

Impact of P119S and/or T165A Mutation of HIV-1 RT on Enzyme Behaviors and Virus Growth

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Abstract

Background: 4'-ethynyl d4T (4'-Ed4T), a novel thymidine analog, exhibits a 5–10-fold higher anti-HIV-1 activity and less cytotoxic than its progenitor (d4T). The triphosphate (4'-Ed4TTP) competitively inhibits HIV-1 reverse transcriptase (RT). 4'-Ed4TTP inhibits the RT M184V mutant with 3-fold less efficiency than the wild type (wt) RT. This is consistent with *in vitro* drug susceptibility studies, which showed that M184V virus (IIB strain) conferred 3 to 10-fold resistance to 4'-Ed4T. Two other mutations (P119S and T165A) in addition to M184V were identified with IIB strain through *in vitro* drug-resistance virus selection. To understand the relevance between the enzymatic behavior of these RT mutants and viral replication, we investigated the inhibition of RT mutants by 4'-Ed4TTP and the drug sensitivity of corresponding viral strains.

Methods: A steady-state single nucleotide incorporation assay was carried out with wt and mutant RTs to determine: (i) the kinetic constants (K_m and k_{cat}) for dTTP incorporation, and (ii) the inhibition constant K_i for 4'-Ed4TTP against dTTP. The K_i/K_m value was used as a measure of the level of resistance. For the antiviral susceptibility studies, TZM-bl cells were infected with the wt or mutant virus in serial concentrations of 4'-Ed4T to calculate EC_{50} .

Results: The DNA polymerase activity of the mutant RTs was comparable to that of wt RT. The P119S, T165A and P119S/T165A mutants showed no resistance to 4'-Ed4TTP, while the M184V mutant showed 3-fold resistance. The P119S/M184V and T165A/M184V mutants showed 8-, and 13-fold resistance, respectively. The P119S/T165A/M184V triple mutant conferred only 11-fold resistance, without any synergistic effect. The P119S, T165A, and P119S/T165A viral strains showed no resistance to 4'-Ed4T, and the M184V strain conferred 3-fold resistance. The P119S/M184V strain achieved 2-fold more resistance than M184V.

Conclusion: P119S in combination with M184V increases resistance to 4'-Ed4T at both RT and virus levels. The T165A/M184V and P119S/T165A/M184V RT mutants showed increased resistance to 4'-Ed4TTP, further investigation is needed to characterize the evolution of 4'-Ed4T resistant mutation and their contribution to viral growth.

Introduction

Nucleoside analogs are inactive prodrugs, require a stepwise intracellular phosphorylation to their triphosphate metabolites, which are preferentially incorporated into HIV DNA and cause premature termination of viral DNA chain elongation. The potency of an NRTI is determined by its ability to inhibit the RNA-dependent DNA or DNA dependent DNA polymerase activity of HIV-1 RT. The adverse effects of NRTIs are mediated by their effects on host DNA polymerase activity. The inhibition of viral and host DNA polymerases act as independent processes. Among the approved nucleoside RTIs (NRTIs), d4T is a highly potent inhibitor of HIV-1 replication *in vitro*. However, the use of d4T *in vivo* has been limited by delayed toxicity, notably peripheral neuropathy and myopathy caused by mitochondrial damage. The cytidine analog NRTIs like lamivudine (3TC) and emtricitabine (FTC) have good anti-HIV-1 activity and less pronounced mitochondrial toxicity. However, the rapid emergence of highly resistant mutants limit their use. The recently discovered 4'-Ed4T is structurally related to d4T (Fig. 1). It is a more potent inhibitor of HIV-1 replication and is much less inhibitory to mitochondrial DNA synthesis and cell growth in cell cultures than its progenitor d4T. It also has a unique resistance profile (Fig. 2) when compared to other thymidine analogs like zidovudine (AZT) and d4T. In this study, we assessed the relative contribution of these mutations to RT activity, viral growth, and antiviral susceptibility.

Methods

4'-Ed4T mutants (119S, 165A, 184V) were introduced by site directed mutagenesis into 5' NL4-3 sequences. Single nucleotide incorporation assay was carried out (i) for the determination of steady-state kinetic constants for dTTP incorporation, and (ii) the inhibition constant K_i for 4'-Ed4TTP against dTTP. The K_i/K_m value was used as a measure of the level of resistance. A DNA/RNA 23/36mer primer/template (P/T) was used in the assay: DNA/RNA23/36mer (5'-TCAGGTCCCTGTTCGGCGCCAC-3'/3'-CGAAGUCCAGGGACAAAGCCCGGUGACGACGACGAC-5'). The steady-state K_m and relative V_{max} values for dTTP incorporation were first determined with 250nM P/T, 2.5nM wt or mutant RT and various dTTP concentrations. Then the K_i values for 4'-Ed4TTP or d4TTP to inhibit dTTP incorporation were determined with 250nM P/T, 2.5nM wt or mutant RT, various concentrations of dTTP and various concentrations of inhibitor. For the antiviral susceptibility studies, 4 × 10⁴ TZM-bl cells (a clone of HeLa-CD4/CCR5 containing a firefly luciferase reporter) were infected with the wild type (NL4-3 or IIB) or 4'-Ed4T mutants at an MOI of 0.01 in serial concentrations of 4'-Ed4T, d4T, 3TC and AZT. The EC_{50} (μ M) of the various RT inhibitors were determined based on luciferase activity of control infection without antiviral agent.

