



STRUCTURAL MECHANISM OF HIV-1 REVERSE TRANSCRIPTASE INHIBITION AND RESISTANCE TO TRANSLLOCATION-DEFICIENT RT INHIBITORS

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Abstract

Background Nucleoside reverse transcriptase inhibitors (NRTIs) are among the most potent therapeutics, and are often considered for first-line therapy. All approved NRTIs act as chain terminators because they lack a 3'OH and it has long been considered that the absence of the 3'OH is essential for antiviral activity. However, this feature can also impart detrimental properties to the inhibitor, such as reduced affinity for RT compared to the analogous dNTP substrate, as well as reduced intracellular conversion to the active nucleoside triphosphate. We have found that certain nucleosides that retain the 3'OH group and have substitutions at the 4' and 2 positions of the deoxyribose sugar and base respectively, have exceptional antiviral properties and are highly potent inhibitors of HIV RT. One of these compounds, 4'-ethynyl-2-fluorodeoxyadenosine (4'E-2FdA) is the most potent RT inhibitor described to date.

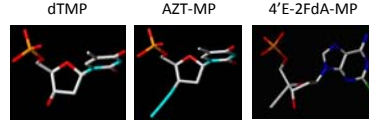
Methods Using enzymology techniques, we characterized the mechanisms of action and resistance of this novel inhibitor of the reverse transcriptase.

Results 4'E-2FdA can inhibit RT by multiple mechanisms. At physiological concentrations of dNTP it acts as a chain terminator despite the presence of an accessible 3'OH. We report here that this apparent chain termination arises from difficulty of the primer 3'-terminus to translocate following incorporation of the compound. Therefore, we propose that 4'E-2FdA is a Translocation-Deficient Reverse Transcriptase Inhibitor (TDRTi) that acts by a novel mechanism.

We show that the M184V mutation in HIV RT confers low-level resistance to 4'-ethynyl modified nucleosides. In a primer extension assay, we observed a ~4 fold increase in the IC₅₀ of 4'E-2FdA-TP, reflecting the viral resistance observed in cell culture assays. Steady-state kinetic experiments demonstrated that resistance is primarily due to a decrease in the affinity of M184V RT for 4'E-2FdA triphosphate. Molecular modeling analysis suggests that the decrease in binding affinity is the result of steric hindrance between the Val184 of the mutant RT and the 4' ethynyl group of the inhibitor.

Conclusion In conclusion, 4'E-2FdA is a highly potent antiviral that acts by a novel mechanism, inhibiting the translocation function of the reverse transcriptase.

Structures of Natural Substrate and Inhibitors of HIV RT



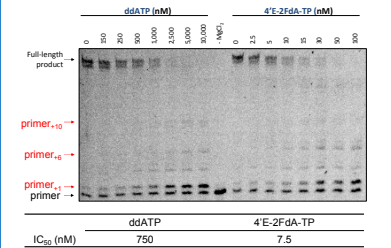
Antiviral activity of 4'E-2FdA against WT and mutant HIV-1

(determined in Dr. Kodama's and Dr. Mitsuya's laboratories)

	EC ₅₀ (μM) ^a		
	AZT	ddl	4'E-2FdA
WT	0.015	4.1	0.0011
K65R	0.004 (X0.3)	ND ^b	0.00023 (X0.2)
L74V	0.019 (X1)	14.6 (X3.5)	0.0048 (X0.4)
M184V	0.003 (X0.2)	ND	0.0083 (X7.5)
M41L/T215Y	0.12 (X8)	ND	0.0016 (X1.5)
M41L/T69S/G7215Y ^c	0.20 (X13)	21 (X5)	0.006 (X6)
MDR ^c	18 (X1,200)	40 (X10)	0.00074 (X0.7)

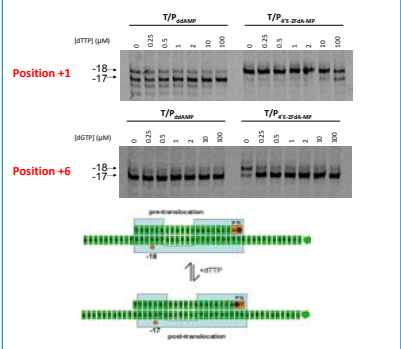
^aAnti-HIV activity was determined with the MAGI assay. Results of at least 3 independent experiments. ^bHIV-1 contains T69S substitution and 6-base pair insertions between 69 and 70 (S-G) and M41L/T215Y. ^cMulti-ddNTP resistant HIV-1: AG29/V75/F77L/F116Y/Q151M. ^dND, not determined.

