HIV-1 MUTAGENESIS DURING ANTIRETROVIRAL THERAPY: IMPLICATIONS FOR SUCCESSFUL DRUG TREATMENT

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1. ABSTRACT

The evolution of antiretroviral drug resistance is a major problem in the treatment of human immunodeficiency virus type 1 (HIV-1) infection. Drug therapy failure is associated with accumulation of mutations and results in the development of drug resistance. Drugs targeted against reverse transcriptase (RT) as well as drug-resistant RT have been shown to increase HIV-1 mutation frequencies. Furthermore, combinations of drug and drug-resistant RT can increase virus mutation frequencies in a multiplicative manner. The evolution of drug resistance also alters virus fitness. The correlation of increased HIV-1 mutation rates with the evolution of antiretroviral drug resistance indicates that drug failure could increase the likelihood of further resistance evolving from subsequent drug regimens. These observations parallel studies from microbial systems that provide evidence for a correlation between drug resistance development and increased pathogen mutation rates. Although increased mutant frequencies may be detrimental to effective therapy, the lethal mutagenesis of the HIV-1 genome may provide a new means for antiretroviral therapy.

2. INTRODUCTION

Drug therapy to HIV infection is typically done with combination therapy that consists of nucleoside reverse transcriptase inhibitors (NRTI’s), non-nucleoside reverse transcriptase inhibitors (NNRTI’s) and protease inhibitors (PI’s), which has dramatically reduced the rate of HIV-1 and AIDS-related morbidity and mortality. The lack of compliance to drug administration may result in suboptimal therapy, which can lead to drug resistance. Drug resistance limits the clinical benefit of drug treatment.

The HIV-1 mutation rate is high (i.e., 4 x 10^5 mutations per target bp per replication cycle, which correlates to about one mutation in every 3 new genomes produced) and likely aids in the rapid development of drug resistance during suboptimal therapy (1). Thus, viral genomes with each possible mutation as well as many with double mutations are likely generated each day, allowing for the rapid selection and fixation of mutations that confer drug resistance. Drug-resistant virus can reside in latently infected cells, which further complicates subsequent drug treatment regimes during initial and salvage therapy. The accumulation of drug-resistance mutations correlates with reduced drug susceptibility and potency of antiretroviral therapy (ART). The continued replication in the presence of drug can select for even greater levels of resistance and typically leads to cross-resistance to drugs of the same class. Transmission of HIV-1 with reduced susceptibility to antiretroviral drugs may compromise the efficacy of drug therapy (2).

3. ANTIRETROVIRAL DRUGS AND INCREASED HIV-1 MUTAGENESIS

The ability of drugs to influence retrovirus mutation rates was first observed over 10 years ago, and can be influenced by alteration of dNTP pools (3-5). The impact of drugs on HIV-1 mutation rates was first studied by testing how the NRTI’s 3’-azido-3’-deoxythymidine (AZT) and 2’, 3’-dideoxy-3’-thiacytidine (3TC), as well as AZT- and 3TC-conferring resistance mutations, influence the HIV-1 mutation rate (6). These analyses used the lacZ alpha peptide gene as a mutation target, which has been used in previous mutation rate studies of HIV-1. AZT increased the HIV-1 mutation rate by 7.6-fold in a...
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Table 1. Drugs and drug-resistant reverse transcriptases that have been shown to increase HIV-1 mutant frequencies

<table>
<thead>
<tr>
<th>Drug</th>
<th>Drug-resistant reverse transcriptase (RT)</th>
<th>Mutant frequency increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>AZT</td>
<td>wt RT</td>
<td>7.6</td>
</tr>
<tr>
<td>3TC</td>
<td>wt RT</td>
<td>3.4</td>
</tr>
<tr>
<td>Hydroxyurea</td>
<td>wt RT</td>
<td>4.5</td>
</tr>
<tr>
<td>Thymidine</td>
<td>wt RT</td>
<td>7.0</td>
</tr>
<tr>
<td>Thioguanine</td>
<td>wt RT</td>
<td>4.0</td>
</tr>
<tr>
<td>ddI</td>
<td>wt RT</td>
<td>6.0</td>
</tr>
<tr>
<td>-</td>
<td>AZT-resistant RT</td>
<td>4.3</td>
</tr>
<tr>
<td>-</td>
<td>3TC-resistant RT</td>
<td>1.0</td>
</tr>
<tr>
<td>AZT</td>
<td>AZT-resistant RT</td>
<td>24.0</td>
</tr>
<tr>
<td>3TC</td>
<td>AZT-resistant RT</td>
<td>13.6</td>
</tr>
<tr>
<td>AZT/3TC</td>
<td>AZT-3TC-dual-resistant RT</td>
<td>22.5</td>
</tr>
<tr>
<td>Hydroxyurea</td>
<td>AZT-resistant RT</td>
<td>21.8</td>
</tr>
<tr>
<td>Thymidine</td>
<td>AZT-resistant RT</td>
<td>16.7</td>
</tr>
</tbody>
</table>

