The 3'-Azido Group Is Not the Primary Determinant of 3'-Azido-3'-deoxythymidine (AZT) Responsible for the Excision Phenotype of AZT-resistant HIV-1*

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The mechanism of human immunodeficiency virus (HIV) 1 resistance to 3'-azido-3'-deoxythymidine (AZT) involves reverse transcriptase (RT)-catalyzed phosphorolytic excision of the chain-terminating AZT-5'-monophosphate (AZTMP). Primers terminated with AZTMP are generally better substrates for this reaction than those terminated with 2',3'-dideoxynucleoside-5'-monophosphate (2',3'-ddNMP) analogs that lack a 3'-azido moiety. This led to the hypothesis that the 3'-azido group is a major structural determinant for maintaining the primer terminus in the appropriate site for phosphorolytic excision of AZTMP by AZT-resistant (AZTR) RT. To test this hypothesis, we evaluated the incorporation, phosphorolytic excision, and antiviral activity of a panel of 3'-azido-2',3'-ddN including 3'-azido-2',3'-ddA (AZdDA), 3'-azido-2',3'-ddC (AZdDC), 3'-azido-2',3'-ddG (AZdDG), AZT, and 3'-azido-2',3'-ddU (AZdDU). The results indicate that mutations correlated with resistance to AZT (D67N/K70R/T215F/K219Q) confer resistance to the 3'-azidopyrimidine nucleosides (AZdDC, AZT, and AZdDU) but not to the 3'-azidoarminucleosides (AZdDA and AZdDG). The data suggest that the presence of a 3'-azido group on the 3'-terminal nucleotide of the primer does not confer increased phosphorolytic excision by AZTR for all 3'-azido-2',3'-ddN analogs. Thus, the 3'-azido group cannot be the only structural determinant important for the enhanced phosphorolytic excision of AZTMP associated with HIV resistance to AZT. Other structural components, such as the base, must play a role in defining the specificity of the excision phenotype arising from AZT resistance mutations.

The replication of human immunodeficiency virus (HIV) 1 is dependent on the enzymatic activities of reverse transcriptase (RT), an RNA- and DNA-dependent DNA polymerase encoded by the viral pol gene. RT synthesizes the double-stranded proviral DNA precursor from the viral (+) RNA genome (1). Because of its essential role in HIV-1 replication, RT is a major target for antiretroviral drug development and two structurally dissimilar classes of RT inhibitors, termed nucleoside RT inhibitors (NRTI) and nonnucleoside RT inhibitors, are routinely used for the clinical treatment of HIV-1-infected individuals (2). NRTI are 2',3'-dideoxyribonucleoside analogs that usually lack a 3'-OH group on the ribose moiety. After intracellular conversion to the active 5'-triphosphate form, NRTI-TP inhibit DNA synthesis by competing with the natural nucleotides both for recognition by RT as a substrate and by incorporation into the nascent viral DNA chain (3). Incorporation of an NRTI into the nascent viral DNA chain by RT results in termination of DNA synthesis.

As is the case with all antiretroviral agents, the emergence of drug-resistant HIV-1 variants limits the efficacy of NRTI. Most NRTI-resistant viruses isolated from patients treated with nucleoside analogs have mutations in the pol gene (4). To date, two major phenotypic mechanisms have been proposed to account for HIV-1 resistance to NRTIs (5, 6). One mechanism is NRTI discrimination in which the mutant RT can preferentially incorporate the natural dNTP over the NRTI-TP analog. The second mechanism involves pyrophosphate- or ATP-dependent phosphorolytic excision of the chain-terminating NRTI from the 3’-end of the primer by HIV-1 RT (7-9). This excision phenotype has primarily been associated with AZT resistance. In this regard, HIV-1 RT having various combinations of AZT resistance mutations (M41L, D67N, K70R, L210W, T215F/Y), and K219Q) shows a significantly enhanced rate of excision of AZT-5'-monophosphate (AZTMP) compared with the wild-type (WT) enzyme (7, 9, 10).

