5 coreceptor; the third variable region (V3) of HIV gp120 is the major determinant of viral tropism \[\text{Figure 29}\]. \[163\] Resistance to CCR5 receptor antagonists can occur by two distinct mechanisms: (1) emergence of R4-tropic HIV or (2) binding of R5-tropic HIV to CCR5 in the presence of maraviroc. \[163,164\] In regard to the first mechanism, because the CCR5 antagonists exert their blockade only against R5-tropic HIV, the emergence of any type of X4-tropic virus (X-4 tropic, dual tropic, or mixed tropic) will allow HIV to bypass the CCR5 receptor blockade. The emergence of X4 tropic virus during treatment can result either from an expansion of preexisting X4-tropic virus that was not detected prior to starting the CCR5 antagonist \(\text{Figure 30}\) or a de novo tropism switch caused by multiple mutations in the HIV gp120 V3 region \(\text{Figure 31}\). \[51,164\] When a true HIV tropism shift from R5-tropic to X4-tropic occurs, it is frequently associated with multiple mutations in the HIV gp120 V3 region, including G11R, P13R, and A25K. \[32,33\] A second type of resistance to maraviroc can occur independent of a change in HIV tropism and this involves binding of the gp120 region R5-tropic HIV to the CCR5 coreceptor in the presence of a CCR5 antagonist; the exact mechanism for how the R5-tropic HIV circumvents the effect of the CCR5 antagonist has not been completely elucidated, but likely involves enhanced affinity of HIV gp120 binding to the N-terminal domain region of the CCR5 coreceptor \(\text{Figure 32}\). \[63,163,165,166\] Resistance involving R5-tropic HIV has been associated with mutations in the HIV envelope gp120 V3 loop that alter the binding properties of HIV, but predictable and characteristic mutations are not well defined. \[163\]

Maraviroc

Maraviroc is the only CCR5 antagonist approved for use by the United States Food and Drug Administration. Most cases of virologic failure that occur in individuals taking maraviroc result from an outgrowth of X4-tropic HIV. \[163,164\] Available data from earlier maraviroc studies have shown that outgrowth of X4 usually occurs from an expansion of preexisting X4-tropic virus that was not originally detected, rather newly formed X4-tropic HIV as a result of mutations causing a switch from R5 to X4 tropism. \[163\] The failure to detect X4-tropic virus in laboratory tropism assays was particularly a problem with the older less sensitive HIV tropism assay. \[164\] Because emergence of X4 virus is the most common reason for virologic failure on maraviroc, performing an HIV tropism test is recommended for all patients who develop virologic failure while taking maraviroc. Two major methods are used to determine HIV tropism: phenotypic testing and genotypic testing. The phenotypic test is performed by first generating laboratory pseudoviruses that express envelope proteins (gp120 and gp41) when combined with viruses obtained from a patient sample. These replication-deficient pseudoviruses are then used to infect laboratory target cell lines that express either CCR5 or CXCR4; the HIV tropism is then determined based on the cells that become infected (CCR5, CXCR4, or both). In contrast, the HIV-1 tropism genotype is performed by sequencing the V3-coding region of the env gene that is known to be the principal determinant of co-receptor usage; with this method, genotypic sequence of the HIV-1 V3-coding region is then used to predict the HIV-1-tropism. In general, the phenotypic tropism assay is preferred over the genotypic tropism assay. \[167\] Currently, there is no recommended modality for determining resistance in R5-tropic HIV that binds to CCR5 in the presence of maraviroc. If virologic failure occurs on maraviroc and the tropism assay result shows pure R5-tropic HIV, then it can be inferred that resistance has likely occurred that is allowing the R5-tropic HIV to bind to CCR5 in the presence of maraviroc.