Single round of replication, while 3TC increased the virus mutation rate by 3.4-fold (Table 1). AZT-resistant RT was also found to influence the mutation rate. In particular, HIV-1 replication with AZT-resistant RTs increased the mutation rate by as much as 4.3-fold, while replication of HIV-1 with a 3TC-resistant RT had no significant effect on the mutation rate. It was observed that only high-level, AZT-resistant RT variants could influence the in vivo mutation rate (i.e., those containing the mutations M41L/T215Y and M41L/D67N/K70R/T215Y).

Further studies of drug-resistant RTs have indicated that other amino acid residues in HIV RT associated with drug resistance can increase virus mutant frequencies when mutated. One example is the Y501F RT mutant, which leads to a 4-fold increase in virus mutant frequencies (7). The Y501 residue is located in the RNase H primer grip region of HIV RT and is associated with resistance to N-(4-tert-Butylbenzoyl)-2-hydroxynaphthaldehyde hydrazone (BBNH), which is a potent inhibitor of RNase H activity (8).

Recent studies with other NRTI’s (i.e., ddI, stavudine, and abacavir) indicate that NRTI drug treatment may generally lead to increased virus mutant frequencies during HIV-1 replication (R. Chen and L.M. Mansky, unpublished observations) (9). How NRTI's increase HIV-1 mutant frequencies is presently not known, but likely involves a similar mechanism. This is supported by the observation that virus mutant frequencies increase in an additive manner during virus replication in the presence of two NRTI’s (i.e., AZT and 3TC, AZT and ddI, and 3TC and ddI) (9).

These observations suggest that when virus replication occurs in the presence of suboptimal concentrations of drug, drug-resistant virus is selected for and that replication of drug-resistant virus in the presence of drug could further increase the virus mutation rate. To test this hypothesis, the combined effects of drug and drug-resistant virus were analyzed (10). It was found that the replication of AZT-resistant HIV-1 in the presence of AZT led to a multiplicative 24-fold increase in the virus mutant frequency to that observed with wild-type virus in the absence of drug (Table 1). This indicates that when drug failure occurs due to the evolution of drug resistance, replication of the drug-resistant virus in the presence of AZT significantly increases HIV-1 mutagenesis. In addition, it was observed that replication of a AZT/3TC dual-resistant virus in the presence of AZT and 3TC also led to a multiplicative 22.5-fold increase in mutant frequencies (Table 1). Thus, each of these drugs tested acted together with drug-resistant RT and increased virus mutant frequencies.

4. IMPACT OF SALVAGE THERAPY ON HIV-1 MUTAGENESIS

When HIV-infected individuals fail potent ART due to the development of drug resistance, salvage therapy is necessary. Drug resistance may occur from a lack of medication compliance or drug discontinuation. Therapy failure can be associated with poor tolerability, persistence of virus in immunological privileged sites, lack of drug potency, and low drug plasma levels. The selection of salvage therapy requires an analysis of the resistance mutations that led to therapy failure, as well as suppressed resistance mutations that could emerge under new therapy. Drug resistance mutations that confer cross resistance to other drugs also needs to be analyzed.