4'E-2FdA-TP inhibits RT at low nM concentrations



Primer extension was observed in the presence of various concentrations of dDATP or 4'E-2FdA-TP. The DNA sequence used in this assay is shown below the gel. A heteropolymeric 43-mer was used as DNA template and a 5' Cy3 labeled 18-mer DNA primer. The template positions where chain-termination may occur are highlighted and numbered in red. The calculated IC₅₀ is indicated below the gel for both dDATP and 4'E-2FdA-TP. Notably, when a longer template is used (100mer), the IC₅₀ is ~1nM.

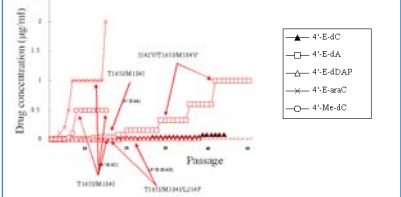
4'E-2FdA-MP is a translocation-deficient RT inhibitor (TDRTi) that blocks RT translocation



The translocation state of RT was observed using Fe²⁺-site-specific footprinting. In this assay, a chain-terminated TTP was incubated with RT and various concentrations of the next incoming nucleotide (dTTP or dGTP). The complex is treated with Fe²⁺ and resolved on polyacrylamide gel. A cut at position -18 is indicative of a pre-translocated complex, while a cut at position -17 represents a post-translocated complex.

Selection of resistance mutation to 4'E-2FdA related inhibitors

(determined in Dr. Kodama's and Dr. Mitsuya's laboratories)



Resistance mutations to 4'-modified nucleosides were selected by serial passage of HIV in cell culture in the presence of increasing concentrations of nucleoside analog.

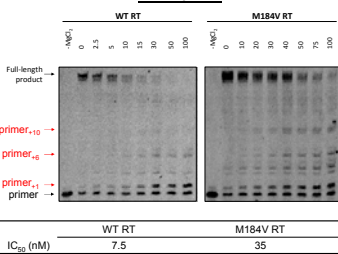
4'E-2FdA selects for mutations M184V, T165R and I142V in the reverse transcriptase of HIV

(determined in Dr. Kodama's and Dr. Mitsuya's laboratories)

	EC ₅₀ (μM) ^a	
	AZT	4'E-2FdA
WT	0.015	0.0011
M184V	0.0021 (X0.14)	0.0083 (X7.5)
T165R	0.011 (X0.7)	0.0016 (X1.5)
I142V	0.016 (X1)	0.001 (X0.9)
T165R/M184V	0.0053 (X0.35)	0.014 (X13)
I142V/T165R/M184V	0.0076 (X0.5)	0.023 (X22)

^aAnti-HIV activity was determined with the MAGI assay. Results of at least 3 independent experiments. ^bND, not determined.

The M184V mutation confers resistance to 4'E-2FdA-TP



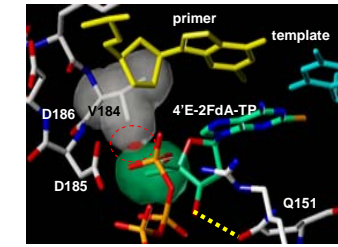
Primer extension by WT and M184V RT was observed in the presence of various concentrations of 4'E-2FdA-TP. The template positions where chain-termination may occur are highlighted and numbered in red. The calculated IC₅₀ is indicated below the gel for both enzymes.