For HIV-1 RT to effectively excise AZT from the 3’-end of a primer, the chain-terminating AZTMP must reside in the nucleotide-binding site (N-site) of the active site of the enzyme (10, 11). Under physiological conditions, the binding of the next correct dNTP can drive the terminating nucleotide into the primer-binding site (P-site) resulting in the formation of a dead-end complex (8, 9, 10). Formation of this complex prevents the excision reaction from occurring. Several studies have shown that the excision of AZTMP by AZT-resistant (AZTR) RT is much less sensitive (>50-fold) to inhibition by the next correct dNTP than other NRTI analogs that lack a 3’-azido-nucleoside reverse transcriptase inhibitor; NRTI-TP, nucleoside reverse transcriptase inhibitor 5’-triphosphate; TAMs, thymidine analog mutations; T/P, template/primer; nt, nucleotide(s); P-site, primer-binding site; N-site, nucleotide-binding site; WT, wild type.
and AZTR RT enzymes, respectively. All experiments described below determined concentrations of RT were calculated from pre-steady-state burst experiments using the hyperbolic Equation 1, where the apparent burst rate constant ($k_{burst}$) and apparent dissociation constant for dNTP ($K_d$) were then obtained by plotting the apparent catalytic rates ($k_{obs}$) against dNTP concentrations and fitting the data to the hyperbolic Equation 2,

$$k_{burst} = \frac{k_{pol}[dNTP]}{K_d + [dNTP]} \quad \text{(Eq. 2)}$$

Assay of RT-catalyzed Phosphorolysis—The 20-nt DNA primer was 5'-labeled with $\gamma$-32P-ATP and then annealed to one of the four DNA templates described above. 3'-Azido-NRTI was added to the primer 3'-terminal RNA:DNA hybridation was initiated by addition of the appropriate 5'-32P-labeled chain-terminated 21-nt primer was purified by elution of the appropriate band following resolution by 7 M urea, 16% acrylamide denaturing gel electrophoresis. The purified chain-terminated primer was then annealed to the appropriate DNA primer for use in phosphorolysis experiments.

The phosphorolytic removal of 3'-azido-2',3'-ddNMP was assayed by incubating WT or AZT(R) RT with the chain-terminated T/P of interest (4:1 ratio of RT:T/P) in 50 mM Tris-HCl (pH 8.0). 32P-labeling with 10 mM MgCl2, Inorganic pyrophosphatase (0.01 unit; Sigma) was included. Aliquots were removed at the defined times and quenched with equal volumes of gel loading dye (98% deionized formamide, 10 mM EDTA, and 1 mg/ml each of bromphenol blue and xylene cyanol). The 21-nt substrate and 20-nt products were separated by denaturing gel electrophoresis and the reduction in substrate and formation of product were analyzed by phosphorimaging. Data were fit to the following single-exponential equation to determine the apparent rate of ATP-mediated excision,

$$[\text{20-nt product}] = A[1 - \exp(-k_{excision}t)] \quad \text{(Eq. 3)}$$

where A represents the amplitude for product formation.

Analytes—WT RT and AZT(R) HIV-1 RT with mutations D67N/K70R/T215F/K219Q were overexpressed and purified to homogeneity as previously described (15). The protein concentration of the purified enzymes were determined spectrophotometrically at 280 nm using an extinction co-efficient ($e_{280}$) of 260,460 $\text{m}^{-1} \text{cm}^{-1}$. The active site concentrations of RT were calculated from pre-steady-state burst experiments using the equation to determine the apparent rate of ATP-mediated excision,

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AZTTP = AZddUTP > AZddCTP > AZddGTP. Consistent with previously published data (22, 23), both the WT and AZTR enzymes incorporated AZTTP with near equivalent catalytic efficiencies. Similarly, the catalytic efficiencies of the WT and AZTR enzymes were nearly equivalent for the other 3'-azido-2',3'-ddNTPs used in this study (Table I).

