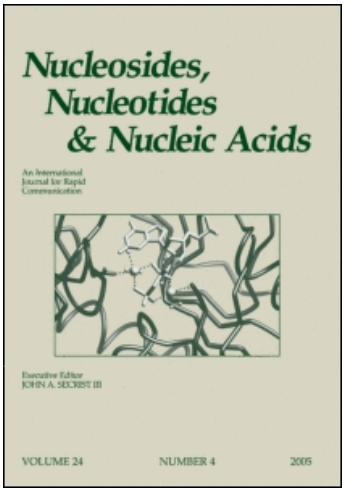

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TENOFOVIR (PMPA) IS LESS SUSCEPTIBLE TO PYROPHOSPHOROLYSIS AND NUCLEOTIDE-DEPENDENT CHAIN-TERMINATOR REMOVAL THAN ZIDOVUDINE OR STAVUDINE

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ABSTRACT

Pyrophosphorolysis, the removal of nucleoside chain-terminators by a pyrophosphate (PPi) acceptor molecule, and a similar mechanism (nucleotide-dependent chain-terminator removal) which uses ATP as an acceptor molecule have been proposed as mechanisms of zidovudine (AZT) resistance. Recombinant HIV-1 wild-type reverse transcriptase (RT) and a mutant RT enzyme containing the AZT/thymidine analog resistance mutations D67N/K70R/T215Y were analyzed for pyrophosphorolysis and nucleotide-dependent chain-terminator removal activities. Our results confirm that pyrophosphorolysis and nucleotide-dependent chain-terminator removal are potential mechanisms of AZT and d4T resistance. However, tenofovir is less efficiently removed by pyrophosphorolysis and by nucleotide-dependent mechanisms. These results are consistent with the minor changes in susceptibility to tenofovir of the AZT/thymidine analog-resistant HIV RT mutants and the corresponding resistance of these mutants to AZT. The inability to remove tenofovir efficiently by these mechanisms may contribute to the durability of the HIV RNA response observed in patients treated with the oral prodrug, tenofovir disoproxil fumarate.

Tenofovir (PMPA) is an adenosine nucleotide analogue RT inhibitor which has activity against HIV-1 and HIV-2. Tenofovir requires only two phosphorylation steps by ubiquitous cellular enzymes to become the active metabolite, tenofovir

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diphosphate [1, 2]. This novel phosphorylation requirement permits activity in a wide variety of dividing and non-dividing cell types [3, 4]. It has a long intracellular half-life which makes once daily dosing possible [5]. The oral prodrug of tenofovir, tenofovir disoproxil fumarate, is currently in phase III trials for the treatment of HIV infection.

A large proportion of the HIV-infected patient population in developed countries have had prior antiretroviral therapy experience and have taken at least one nucleoside RT inhibitor. Many of these patients have developed resistance mutations against the nucleoside RT inhibitors. This is demonstrated by a recent clinical trial where baseline genotypes of 184 patients showed that 94% of patients had nucleoside RT inhibitor-associated mutations [6]. 74% had AZT-associated resistance mutations and 50% had the T215Y/F mutation which is associated with high-level AZT resistance. In addition, 66% had the lamivudine-associated mutation, M184V, which has been previously shown to be hypersusceptible to tenofovir [7]. Any new anti-HIV drug therapy will be facing a highly antiretroviral-experienced patient population and will have to contend with possible cross-resistance issues.

We have focused on the AZT-associated resistance mutations in RT, T215Y (215), D67N/K70R (67/70), and D67N/K70R/T215Y (67/70/215), and have analyzed HIV with these RT mutations for their susceptibility to tenofovir and AZT in an antiviral assay in MT-2 cells (Fig. 1). The 215 and 67/70 mutant viruses had 6-7-fold decreased susceptibility to AZT, and the 67/70/215 mutant virus demonstrated a 25-fold decrease in susceptibility to AZT. These mutant viruses have only minor shifts in susceptibility to tenofovir with at most a 2.5-3-fold change in tenofovir susceptibility for the 67/70/215 mutant virus.