The M184V mutation confers resistance by decreasing the binding affinity for 4'E-2FdA

	K _m (nM)	k _{cat} (min ⁻¹)	k _{cat} /K _m (min ⁻¹ μM ⁻¹)
WT RT	1.6	0.19	0.12
M184V RT	10.7	0.22	0.021

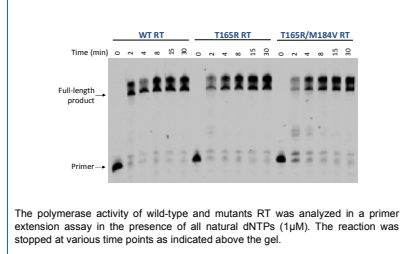
Kinetic constants for 4'E-2FdA-TP incorporation by WT and M184V RT were measured in a single nucleotide incorporation assay. The products were resolved on denaturing polyacrylamide gel and quantified. The Michaelis-Menten equation was used to calculate K_m and k_{cat} from the observed reaction velocities.

Valine at position 184 in RT causes steric hindrance with 4'E-2FdA-TP



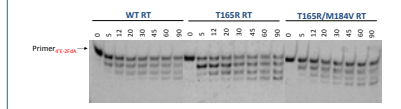
Molecular model of M184V RT bound to a DNA/DNA Template/Primer and an incoming 4'E-2FdA-TP. Small steric conflict (encircled red volume) between the ethynyl group (green Van der Waals volume) of the 4'E-2FdA-TP and the V184 side chain (grey Van der Waals volume) of M184V RT can be seen, which results in moderate decrease in binding and inhibitor resistance.

Mutations T165R and M184V decrease the polymerase activity of RT



The polymerase activity of wild-type and mutants RT was analyzed in a primer extension assay in the presence of all natural dNTPs (1μM). The reaction was stopped at various time points as indicated above the gel.

Mutations T165R and M184V affect the excision activity of RT



RT was incubated with a 4'E-2FdA terminated DNA primer annealed to a DNA template and incubated with 150μM pyrophosphate as a substrate for excision. The reaction was allowed to proceed at 37°C for various time as indicated above the gel.

Conclusions

1. **TDRTi**s are ultra-potent inhibitors of HIV
2. 4'E-2FdA-TP inhibits RT approximately 100 times more efficiently than dATP
3. 4'E-2FdA-MP blocks elongation mainly at the site of incorporation
4. 4'E-2FdA-MP decreases binding of the incoming nucleotide by stabilizing RT in the pre-translocation state
5. The M184V mutation appears to cause resistance to 4'E-2FdA-TP by decreasing the binding affinity for the inhibitor
6. Mutations I142V, T165R and M184V are selected in cell culture by 4'Ethynyl compounds
7. Mutations T165R and M184V reduce the polymerization and excision activities of RT

Acknowledgments

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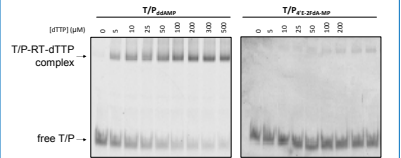
Antiviral activity of 4'- and 2-halo-substituted adenosine analogs

(determined in Dr. Kodama's and Dr. Mitsuya's laboratories)

Compound	EC ₅₀ (μM) ^a	CC ₅₀ (μM) ^b	Selectivity Index ^c
4'E-dA	0.01 ± 0.0027 ^d	104 ± 6	11,000
4'E-2FdA	0.00007 ± 0.00001	10 ± 3	>130,000
4'E-2FdA	1.17 ± 0.29	230 ± 33	196
4'E-2FdA	0.11 ± 0.033	98 ± 26	899
4'CN-2FdA	0.1 ± 0.03	>340	>3,300
4'E-2CldA	0.0007 ± 0.0002	230 ± 16	>330,000
ddl	28 ± 12	>100	>4
AZT	0.0028 ± 0.0006	30 ± 7	10,800

^aEC₅₀ concentration that blocks replication by 50%. ^bCC₅₀ concentration that suppresses the viability of HIV-unspliced cells by 50%. ^cSelectivity Index=CC₅₀/EC₅₀. ^dData shown are mean values with standard deviations for at least three experiments.

Incorporation of 4'E-2FdA-MP affects the binding of the next incoming nucleotide



The stability of the template/primer-RT-dNTP ternary complex was analyzed by incubating RT and chain-terminated T/P in the presence of increasing dTTP concentrations and heparin, acting as an enzyme trap. In the absence of dTTP, the T/P-RT complex is unstable. RT can fall off the T/P and get trapped by heparin. Binding of dTTP to T/P-RT stabilizes the complex which can be observed on a native polyacrylamide gel.