Pre-steady-state Excision of 3'-Azido-2',3'-ddNMP by WT and AZTR HIV-1 RT—Transient kinetic analyses of ATP-mediated excision by WT and AZTR HIV-1 RT were carried out to determine the apparent rates for phosphorolytic removal of the 3'-azido-2',3'-ddNMP analogs (Table II). The relative rates of ATP-mediated phosphorolysis by WT and AZTR RT (in the absence of dNTP) were AZddAMP > AZddUMP > AZddATP > AZddGMP. In contrast to the incorporation data, there were significant differences in the rates of ATP-mediated phosphorolytic excision of the various 3'-azido-2',3'-ddNMP analogs by WT and AZTR RT. Consistent with previously published data (9, 10, 13, 24), AZTR RT was more effective then the WT enzyme in removing AZTMP from a chain-terminated primer. The AZTR enzyme was also significantly more efficient (9-fold) in removing AZddUMP than the WT enzyme. However, AZTR RT showed only a moderately improved ability (3-fold) to remove AZddAMP and AZddCMP compared with the WT enzyme. AZTR and WT RT were equally effective at removing AZddGMP (Table II).

Previous studies have shown that the next complementary nucleotide can inhibit ATP-mediated chain terminator removal (8–10, 12). Furthermore, it has been suggested that the relative insensitivity of AZTMP excision to inhibition by the next nucleotide compared with other NRTI-MP may account for the greater degree of resistance to AZT conferred by TAMs than to other NRTIs (10, 12). To determine the effect of AZT resistance mutations on the magnitude and nature of inhibition by the next complementary nucleotide during the phosphorolytic removal of 3'-azido-2',3'-ddNMP analogs, 50% inhibitory concentrations (IC_{50}) of the next complementary nucleotide for the ATP-dependent removal of the primer 3'-terminal NRTI-MP were determined for each of the 3'-azido-2',3'-ddNMP analogs (Table II). For both the WT and AZTR enzymes, the inhibitory concentrations of the next complementary dNTP were substantially higher than putative intracellular dNTP concentrations. IC_{50} values for the AZTR enzyme were ~3-fold higher than for the WT enzyme.

Comparison of the Incorporation and Excision Efficiencies of 3'-Azido-2',3'-ddNTP by WT and AZTR RT—During HIV-1 replication there are multiple opportunities for RT to incorporate and excise NRTI-MP. The capacity for WT or mutant RT to excise any given NRTI is therefore best considered as the ratio of the efficiency of RT-catalyzed NRTI excision to the catalytic efficiency of RT-catalyzed NRTI incorporation. In this context, analysis of the data from Tables I and II shows that AZTR RT exhibits a preference to incorporate and then excise AZddUMP (Fig. 2). Of note, AZTR RT showed no increased ability to excise AZddGMP compared with the WT enzyme. It should be noted that the catalytic efficiency of ATP-mediated 3'-azido-2',3'-ddNMP excision was calculated by dividing the observed k_{excision} (Table II) by the concentration of ATP used in the assay (3000 μM), rather than the K_{D} for ATP. We used this approach because difficulty in obtaining saturating ATP concentrations in these assays precludes accurate estimation of the K_{D} for ATP. However, we feel our approach provides a reasonable comparative estimate of the catalytic efficiency of nucleotide excision because previous studies have suggested that there is no significant difference in the affinity for binding of ATP by WT and AZTR reverse transcriptase (25).

To further evaluate the differences in incorporation and excision of the various 3'-azido-2',3'-ddNTP, we analyzed steady-state DNA synthesis by both WT and AZTR RT in the presence of each of the 3'-azido-2',3'-ddNTP and 3 mM ATP, using a heteropolymeric RNA template corresponding to the HIV-1 sequence used for (+) strong stop DNA synthesis, primed with a DNA oligonucleotide (7). The 173-nt incorporation events needed to produce the full-length DNA product in this assay system allow multiple 3'-azido-2',3'-ddNTP incorporation and excision events during the formation of the full-length final product.