Our objective was to explore the mechanistic basis for this lack of cross-resistance of the AZT-resistant RT mutants to tenofovir. Mutations in RT might lead

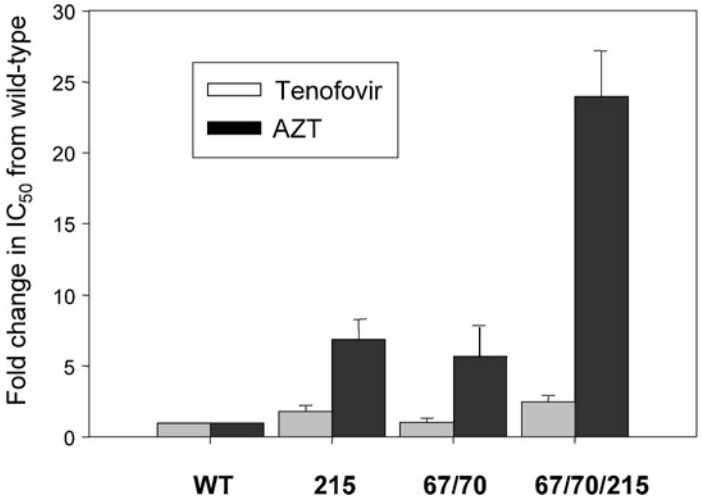

Figure 1. Susceptibility of RT Mutants.

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Table 1. Relative Binding Affinities of Recombinant Wild-type and Mutant HIV-1 RT Enzymes

	Tenofovir Diphosphate		AZT Triphosphate	
Recombinant HIV-1 RT	K_i (μM)	K_i/K_m	K_i (μM)	K_i/K_m
Wild-type	0.52	1.7	0.12	0.26
T215Y	0.59	1.2	0.20	0.38
D67N/K70R	0.44	1.1	0.11	0.18
D67N/K70R/ T215Y	0.63	1.9	0.22	0.28

K_i values are averages of 2-3 experiments with an average standard error of 0.05 for tenofovir diphosphate and 0.03 for AZT triphosphate.

to resistance by decreasing incorporation of chain-terminators or by increasing the removal of chain-terminators after they are incorporated by mechanisms such as pyrophosphorolysis.

The relative binding affinities of recombinant and mutant HIV-1 RT enzymes were analyzed by determining the K_i and K_i/K_m ratios of each of the enzymes (Table 1). The K_i values for tenofovir diphosphate of each of the mutant enzymes was similar to the K_i value for the wild-type enzyme. Furthermore, there was no major difference in the relative tenofovir binding to natural substrate binding (K_i/K_m ratios). Similar results were seen for AZT triphosphate with less than 2-fold changes in the K_i value for each of the mutants compared to wild-type enzyme and no significant changes in the K_i/K_m ratio. Therefore, changes in relative binding affinities are not sufficient to explain the changes in susceptibilities to AZT and tenofovir. This has led to exploration of other mechanisms that might contribute to resistance.

Chain-terminators might be removed by a mechanism called pyrophosphorolysis, or reverse nucleotide polymerization, where pyrophosphate acts as acceptor molecule for the removal of the chain-terminator. Removal of the chain-terminator would free the RT to incorporate the natural nucleotide substrate and rescue viral replication. ATP has also been proposed as an acceptor molecule for the removal of chain-terminators and is referred to as ATP-dependent primer unblocking. Both pyrophosphate and ATP have been shown to act as acceptor molecules for the removal of AZT [8, 9]. Moreover, these groups have demonstrated that RT enzyme with AZT-resistance mutations has increased removal of AZT by pyrophosphorolysis and ATP-mediated primer unblocking compared to wild-type RT [9, 10].