Interestingly, different drugs used in conjunction with the AZT-resistant virus led to a similar multiplicative increase in virus mutant frequencies (10). This indicates that when new drugs are added in drug therapy regimens they could also act with the drug-resistant virus to further increase virus mutant frequencies even though the drug-resistance phenotype is associated with another drug. For example, 3TC increased mutant frequencies of AZT-resistant virus to 13.6-fold compared to that with wild-type virus in the absence of drug (Table 1). Hydroxyurea, a well-documented drug used in HIV-1 treatment, is known to alter intracellular dNTP pools by inhibiting ribonucleotide reductase and results in a depletion of all dNTPs. AZT-resistant HIV-1 replication in the presence of hydroxyurea resulted in a 21.8-fold increase in mutant frequencies compared to that observed in the absence of drug (Table 1). Like hydroxyurea, thymidine has also been shown to alter intracellular dNTP pools and in addition has been shown to increase retrovirus mutation rates. AZT-resistant HIV-1 replication in the presence of thymidine increased mutant frequencies by 16.7 fold (Table 1). Thioguanine (an antileukemic agent that has been reported to inhibit RNase H activity) has been shown to increase HIV-1 mutant frequencies by 4-fold and to significantly alter mutant frequencies during virus replication with RTs containing mutations not associated with the drug (7). These data suggest that subsequent therapies could lead to increased HIV-1 mutagenesis even though the drug-resistant phenotype is not directed against the new drug(s) used in the drug therapy regimen.

5. THERAPEUTIC APPLICATION OF INCREASED HIV-1 MUTAGENESIS

The data discussed above predict that when drug failure occurs during NRTI HIV-1 chemotherapy, there is
The HIV-1 Vif protein is essential for viral replication in non-permissive cells such as primary T cells and macrophages as well as some CD4+ transformed T cell lines. Recently, a cellular protein, CEM 15 (also known as APOBEC3G) was identified as a specific antiviral factor in nonpermissive cells whose antiviral activity was overcome by the presence of Vif (17). APOBEC3G is a member of the family of RNA editing enzymes that deaminate cytosine residues to uracil in DNA or mRNA (18). The members of this family (e.g., activation-induced deaminase, AID, APOBEC1, and APOBEC3G) may function as a DNA mutator in *E. coli* (19). When ∆vif-viruses are produced from cells expressing APOBEC3G contain G-to-A hypermutations in newly synthesized plus-strand of viral DNA, which suggests an activity of APOBEC3G that results in the deamination of cytosine to uracils in minus-strand DNA during reverse transcription. Furthermore, Vif expression in virus producer cells prevented the accumulation of G-to-A mutations, suggesting that APOBEC3G is the critical component of innate defense mechanism for HIV-1 by causing instability of incoming nascent viral reverse transcripts or by inducing lethal mutagenesis (20-23).

Vif can complex with APOBEC3G, and this interaction can prevent the encapsidation of ABOBEC3G into HIV-1 particles (24). However, HIV-1 Vif does not inhibit the incorporation of mouse or African green monkey (AGM) APOBEC3Gs into HIV-1 virions and the antiviral activity of these APOBEC3Gs was maintained (25). This indicates that the mouse and AGM APOBEC3Gs can be potent inhibitors of HIV-1 replication, even in the presence of Vif. Therapeutic intervention could be envisioned by the development of a new class of antiviral drugs that exploit the properties of APOBEC3G virion incorporation to induce viral DNA damage and/or HIV-1 lethal mutagenesis. Cytosine deamination and the creation of uracil residues during minus-strand DNA synthesis can block virus replication in at least three different ways. First, the presence of uracil can destabilize DNA, causing it to degrade before integration. Second, uracils in the minus-strand DNA can impair the initiation of plus-strand DNA synthesis during reverse transcription (26). Third, the deamination of many cytosines in minus-strand DNA will cause G-to-A substitutions in plus-strand of viral DNA that results in amino-acid changes and the aberrant introduction of stop codons in viral proteins which will reduce viral fitness. In summary, the use of APOBEC3G by the cell as an innate immune response to HIV-1 infection may lead to the development of a novel class of antiretroviral drugs.

6. HIV DRUG RESISTANCE AND VIRAL FITNESS

Studies on HIV-1 fitness of drug-resistant mutants under different selective pressures have led to a better understanding of emergence of specific drug resistance mutations during therapy, as well as risk/benefit of these mutations for HIV-infected individuals. Fitness is a parameter defining the replicative adaptation of an organism to its environment (reviewed in (27)), and survival of the fittest is the concept that drives evolution in a complex population. For example, in HIV quasispecies...
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Each clone has a fitness value, representative of those viral properties undergoing selection in that particular environment. Therefore, positive selection implies that one or more members of the quasispecies are better suited to a given environment while negative selection eliminates unfit variants (27).