Fig. 3 shows that in the presence of 3 mM ATP, AZTR RT synthesized significantly greater amounts of full-length DNA than WT RT in reactions containing AZTTP (Fig. 3E) or AZddUTP (Fig. 3F), and lesser but still significant amounts of full-length DNA in reactions containing AZddCTP (Fig. 3D). Similarly, much greater levels of small termination products were noted in reactions catalyzed by WT RT in the presence of the 3'-azidopyrimidine compounds compared with AZTR RT. In contrast, there were essentially no differences in either the amount of full-length final product or premature termination products produced by WT and AZTR RT in the presence of AZddATP (Fig. 3B) or AZddGTP (Fig. 3C).

Antiviral Activity of 3'-Azido-2',3'-ddN—WT or AZTR virus (encoding the mutations D67N/K70R/T215F/K219Q in RT) were tested for susceptibility to inhibition by 3'-azido-2',3'-ddN in virus spread assays in lymphocytoid MT2 cells. The antiviral activity of the various 3'-azido-2',3'-ddN was consistent with the enzyme studies, in that AZTR virus showed demonstrable resistance to the 3'-azidopyrimidine nucleosides AZT and AZddC, but not to the 3'-azidopurine nucleosides AZddA or AZddG (Table III). In our experiments, AZddU did not exhibit antiviral activity in the MT2 cell line at concentrations up to 200 μM. Previous studies have shown that AZddU is not phosphorylated in certain cells, and hence is not effective against HIV in these cell systems (26).
respectively.

ddCTP, 3\(^{-}\text{H}11032\), 3\(^{-}\text{H}9262\)

Catalytic efficiencies for ATP-mediated 3\(^{-}\text{H}11032\) HIV-1 RT.

It is increasingly evident that TAMs are associated with varying degrees of resistance to d4T (27–29). It is increasingly evident that TAMs are associated with varying degrees of resistance to d4T (27–29). It is increasingly evident that TAMs are associated with varying degrees of resistance to d4T (27–29).

TABLE I

| ddNTP Incorporation for WT (\(\mu M\)) | Catalytic Efficiency Ratio AZT<sup>im</sup>/WT | Data are the mean ± S.D. determined from three independent experiments. |
|--------------------------------------|--------------------------------------------|---------------------------------------------------------------------|
| M = 3.1 s<sup>−1</sup> | | |
| WT RT                               | 1.32 ± 0.05                               | 1.32 ± 0.05 |
| AZT<sup>im</sup> RT                 | 3.68 ± 0.05                               | 3.68 ± 0.05 |

ATP-dependent excision of chain-terminating 3\(^{-}\text{H}11032\) ddNMP catalyzed by WT and AZT<sup>im</sup> HIV-1 reverse transcriptase

Data are the mean ± S.D. determined from three independent experiments.

| Excision (×10<sup>−3</sup> s<sup>−1</sup>) | Ratio AZT<sup>im</sup>/WT | IC<sub>50</sub> (\(\mu M\)) for inhibition of excision by TTP | Ratio IC<sub>50</sub> AZT<sup>im</sup>/WT |
|------------------------------------------|---------------------------|------------------------------------------------------|--------------------------------------|
| 3\(^{-}\text{H}11032\) ddATP             |                           |                                                      |                                      |
| WT RT                                   | 3.02 ± 0.33               | 70                                                   | 3.02 ± 0.33                          |
| AZT<sup>im</sup> RT                     | 9.66 ± 0.70               | 235                                                  | 9.66 ± 0.70                          |
| 3\(^{-}\text{H}11032\) ddCTP             |                           |                                                      |                                      |
| WT RT                                   | 0.23 ± 0.11               | 3.2                                                  | 0.23 ± 0.11                          |
| AZT<sup>im</sup> RT                     | 0.64                      | 3.0                                                  | 0.64                                 |
| 3\(^{-}\text{H}11032\) ddGTP             |                           |                                                      |                                      |
| WT RT                                   | 0.10 ± 0.07               | 2.8                                                  | 0.10 ± 0.07                          |
| AZT<sup>im</sup> RT                     | 0.11 ± 0.05               | 1.0                                                  | 0.11 ± 0.05                          |
| AZTTP                                   | 0.54 ± 0.09               | 7.0                                                  | 0.54 ± 0.09                          |
| WT RT                                   | 3.79 ± 0.65               | 200                                                  | 3.79 ± 0.65                          |
| AZT<sup>im</sup> RT                     | 0.89 ± 0.11               | 60                                                   | 0.89 ± 0.11                          |
| AZT<sup>im</sup> RT                     | 8.46 ± 0.35               | 210                                                  | 8.46 ± 0.35                          |

