

THE FITNESS AND ANTIVIRAL SUSCEPTIBILITY OF UNIQUE HIV-RT RESISTANCE MUTATIONS OF A NOVEL THYMIDINE ANALOG (4'-Ed4T)

Elijah Paintsil^{*1,2}, Ginger Dutschman¹, Susan Grill¹, Masanori Baba³, Hiromichi Tanaka⁴, Guangwei Yang¹, and Yung-Chi Cheng¹.

Affiliations: Departments of Pharmacology¹ and Pediatrics², Yale University School of Medicine, New Haven, CT; Faculty of Medicine³, Kagoshima University, Kagoshima, Japan; School of Pharmaceutical Sciences⁴, Showa University, Tokyo, Japan

Abstract

Background: 2',3'-didehydro-3'-deoxy-4'-ethynylthymidine (4'-Ed4T), a novel thymidine analog is active against multi-drug resistant HIV-1. *In vitro* selection of HIV resistant to 4'-Ed4T revealed that the M184V mutation confers some resistance (3-10-fold) to 4'-Ed4T, yet additional mutations (P119S and T165A) were required to achieve phenotypically higher resistance (40-fold). In this study, we assessed the relative contribution of these mutations (i.e., increasing replication capacity or resistance) to the evolution of 4'-Ed4T resistance.

Methods: The mutations were engineered by site directed mutagenesis into NL4-3 background. Standard growth assay was used to study the replication capacity. Viral RNA concentrations were determined by real time RT-PCR starting on post infection day 4, and then plotted on a log-scale. The HIV-1 RT region was amplified from culture supernatants obtained from day 8 post infection, and then sequenced for analysis of changes at 119, 165, and 184 codons. For the antiviral susceptibility studies, 4 x 10⁶ T2M-bl cells (containing a firefly-luciferase reporter) were infected with the wild type NL4-3 or the 4'-Ed4T mutants at an MOI of 0.01 in serial concentrations of 4'-Ed4T, d4T, 3TC and AZT. The EC₅₀ (μM) of the various RT inhibitors were then calculated.

Results: The 119S, 165A, 184V, and 119S/184V mutants replicated with kinetics similar to that of the wild type NL4-3. There was delay in replication of the double mutant 165A/184V, and the 119S/165A/184V triple mutant. All the single mutants and double mutant (119S/184V) had their mutations intact at post-infection day 10. However, the 165A/184V mutant had reverted to wild type codon at the 184 locus and the triple mutant 119S/165A/184V had reverted to wild type codons at all loci. The reversion of the triple mutant coincided with increased replication at day 10. The reversion of the double mutant 165A/184V at the 184 locus did not increase the replication until day 12 when the 165A/184V acquired the 119S mutation. In general, 4'-Ed4T, had the lowest EC₅₀ for all the strains tested in comparison to d4T and 3TC. The EC₅₀ of 4'-Ed4T and 3TC for the 184V strain were 3.4 μM and >40 μM, respectively.

Conclusion: Our data suggest that the 184V mutation is acting as a primary mutation (increasing resistance to 4'-Ed4T), and the 119S as a secondary mutation (increasing viral fitness). Addition of the 165A mutation to virus with the 184V mutation compromised the viral fitness.

Introduction

All currently recommended ART protocols employ Reverse Transcriptase Inhibitors (RTIs). Mutations within the RT domain leads to resistance to these agents and treatment failure. Long term toxicities further limit therapeutic options at treatment failure. Therefore, there is an urgent need to design therapeutic regimens and/or develop novel inhibitors which can inhibit drug resistant HIV-1 replication while displaying a favorable toxicity profile. 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 when compared to other thymidine analogs like zidovudine (AZT) and d4T. In this study, we assessed the relative contribution of these mutations (i.e., increasing replication capacity or resistance) to the evolution of 4'-Ed4T resistance.

Methods

4'-Ed4T mutants; 119S, 165A, 184V, 119S/165A, 119S/184V, 165A/184V and 119S/165A/184V were introduced by site directed mutagenesis into 5' NL4-3 sequences using Quick Change Site-directed Mutagenesis Kit. Standard growth assay was used to study the replication capacity. Viral RNA concentrations were determined by real time RT-PCR starting on post infection day 4, and then plotted on a log-scale (Fig. 2). The HIV-1 RT region was amplified from culture supernatants obtained from day 8 post-infection, and then sequenced for analysis of changes at 119, 165, and 184 codons. For the antiviral susceptibility studies, 4 x 10⁶ T2M-bl cells (containing a firefly-luciferase reporter) were infected with the wild type NL4-3 or the 4'-Ed4T mutants at an MOI of 0.01 in serial concentrations of 4'-Ed4T, d4T, 3TC and AZT. The EC₅₀ (μM) of the various RT inhibitors were then calculated.