The current goal of any antiretroviral therapy regimen is to maintain low-to-undetectable plasma HIV-RNA levels, which minimizes the possible emergence of drug-resistant HIV-1 variants and prevents progression to AIDS (28). In the absence of antiretroviral therapy, strains containing drug-resistance mutations have a reduced fitness compared to the wild-type (wt) quasispecies within the population (29). Therefore, selective pressure in the form of drug therapy leads to selection and competition of several drug-resistant variants within the viral quasispecies, which will pass through the drug-induced bottleneck and initiate a new quasispecies distribution that will be governed again by replication efficiency (29, 30).

During antiretroviral therapy two types of mutations are associated with drug resistance and respective changes in viral fitness. An initial decrease in fitness coincides with the appearance of primary substitutions conferring direct drug resistance. Continued drug pressure allows the selection of secondary or compensatory mutations to restore the enzymatic activity of the drug-targeted resistant enzyme (protease, PR or reverse transcriptase, RT) leading to a rebound in fitness, similar if not greater than the fitness of the quasispecies prior to treatment (28, 31, 32). The appearance of specific mutations is often highly dependent on the baseline sequence and the sequential selection of “de novo” compensatory mutations that contribute to viral fitness (33-35). Finally, it is important to note that, in the human host, the most fit wild-type HIV-1 sequence differs from the drug-resistant virus (28, 31, 32).

5. FITNENESS OF REVERSE TRANSCRIPTASE INHIBITOR (RTI)-RESISTANT HIV VARIANTS

Although amino acid changes conferring RTI-resistance do not appear to reduce viral fitness to the same extent as PI-resistant mutations (31, 32), multiple studies have explored how RTI-resistant mutations affect HIV-1 replication capacity (reviewed in 32, 36, 37)). NRTIs were the first class of antiretroviral drugs to be approved for anti-HIV-1 therapy (38). Thus, it is not surprising that some of the first studies showing the effect of drug-resistance mutations on viral replication fitness were related to AZT (39). A stepwise accumulation of AZT resistance mutations (70R, 215Y, and 41L) during in vitro selection was similar to that observed in vivo (40). Interestingly, ordered accumulation of resistant variants was also predictive of relative fitness (i.e., wt > 70R >> 215Y = 41L/215Y >> 41L). Similarly, lamivudine (3TC)-resistant viruses, harboring the 184V mutation, appear to have diminished RT processivity and reduced replication capacity (41, 42).

Resistance mutations associated to NNRTIs, non-competitive inhibitors that bind to a hydrophobic pocket adjacent to the polymerase active site of RT (43), results in a slight decrease in RT polymerase activities relative to RNase H activity of the enzyme (44, 45). However, the viral replicative capacity of NNRTI-resistant viruses has not been extensively studied. Available data suggest that single-point mutations such as 103N or 181C, selected during NNRTI treatment, have limited effects on viral fitness but confer high level resistance and persist in the absence of drug pressure. Unlike other antiretroviral drugs, the genetic barrier to NNRTI resistance is minimal and is found at high frequencies in the HIV-1 quasispecies in the absence of therapy. Interestingly, related lentiviral polymerases (e.g., HIV-1 group O and HIV-2) sharing conserved structural RT domains are resistant to NNRTIs (37, 46, 47). Most HIV-1 group O isolates are intrinsically resistant to NNRTIs due to the presence of three amino acid substitutions (i.e., 98G, 179E, and 181C) in RT (37), which obviously do not affect the wild-type fitness.

8. VIRAL FITNESS AND ITS POTENTIAL INFLUENCE ON ERROR CATASTROPHE

There is little information on the effects of viral load, viral fitness, and the types/numbers of mutations associated with a loss of viral infectivity of RNA viruses related to increased mutagenesis. VSV fitness gain during population passages in cell culture was severely limited by the presence of mutagenic agents (48). Using FMDV as a model, both low viral load and low viral fitness contributed to FMDV extinction in the presence of the mutagenic base analogs FU and AZC (49, 50). In the case of HIV-1, drug treatment often led to a decrease in viral load, while selection of drug-resistant HIV-1 commonly decreased viral fitness (51). During antiretroviral therapy this initial decrease in fitness coincides with the appearance of primary substitutions conferring direct drug resistance (51). In addition, previous studies have suggested that both reverse transcriptase inhibitors (e.g., AZT) and AZT-resistant viruses can increase the mutation rate of HIV-1 (6) and feline immunodeficiency virus (FIV) (52). Progressive accumulation of mutations throughout the HIV-1 genome, some of which would diminish viral replication and fitness, may eventually lead to exceed the error threshold for maintenance of the viral quasispecies, resulting in viral extinction. Thus, it seems plausible that HIV-1 quasispecies would be more susceptible to extinction by lethal mutagenesis at low viral loads and impaired viral fitness.