Fusion Inhibitors (Enfuvirtide)

Enfuvirtide is the only fusion inhibitor approved for use in the United States. Enfuvirtide is a synthetic 36-amino-acid peptide that mimics amino acids 127-162 in the HR2 region of HIV-1 gp41 \(\text{Figure 33}\). \[168,169\]
In the HIV cell entry process, the HR2 and HR1 regions of HIV gp41 fold over each other in a hairpin-like loop to form a 6-helix bundle; the enfuvirtide peptide acts as a competitive decoy by binding to the HR1 region of gp41 in place of HR2 and thus prevents the binding of HR1 and HR2. Resistance to enfuvirtide is associated primarily with mutations in the HIV gp41 component of the HIV envelope; specific drug-resistant mutations have been identified in the HR1 region of the HIV gp41 envelope gene; these mutations correspond with codons 36 to 45 in the HR1 region of gp41 at the site where enfuvirtide binds. Less often, resistance is associated with mutations in the envelope gene that codes for a region of HR2 outside of amino acids 36-45 or gp120.[51,63] The mutations most often identified correspond to amino acids 36, 37, 38, 39, 40, 42, and 43.[51] A single one of these HR1 mutations is associated with about a 10-fold decreased susceptibility to enfuvirtide, which increases to 100-fold with a second mutation.[63] Enfuvirtide-resistance mutations reduce HIV replication capacity and accessory mutations can develop that help restore viral fitness.[63] As might be expected, HIV strains that emerge with resistance will often rapidly revert to wild-type virus soon after discontinuation of enfuvirtide.[170] Some data suggest an association between the development of enfuvirtide-induced gp41 resistance mutations and an increased CD4 cell count, possibly due to the mutation-induced decreased HIV replicative capacity, or to increased susceptibility of gp41 to neutralizing antibodies that target the fusion step in the HIV life cycle. [63]
Therapeutic Drug Monitoring

The clinical interest in HIV antiretroviral therapeutic drug monitoring most often arises in the setting of virologic failure in a patient who reports excellent adherence. In this situation, therapeutic drug monitoring may help to determine whether poor drug absorption has occurred or the history for adherence is not accurate. These issues can be sorted out by obtaining a drug level in two situations: (1) a random drug level at a visit linked to patient reported last dose of medication, and (2) directly observed administration of the medication in the clinic followed by a drug level at the appropriate time. Several antiretroviral medications demonstrate variability in drug-concentration among patients, and this may affect both the efficacy and toxicity of the medication in any given patient. For many of the available antiretroviral medications, however, the therapeutic threshold is uncertain and there is no evidence that therapeutic drug monitoring improves clinical outcomes. In addition, therapeutic drug monitoring for monitoring and determining toxicity is rarely performed in clinical practice. Consequently, the Adult and Adolescent ARV Guidelines do not recommend the routine use of therapeutic drug monitoring in the management of adults with HIV infection, but indicate therapeutic drug monitoring may have a role in the following situations:

- Suspicion for clinically significant drug-drug or drug-food interactions that may result in reduced efficacy or increased dose-related toxicities;
- Changes in pathophysiologic states that may impair gastrointestinal, hepatic, or renal function, thereby potentially altering drug absorption, distribution, metabolism, or elimination;
- Among pregnant women who have risk factors for virologic failure (e.g., those not achieving viral suppression during earlier stage of pregnancy)—during the later stages of pregnancy, physiologic changes may result in reduced drug exposure and thus further increase the risk of virologic failure;
- Heavily pretreated patients experiencing virologic failure and who may have viral isolates with reduced susceptibility to antiretroviral medications;
- Use of alternative dosing regimens and antiretroviral combinations for which safety and efficacy have not been established in clinical trials;
- Concentration-dependent, drug-associated toxicities; and
- Lack of expected virologic response in medication-adherent patients.

If therapeutic drug monitoring is used for patient management, providers should consult the most up-to-date literature on target drug concentrations, timing of when to obtain the drug level in relation to the last dose of medication, ideally in consultation with a clinical pharmacologist or clinical pharmacist. Drug levels alone should not dictate patient management but rather should complement other relevant clinical information, such as patient adherence and antiretroviral treatment history.
General Approach to Management of Confirmed Virologic Failure

Management of virologic failure can be challenging, and expert consultation is advised. The Adult and Adolescent ARV Guidelines outline appropriate steps to take when virologic failure is suspected, including assessing adherence and medication tolerability as well as evaluating possible pharmacokinetic issues (e.g. drug-drug or drug-food interactions), and has also outlined recommendations based on whether the patient is failing a first or subsequent regimen.[12] Since the goal is to suppress the patient’s viral load below the threshold of detection, a new antiretroviral therapy regimen should be designed using at least 2, and preferably 3, active agents; adding a single additional agent to a failing regimen is not recommended as it can risk the development of additional resistance mutations.[12] Given the possibility of archived or low frequency variant resistance mutations, which are not reliably detected with standard drug-resistance tests, the choice of antiretroviral medications should be based on both past and current drug-resistance test results as well as on treatment history.[12] In rare cases of very extensive drug resistance, a fully suppressive antiretroviral regimen may not be possible to achieve; in these cases, investigational therapies should be pursued.
Summary Points