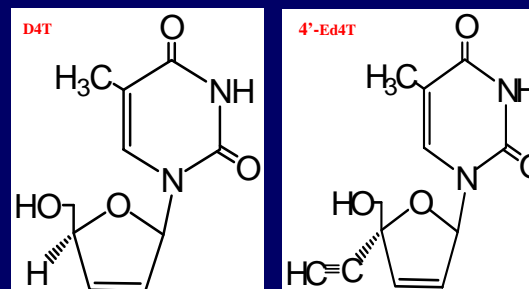


FIG. 1. Structure comparison of 4'-Ed4T and D4T

Results

Growth analysis of 4'-Ed4T mutants: All the mutants replicated with kinetics similar to that of wild type, except for the double mutant 165A/184V, and the 119S/165A/184V triple mutant (Fig. 2). The triple mutant on day 8 post-infect had a mixture of wild type and mutant strains at codon 119. However, it completely reverted to wild type codons at all the three loci by day 10 post-infection. There was a delay in replication of the 165A/184V and triple mutant. On day 12 post infection, the 165/184 mutant strain acquired the 119 mutation which increased its replication remarkably. The 165/184 and triple mutant strains were less fit.

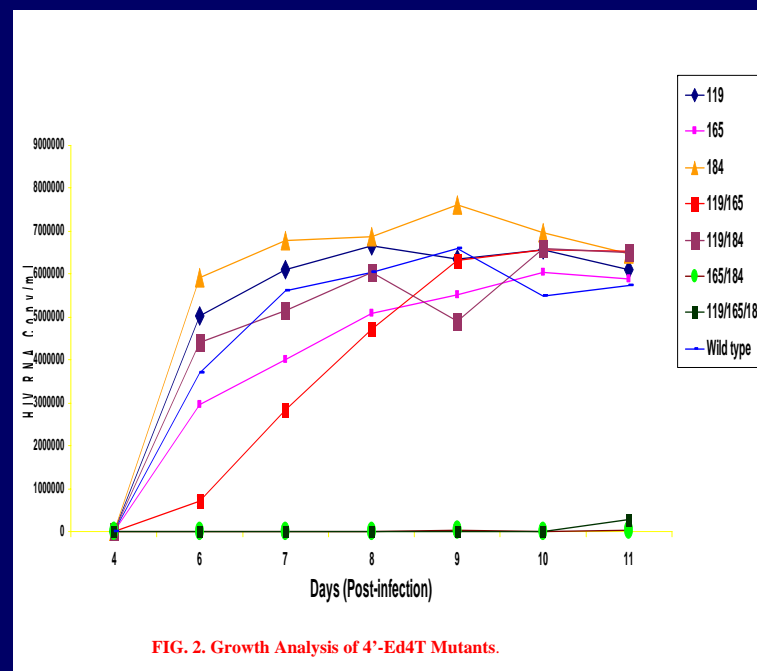


FIG. 2. Growth Analysis of 4'-Ed4T Mutants.

Mutant	Post-Infection Day	Codon 119 (CCC→TCC)	Codon 165 (ACA→GCA)	Codon 184 (ATG→GTG)
119S	10	TCC		
165A	10		GCA	
184V	10			GTG
119S/165A	10	TCC	GCA	
119S/184V	10	TCC		GTG
165A/184V	10		ACA	ATG
	12	TCC	ACA	ATG
119S/165A/184V	8	NCC	ACA	ATG
	10	CCC	ACA	ATG

Table. 1. Time to Reversion of Mutations

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. The assay was performed in T2M-bl cells (a clone of HeLa-CD4/CCR5 containing a firefly luciferase reporter). EC₅₀ was based on the percentage of control. The 184V, and 119/184 strains had EC₅₀ of 3.4 and 2.15 μM to 4'-Ed4T, respectively. These values were about 10-20-fold less than their EC₅₀ to 3TC. The single mutants (119S and 165A) did not confer any significant resistance to 4'-Ed4T. In general, 4'-Ed4T, had the lowest EC₅₀ for all the strains tested in comparison to D4T, and 3TC.

SUMMARY AND CONCLUSIONS

- The general fitness order of the 4'-Ed4T mutants was; Single > Double > Triple mutants
- 184V and 119S seem to act as primary and secondary mutations, respectively
- 165A mutation on its own did not affect viral replication, however, when added to the virus with 184V mutation fitness was compromised
- 4'-Ed4T had the lowest EC₅₀ for all the strains tested in comparison to D4T and 3TC
- Strains with the 184V mutation were still susceptible to 4'-Ed4T in comparison to 3TC