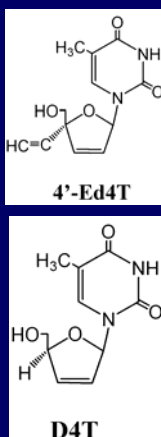


FIG. 1. Structure comparison of 4'-Ed4T and d4T

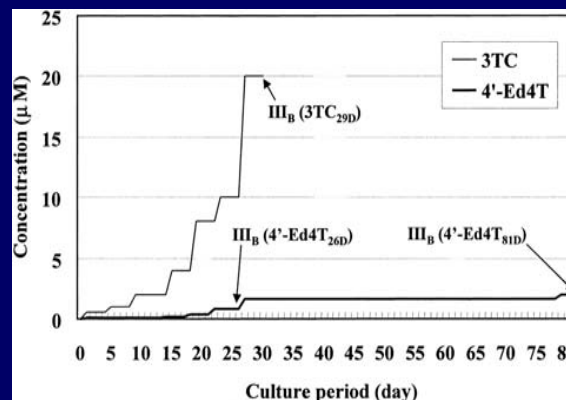


FIG. 2. Selection of HIV-1 strains resistant to 4'-Ed4T and 3TC in vitro (Nitanda *et al*, Antimicrob. Agents Chemother. (2005) 49:3355-3360

Results

RT Activity of wild type and 4'-Ed4T mutants: The DNA polymerase activity of the mutant RTs was comparable to that of wt RT (Table 1). The P119S, T165A and P119S/T165A mutants showed no resistance to 4'-Ed4TTP, while the M184V mutant showed 3-fold resistance. The T165A/M184V and P119S/M184V mutants showed 13-, and 8-fold resistance, respectively. The P119S/T165A/M184V triple mutant conferred only 11-fold resistance, without any synergistic effect.

| | Enzyme | wt RT | P119S | T165A | M184V | P119S/ T165A | T165A/ M184V | P119S/ M184V | P119S/ T165A/ M184V |
|-----------|------------------------------------------|-------|-------|-------|-------|-----------------|-----------------|-----------------|---------------------------|
| dTTP | $K_m(\text{dTTP})$ (μ M) | 0.12 | 0.13 | 0.10 | 0.12 | 0.03 | 0.11 | 0.18 | 0.17 |
| | Relative V_{max} | 1.0 | 1.1 | 1.5 | 1.0 | 1.5 | 0.6 | 1.7 | 0.3 |
| 4'-Ed4TTP | K_i (μ M) | 0.054 | 0.11 | 0.08 | 0.21 | 0.024 | 0.66 | 0.62 | 0.73 |
| | $K_i(4'-\text{Ed4TTP})/K_m(\text{dTTP})$ | 0.45 | 0.85 | 0.8 | 1.7 | 0.8 | 6.0 | 3.6 | 4.9 |
| D4TTP | K_i (μ M) | 0.63 | N.D. | N.D. | 0.79 | N.D. | 0.3 | N.D. | 1.05 |
| | $K_i(\text{D4TTP})/K_m(\text{dTTP})$ | 5.3 | N.D. | N.D. | 6.6 | N.D. | 2.7 | N.D. | 7.0 |

Table 1. RT Activity. K_i and K_m values represent means from at least three independent experiments with standard deviation less than 20%. N.D.: Not determined.

Antiviral susceptibility of 4'-Ed4T mutants: Since 4'-Ed4T shares resistance mutation (184V) with the cytidine nucleoside analogs, we tested the susceptibility of these mutant strains to 4'-Ed4T, d4T, 3TC, and AZT (Table 2). The 184V, and 119S/184V strains had EC_{50} of 3.5 and 6.4 μ M to 4'-Ed4T, respectively. These values were about 10-20-fold less than their EC_{50} to 3TC. The single mutants (119S and 165A) did not confer any significant resistance to 4'-Ed4T. The double 165A/184V and triple 119S/165A/184V strains were not tested.

| Virus | EC_{50} (μ M) | | | |
|-------------------|----------------------|------|-----|--------|
| | 4'Ed4T | D4T | 3TC | AZT |
| Wild Type (NL4-3) | 0.9 | 0.9 | 1.0 | 0.009 |
| P119S | 1.0 | <0.5 | 1.2 | 0.002 |
| T165A | 1.4 | 1.0 | 1.4 | 0.003 |
| M184V | 3.5 | 0.8 | 23 | 0.005 |
| P119S/M184V | 6.4 | <0.5 | >50 | 0.003 |
| P119S/T165A | 1.2 | <0.5 | 1.9 | 0.0008 |
| Wild Type (IIB) | 1.1 | 1.0 | 1.7 | 0.006 |

Table 2. Antiviral Susceptibility. Cells were pretreated with antiviral agent 24 h prior to viral infection. EC_{50} was based on percent of control. Values are the average of two independent experiments.

SUMMARY AND CONCLUSIONS

- 4'-Ed4T mutants did not have cross-resistance to other thymidine analogs (D4T and AZT)
- M184V acts as primary mutation and P119S and T165A may be secondary mutations
- The behaviors of 4'-Ed4TTP toward RT with defined resistant mutations were consistent with the results from drug susceptibility assay
- RT mutants P119S or T165A alone did not create resistance to 4'-Ed4TTP; however, both mutants increased resistance to 4'-Ed4T when in combination with M184V

Acknowledgment

4'-Ed4T is in preclinical development by Oncolys, Japan