- Among persons with HIV infection who are not on antiretroviral therapy, HIV replicates at a high rate, and a high mutation rate occurs during the replication process.
- The presence of resistant strains of HIV can lead to suboptimal response to antiretroviral therapy and subsequently, to virologic failure and the need for new antiretroviral regimens.
- Testing for antiretroviral resistance mutations is recommended at the time of entry into HIV care (including in the setting of acute HIV, if the patient presents at that time), at the time of antiretroviral therapy initiation (if not started at entry into HIV care), and in cases of virologic failure.
- Resistance mutations can be acquired through suboptimal virologic suppression (due to a variety of patient-related or antiretroviral regimen-related causes) or transmitted at the time of initial infection. The rate of transmitted drug resistance to at least one antiretroviral drug is between 6 and 21%.
- Three types of resistance tests are available to help guide clinicians in the choice of an appropriate antiretroviral regimen: genotype, phenotype, and virtual phenotype.
- Resistance tests provide the most accurate information when performed while the patient is on therapy and they most accurately reflect resistance to the medications currently being taken. When a patient stops antiretroviral therapy, wild-type virus tends to outgrow resistant virus, usually within 12 weeks of stopping therapy.
- Resistance testing is most successful when the HIV RNA level is above 1000 copies/mL.
- Resistance testing does have significant limitations; in particular, all types of resistance assays may fail to detect mutant strains that constitute a minority of the patient's HIV population.
- Resistance mutations affect drugs in all six classes of FDA-approved antiretroviral medications, with signature mutations associated with particular drugs.
- Management of virologic failure can be challenging, and expert consultation is advised to help assess causes for failure and design a new antiretroviral regimen, taking into account prior and current drug-resistance testing results as well as treatment history.
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Figures

Figure 1 High Error Rate with HIV Reverse Transcription

Illustration by David Spach, MD and David Ehlert, Cognition Studio

Error rate: 1 misincorporation in every 5,000-7,000 nucleotides polymerized
Figure 2 HIV Resistance Basic Concepts

This graphic illustrates the basic concept that with suboptimal antiretroviral therapy, as may occur with poor adherence, drug-resistant strains of HIV have a selective advantage and can emerge to become the dominant circulating strains of HIV.

Illustration by David Spach, MD
**Figure 3 Virologic Suppression**

Virologic suppression is defined as a confirmed HIV RNA level below the limit of assay detection (e.g., less than 48 copies/mL).
Incomplete virologic response is defined as two consecutive plasma HIV RNA levels greater than 200 copies/mL after 24 weeks on an ARV regimen. Note that baseline HIV RNA may affect the time course of response, and some regimens will take longer than others to suppress HIV RNA levels. Most assays can typically quantitate HIV if the plasma HIV RNA level is greater than 40 copies/mL.
Virologic rebound is defined as confirmed detectable HIV RNA (greater than 200 copies/mL) after virologic suppression.
Figure 6 Virologic Blip

In an individual with suppression of HIV RNA levels, a virologic blip is defined as an isolated detectable HIV RNA level followed by return to virologic suppression.
Figure 7 Low-Level Viremia

Low-level viremia is defined as HIV RNA levels above the level of detection of the assay but below 200 copies/mL.
Figure 8 Detectable HIV RNA Below the Limit of Quantification

As shown in the sample from patient 2, in some individuals with HIV infection, HIV RNA is detectable in a plasma sample but the amount of HIV RNA is so low (less than 40 copies/mL) that the laboratory assay cannot accurately quantitate the HIV RNA level. In this situation, the laboratory report typically states HIV-1 RNA was detected in this sample but below the assay’s limit of quantitation. This contrasts with the sample from patient 1 that corresponds with a quantitative HIV-1 RNA level since it is above 40 copies/mL. For the sample from patient 3, the HIV RNA level is extremely low and would not be detected on most standard commercial assays.
Conventional HIV drug resistance genotypes require a patient plasma sample and HIV is isolated from the plasma sample.