We have analyzed the removal of tenofovir by these mechanisms compared to the removal of AZT and d4T. A primer was end-labeled with γ-[^{32}P] and annealed to a heteropolymeric RNA template. Wild-type RT was used to incorporate tenofovir, AZT, or d4T. The end-labeled terminated primer was gel purified and annealed

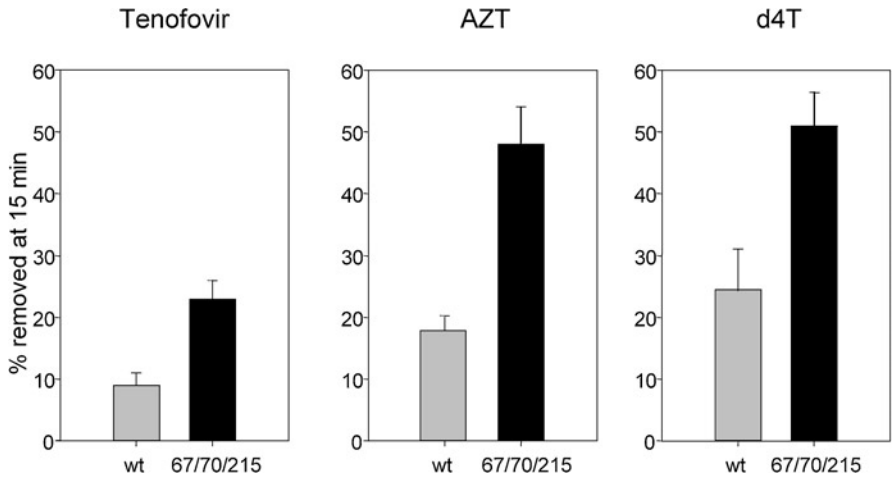

Figure 2. Quantitation of Pyrophosphate-Mediated Chain-Terminator Removal.

to an RNA template. The RT enzyme to be analyzed was added and NaPPi and ATP added for varying times. Tenofovir was less efficiently removed than AZT or d4T by pyrophosphorolysis (Fig. 2). The 67/70/215 mutant demonstrated increased removal by pyrophosphorolysis for each of the chain-terminators compared to wild-type RT (Fig. 2). With ATP as an acceptor molecule, minimal removal of tenofovir by wild-type RT or the mutant was detected following addition of ATP, while both AZT and d4T are readily removed by both wild-type and mutant enzyme following addition of ATP by 15 min. (Fig. 3). Again, the 67/70/215 mutant enzyme showed increased removal of AZT and d4T by the ATP-mediated primer unblocking mechanism compared to wild-type enzyme (Fig. 3).

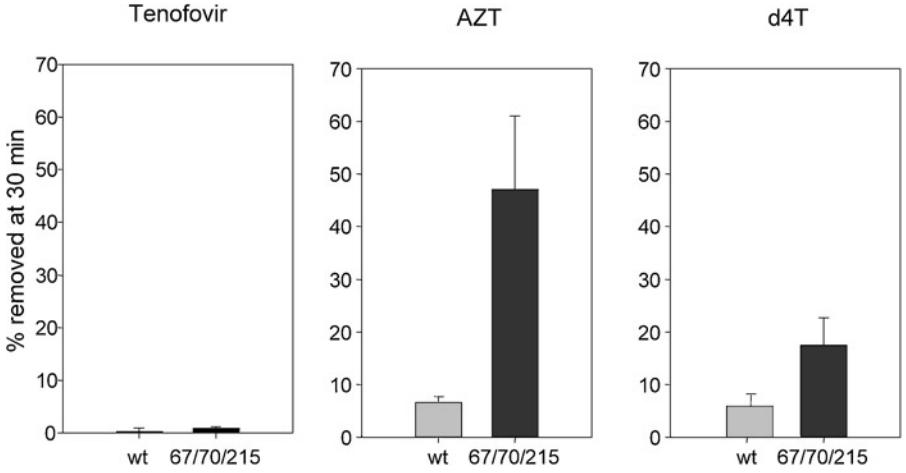

Figure 3. Quantitation of ATP-Mediated Chain-Terminator Removal.

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We have provided a mechanistic explanation for the favorable resistance profile of tenofovir against AZT-resistant RT mutants. Incorporation of tenofovir by these mutants was similar to wild-type RT. In addition, tenofovir was less efficiently removed compared to AZT and d4T. This reduced removal of tenofovir might result from the unique phosphonate bond of tenofovir or reduced accessibility of the phosphorus to attack by pyrophosphate and ATP because of the unique structure of tenofovir.

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