9. ANTIMICROBIAL DRUG RESISTANCE AND INCREASED PATHOGEN MUTATION RATES

There is a growing body of literature indicating that mutator alleles are selected for in microbial populations, particularly in response to environmental stress (53, 54). For instance, the emergence of antimicrobial resistance during drug therapy can increase the likelihood of selection for mutator alleles, as well as increase the probability of failure of subsequent drug therapies (55, 56) (Table 2). The generation of drug resistance depends on the rate of emergence of resistant mutants which is defined by the mutation rate. In bacteria,
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| Table 2. Examples of antimicrobial resistance associated with increased pathogen mutation rates |
| Example | Drug Resistance | Reference |
| E. coli | Rifampin | 62 |
| E. coli | Streptomycin | 60 |
| E. coli | Fluoroquinolone | 71 |
| S. aureus | Vancomycin | 72 |
| S. pneumoniae | Cefotaxime | 61 |
| P. aeruginosa | Rifampicin | 57 |
| H. pylori | Rifampicin | 73 |

| Table 3. Enzyme-encoding genes that can act as mutator alleles |
| 1. Enzymes that protect DNA from various DNA damaging agents |
| 2. Enzymes that degrades modified nucleotides |
| 3. DNA polymerases |
| 4. Post replicative methyl-directed mismatch repair enzymes |

there are many examples indicating that antibiotic treatment not only selects for antibiotic-resistant bacteria, but also selects for mutator alleles which confer a higher mutation rate (57-65). Correlations between mutation rate and the efficacy of antimicrobial drug treatment have been recently observed (66). Error-prone polymerases and repairs of the mismatch repair system, along with mutations of enzymes that protect DNA from DNA damaging agents and enzymes that degrade modified nucleotides, have been implicated as the ultimate mechanisms responsible for these mutator phenotypes (Table 3) (57, 67-69). A recent study of *Mycobacterium tuberculosis* has identified a DNA polymerase, DNA polymerase E2, which is upregulated when *M. tuberculosis* is exposed to DNA damaging agents (i.e., UV irradiation, mitomycin C, and hydrogen peroxide) (70). A *M. tuberculosis* DnaE2 mutant was severely attenuated for long-term murine infections, suggesting that continued repair of DNA damage during infection is essential for survival. An implication of this work is that DnaE2 contributes to the creation of mutations that are better adjusted for survival during infection and/or antimicrobial drug therapy. This suggests that ongoing DNA repair is an essential process during persistent infection and provides a new potential target for preventing the emergence of drug-resistant *M. tuberculosis*.

10. SUMMARY

In summary, increased HIV-1 mutation rates can be associated with the evolution of drug resistance, and this observation correlates with observations made in bacterial systems with antimicrobial drug resistance. The current management of HIV-1 infection involves combinations of NRTI, NNRTI, and protease inhibitors that are changed over time when drug resistance occurs. The transmission of drug-resistant HIV-1 along with the development of drug-resistant virus raises concerns about the efficacy of drug regimens due to the presence of mutator phenotypes. Future studies should be directed at determining the risk of these mutator phenotypes with the potential for more rapid development of HIV-1 drug resistance to NRTI’s. In addition, the unintentional increase in HIV mutagenesis by NRTIs could be used for improving the efficacy of drug therapy by the rational selection NRTI combinations that either minimize the potential for HIV mutagenesis or intentionally increase HIV mutagenesis to induce (perhaps along with a mutagen or ABOBEC3G) lethal mutagenesis. It has been argued that HIV-1 population sizes in infected individuals is very large (29), but recent studies have suggested that that population sizes may be relatively small (74, 75). Small population sizes would predict that the relatively small changes in mutation rate caused by HIV-1 mutagenesis during drug therapy would influence HIV-1 evolution.

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