DISCUSSION

Antiretroviral therapy with thymidine nucleoside analogs such as AZT or d4T selects for several mutations (generally referred to as thymidine analog mutations or TAMs) in the RT gene that confer high-level resistance to AZT, and moderate levels of resistance to d4T (27–29). It is increasingly evident that TAMs are associated with varying degrees of cross-resistance to other “non-thymidine” NRTI including abacavir, ddI, ddC, and tenofovir (30–33). TAMs in RT do not enable discrimination between the natural dNTP substrate and the analogous NRTI-TP during mutant RT-catalyzed DNA synthesis (22, 23). Instead, TAMs increase the ability of RT to remove or excise the incorporated NRTI by a phospho-

hydrolytic enzymatic reaction (7–10).

The 3′-terminal nucleotide of the growing viral DNA strand resides in two different positions in the polymerase active site of the RT-nucleic acid complex during polymerization (10, 11, 33). The N-site, or the nucleotide site, is that where the primer 3′-terminal nucleotide is located immediately after addition of a dNTP or NRTI-TP. The P-site is that in which the primer 3′-terminal nucleotide is moved by a single register from the N-site. This translocation event positions the next template base to allow hydrogen bond stabilization with the base of the incoming cognate dNTP, thereby facilitating formation of the appropriate ternary complex for additional dNTP incorporation.

RT-catalyzed phospho-
ylolytic excision of an NRTI-TP from the primer 3′ terminus requires that the terminal NRTI reside in the N-site (10, 11). Binding of the next complementary dNTP seems to promote translocation of the primer 3′ terminus into the P-site (34). If the 3′-terminal nucleotide is an NRTI, this translocation effectively forms a “dead-end complex” in which phospho-
ylolytic excision cannot occur (8, 9), because phosphodiester bond formation is precluded. Excision of AZTMP by HIV-1 AZT<sup>im</sup> RT is much less sensitive to inhibition by the next complementary dNTP...
DNA polymerization reactions were carried out by incubating RT and T/P (4:1 molar ratio) for 5 min, then initiating the reaction by addition of a mixture providing final concentrations of 5 μM of each dNTP, 1 μM of the desired 3'-azido-2',3'-ddNTP, 3 mM ATP, 10 mM MgCl₂, and 0.01 unit of inorganic pyrophosphatase. Aliquots were removed at various times and quenched with gel loading buffer. Polymerization products were resolved by electrophoresis and quantified by phosphorimaging. A, DNA synthesis in the absence of 3'-azido-2',3'-ddNTP; B, DNA synthesis in the presence of 1 μM 3'-azido-2',3'-ddATP; C, DNA synthesis in the presence of 1 μM 3'-azido-2',3'-ddCTP; D, DNA synthesis in the presence of 1 μM 3'-azido-2',3'-ddGTP; E, DNA synthesis in the presence of 1 μM AZTTP; F, DNA synthesis in the presence of 1 μM 3'-azido-2',3'-ddUTP. Major chain-termination events during DNA synthesis are indicated using A, G, C, T, or U to indicate positions of incorporation of 3'-azido-2',3'-ddATP, 3'-azido-2',3'-ddGTP, 3'-azido-2',3'-ddCTP, AZTTP or 3'-azido-2',3'-ddUTP, respectively. FP, full-length 173-nt polymerization product. The figure is intended to provide quantitative comparison only between WT and AZTₖRT for any given chain-terminating 3'-azidonucleotide. Comparisons among the various 3'-azidonucleotides are qualitative only, as panels A–C and D–F are data from different electrophoretic separations and phosphorimaging exposures.