Illustration by David H. Spach, MD
The HIV is first isolated from the plasma sample, then reverse transcribed in the laboratory to form HIV DNA. The HIV DNA sample is amplified using PCR techniques and then sequenced. Conventional HIV genotype assays routinely sequence DNA for the reverse transcriptase and protease genes. Assays are also available that can sequence the HIV integrase and envelope genes.

Illustration by David H. Spach, MD
This image represents a schematic of normal HIV protein synthesis. In this process, HIV DNA is transcribed to HIV RNA, which is then translated into polypeptides that are eventually processed into proteins.

Illustration by David H. Spach, MD
Figure 9 (Image Series) - Conventional HIV Drug Resistance Genotypic Assay
Image 9D: Mutation in HIV DNA Leading to Amino Acid Substitution

Mutations in the HIV DNA nucleotides can result in amino acid substitutions that may impact specific regions or functions of HIV proteins.

Illustration by David H. Spach, MD
In contrast to a conventional HIV drug resistance assay, which is performed on a patient plasma sample and typically requires HIV RNA levels of at least 500 copies/mL or more, a HIV DNA drug resistance assay is performed on whole blood and it detects proviral DNA that is incorporated into host DNA in cells infected with HIV. The HIV DNA resistance assay can be performed in patients who have undetectable plasma HIV RNA levels.

Illustration by David H. Spach, MD
**Figure 11 Indication for HIV Drug Resistance Testing**

The Adult and Adolescent ARV Guidelines recommends routine HIV drug resistance testing in the situations shown in this figure.


<table>
<thead>
<tr>
<th>Clinical Setting for Ordering HIV Drug Resistance Testing</th>
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<tr>
<td><strong>Acute HIV infection</strong></td>
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<tr>
<td><strong>Entry into care regardless of timing for starting ARV therapy</strong></td>
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<tr>
<td><strong>Virologic failure and HIV RNA &gt;1,000 copies/mL</strong></td>
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<tr>
<td>(consider if HIV RNA &gt; 500 but &lt; 1,000 copies/mL)</td>
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<tr>
<td><strong>Patients with suboptimal HIV RNA suppression</strong></td>
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<tr>
<td><strong>Pregnant women with HIV</strong></td>
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In situations where HIV drug resistance has developed while an individual is taking antiretroviral therapy, the discontinuation of the antiretroviral therapy regimen will remove the selective pressure on HIV and some drug-resistant mutants may back mutate to wild-type HIV. In addition, in this situation, wild-type HIV may have greater fitness than mutated strains and thus growth of wild-type strains may outpace drug-resistant strains. Accordingly, it is optimal to obtain resistance testing while the patient is on antiretroviral therapy or promptly after discontinuation.

Illustration by David H. Spach, MD
Figure 13 HIV DNA Mutations Resulting in Amino Acid Changes

Mutations in the DNA sequence are analyzed to predict amino acid substitutions in the HIV polypeptide.

Illustration by David H. Spach, MD
**Figure 14 Amino Acid Substitution at Position 103**

In this example, the amino acid lysine (K) has been replace by asparagine (N) at amino acid position 103 in the reverse transcriptase protein. The amino acid position 103 is located in the outer rim of pocket where NNRTIs bind.

Illustration by David H. Spach, MD
Figure 15 Interpretation of HIV Drug Resistance Genotype Report

The HIV genotype provides information based on the inferred amino acid substitutions predicted by the HIV DNA sequence and these substitutions are compared with wild-type HIV amino acid sequences. The genotype resistance report lists the wild type amino acid, followed by the position of this amino acid, followed by the amino acid that has replaced the wild type amino acid at the position listed.

103 = codon (amino acid position)

K = Wild-type amino acid  N = Mutant amino acid
Figure 16 Method for Calculating Level of Phenotypic Resistance

This graph shows the method for calculating the level of phenotypic resistance of a single antiretroviral medication. The antiretroviral drug is tested on a patient's HIV isolate and a laboratory reference (wild-type strain). The IC50 represents the concentration of the antiretroviral drug required to cause 50% inhibition of HIV replication. The fold change is calculated by dividing the IC50 of the patient's isolate by the IC50 of the wild-type laboratory strain. As shown, as the curve shifts to the right, a higher concentration of drug would be required to inhibit HIV replication and thus the strain of HIV would be more resistant. The further the curve shifts to the right (for the patient's HIV strain tested), the greater the level of resistance.
This graph shows a phenotypic susceptibility curve comparing the effect of a single antiretroviral drug on the patient's HIV and a laboratory reference (wild-type strain). The wild-type strain is known to be susceptible to the drug tested. The graph shows a similar IC50 for both the patient and wild-type HIV and this would be interpreted that the patient's HIV is susceptible to the drug tested in this assay.
Image 17B: Drug-Resistant HIV

This graph shows a phenotypic susceptibility curve comparing the effect of a single antiretroviral drug on the patient's HIV and a laboratory reference (wild-type strain). The wild-type strain is known to be susceptible to the drug tested. The graph shows a significant shift to the right for the patient's HIV isolate compared with the wild-type strain, thus a higher concentration of drug is required to inhibit replication of the patient's HIV. Conceptually, this graph is showing the patient's HIV strain is resistant to the medication tested. In the actual phenotypic assay, the exact level of resistance is calculated by dividing the IC50 of the patient's isolate by the IC50 of the wild-type laboratory strain.
This graph shows a phenotypic susceptibility curve comparing the effect of a single antiretroviral drug on the patient's HIV and a laboratory reference (wild-type strain). The wild-type strain is known to be susceptible to the drug tested. The graph shows a significant shift to the left for the patient's HIV isolate compared with the wild-type strain, thus a lower concentration of drug is required to inhibit replication of the patient's HIV. Conceptually, this graph is showing the patient's HIV strain is hypersusceptible to the medication tested. In the actual phenotypic assay, the exact level of hypersusceptibility is calculated by dividing the IC50 of the patient's isolate by the IC50 of the wild-type laboratory strain.
Figure 18 Discrimination (Decreased Incorporation) Mechanisms for HIV Resistance to NRTIs

Illustration by David Spach, MD
Figure 19 Excision (Primer Unblocking) Mechanisms for HIV Resistance to NRTIs

Illustration by David Spach, MD
**Figure 20 Thymidine Analog Mutations (TAMs)**

The thymidine analog mutations arise in the setting of inadequate virologic suppression with an antiretroviral therapy regimen that contains either zidovudine or stavudine.

Figure 21 Thymidine Analog Mutation (TAM) Resistance Pathway

Figure 22 Interaction of NNRTI Medication with the Binding Pocket of Reverse Transcriptase

The NNRTIs exert their action by attaching to a region of the binding pocket of reverse transcriptase.

Illustration by David Spach, MD and David Ehlert, Cognition Studio
Resistance to NNRTIs can result from mutations that impact amino acids surrounding the binding site thereby preventing the NNRTI from entering into the binding pocket. This is referred to as the altered binding mutation.

Illustration by David Spach, MD and David Ehlert, Cognition Studio
Resistance to NNRTIs can result from mutations that impact amino acids surrounding the binding site thereby preventing the NNRTI from entering into the binding pocket. This is referred to as the reduced access mutation.

Illustration by David Spach, MD and David Ehlert, Cognition Studio
**Figure 25 Major Primary Integrase Resistance Mutations**

HIV integrase enzyme is a 288-amino acid enzyme comprised of three structural domains: C-terminal domain, N-terminal domain, and the catalytic core domain. Eight major primary integrase resistance mutations have been identified. Note that all of these major primary resistance mutations are located in the catalytic core domain region of the integrase enzyme.

Illustration by David Spach, MD and David Ehlert, Cognition Studio

### Major Primary Integrase Mutations

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**HIV Integrase**

- **C-terminal domain (CTD)**
- **N-terminal domain (NTD)**
- **Catalytic core domain (CCD)**

1 47 59 202 223 270 288
Figure 26 Raltegravir Resistance Pathways

Drug resistance to raltegravir occurs most often in one of three pathways: Q148, N155, and Y143. As shown by the relative sizes of the arrows, the Q148 pathway is the most common pathway and it has the greatest impact on raltegravir-associated resistance. The N155 pathway is the next most common pathway, but because this mutation does not impact raltegravir nearly as much as the Q148, there is frequent cross-over from the N155 pathway to the Q148 pathway.