**TABLE III**

Antiviral activity of 3'-azido-2',3'-dideoxynucleosides

| Virus | 3'-Azido-ddA | 3'-Azido-ddC | 3'-Azido-ddG | 3'-Azido-ddC | 3'-Azido-ddG |
|-------|--------------|--------------|--------------|--------------|--------------|
| WT    | 7.9 ± 3.4    | 15.6 ± 10.7  | 7.9 ± 4.6    | 0.031 ± 0.02 | —³           |
| AZTₖRT | 8.6 ± 5.4    | 160.8 ± 24.1 | 10.3 ± 8.3   | 0.36 ± 0.2   | —³           |

Fold resistance: 1.2, 10.7, 1.3, 11.7, NA ⁶

³ No inhibition noted at highest concentration tested (200 μM).
⁶ NA, not applicable.

than is that of other NRTI that lack a 3'-azido group (9, 10, 12–14). This observation has led to the hypothesis that the 3'-azido group is an important, perhaps the major, determinant for enabling the phosphorolytic excision process (10, 11). Our results, however, do not support this hypothesis.

WT and AZTₖRT RT showed virtually identical catalytic efficiencies for the incorporation of all the 3'-azido-2',3'-ddNTPs tested (Table I). In contrast, AZTₖRT RT was more efficient than WT RT for ATP-dependent phosphorolytic removal of incorporated 3'-azido-2',3'-ddNMPs, in particular AZTPM and AZddUMP. The differences between 3'-azidopyrimidine and 3'-azidopurine NRTI were further magnified in the steady-state DNA synthesis that enabled multiple events of 3'-azido-2',3'-ddNTP incorporation and 3'-azido-2',3'-ddNMP excision (Fig. 3). Differences in the full-length DNA product synthesized by WT and AZTₖRT RT were only noted in reactions containing pyrimidine compounds AZTTP, 3'-azido-2',3'-ddUTP, and 3'-azido-2',3'-ddCTP (Fig. 2).

The inability of AZTₖRT RT to efficiently excise 3'-azido-2',3'-ddAMP and 3'-azido-2',3'-ddGMP in the steady-state DNA synthesis experiments was not a result of these analogs being more sensitive to dead-end complex formation by the next complementary nucleotide. AZTₖRT RT showed ~3–5-fold increases in IC₅₀, relative to WT RT for inhibition of all of the 3'-azido-dNMPs evaluated (Table II). In all cases, the levels of the next complementary dNTP needed to inhibit AZTₖRT RT-mediated excision were much greater than putative intracellular dNTP concentrations (35).

HIV-1 susceptibility to the 3'-azido-2',3'-dN (Table III) was consistent with biochemical data. AZTₖRT virus exhibited at least 10-fold resistance to AZT and AZddC. AZddU was inactive in the MT2 cell line used in these studies, possibly because of inadequate intracellular conversion to the triphosphate (25). Interestingly, AZTₖRT HIV showed no cross-resistance to either AzzddA or AzzddG. This result suggests that 3'-azidopurine nucleoside analogs that exhibit good antiviral activity and cytotoxicity profiles may be useful for the treatment of AZT-resistant HIV.
Our data clearly show that the nucleotide base component, not just the sugar 3'-azido moiety, is important for the excision phenotype. In this regard, TAMs in RT enable the enzyme to efficiently excise chain-terminating 3'-azidopirimidines, but are less effective for excision of 3'-azidopyrimidines. These data suggest that careful consideration be given to the choice of NRTI in experiments characterizing the phosphorolysis phenotype conferred by specific combinations of TAMs.

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