In this study, investigators followed 29 individuals who had incomplete virologic suppression on a regimen that contained raltegravir. With each subsequent visit, a progressive number of integrase mutations developed, translating to higher levels of resistance to raltegravir and to other integrase strand transfer inhibitors.

**Figure 28 HIV Protease and Location of Amino Acid Resistance Mutations**

Illustration by David Spach, MD and David Ehlert, Cognition Studio

![HIV Protease and Location of Amino Acid Resistance Mutations](image-url)
**Figure 29 CCR5 Antagonists**

The binding of the CCR5 antagonist maraviroc causes a conformation change in the extracellular loop region of the CCR5 coreceptor. The changes in the CCR5 coreceptor that occur do not involve significant changes in the N-terminal region of the CCR5 coreceptor.

Illustration by David H. Spach, MD
**Figure 30 Emergence of Preexisting X4-Tropic Virus**

This illustration shows the emergence of preexisting minority variants of X4-tropic virus that are preferentially selected out from the use of a CCR5 antagonist.

Illustration: David H. Spach, MD
Figure 31 Emergence of New X4-Tropic Virus

This illustration shows emergence of newly formed X4-tropic HIV as a result of mutations in the HIV gp120 region. This contrasts with emergence of preexisting minority variants of X4-tropic virus.

Illustration: David H. Spach, MD
Resistance to maraviroc can occur when R5-tropic HIV-1 develops mutations that facilitate the gp120-CCR5 coreceptor binding despite maraviroc attachment to the CCR5 coreceptor and receptor conformational changes. When this type of resistance occurs, the binding of HIV-1 gp120 occurs with enhanced affinity at the CCR5 N-terminal domain region.

Illustration: David H. Spach, MD
The antiretroviral medication enfuvirtide is a synthetic 36-amino-acid peptide that mimics a segment of the HR2 region of HIV-1 gp41. The medication binds to the corresponding HR1 region and thus prevents the normal HR1-HR2 binding that is critical for HIV-1 to form the 6-helix bundle.
Table 1. **Guidelines for the Use of Antiretroviral Agents in Adults and Adolescents with HIV**

### Causes of Virologic Failure

<table>
<thead>
<tr>
<th><strong>Patient/Adherence-Related Factors</strong></th>
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<tr>
<td>• Comorbidities that may affect adherence (e.g. active substance abuse, mental health disorders, neurocognitive impairment)</td>
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<td>• Unstable housing and other psychosocial factors</td>
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<td>• Missed clinic appointments</td>
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<td>• Interruption of or intermittent access to antiretroviral therapy</td>
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<tr>
<td>• Cost and affordability of ARVs (i.e. these factors may affect the ability to access or continue therapy)</td>
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<tr>
<td>• Adverse drug effects</td>
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<td>• High pill burden and/or dosing frequency</td>
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<table>
<thead>
<tr>
<th><strong>HIV-Related Factors</strong></th>
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<tbody>
<tr>
<td>• Presence of transmitted or acquired drug-resistant virus documented by current or past resistance test results</td>
</tr>
<tr>
<td>• Prior treatment failure</td>
</tr>
<tr>
<td>• Innate resistance to antiretrovirals due to viral tropism or the presence of HIV-2 infection/coinfection</td>
</tr>
<tr>
<td>• Higher pretreatment HIV RNA level (some regimens may be less effective at higher levels)</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th><strong>Antiretroviral Regimen-Related Factors</strong></th>
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<tr>
<td>• Suboptimal pharmacokinetics (PKs) (e.g. variable absorption, metabolism, or possible penetration into reservoirs)</td>
</tr>
<tr>
<td>• Suboptimal virologic potency</td>
</tr>
<tr>
<td>• Low genetic barrier to resistance</td>
</tr>
<tr>
<td>• Reduced efficacy due to prior exposure to suboptimal regimens (e.g. monotherapy, dual nucleoside reverse transcriptase inhibitor (NRTI) therapy, or the sequential introduction of drugs)</td>
</tr>
<tr>
<td>• Food requirements</td>
</tr>
<tr>
<td>• Adverse drug-drug interactions with concomitant medications</td>
</tr>
<tr>
<td>• Prescription errors</td>
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Source:
