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Mutational Patterns Associated with Reduced and Increased Susceptibility to NcRTIs (Nucleotide-competing Reverse Transcriptase Inhibitors) in Over 6000 Clinical HIV-1 Isolates

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ABSTRACT

Nucleotide-competing reverse transcriptase inhibitors (NcRTI) bind to the active site of HIV reverse transcriptase (RT) in competition with the next incoming nucleotide. To further investigate the impact of RT inhibitor resistance mutations on the activity of NcRTIs, the susceptibility of > 6000 recent clinical isolates for a prototype compound, NcRTI-1, was determined. Over 80% of the profiled clinical isolates remained susceptible for NcRTI-1 (FC < 4). No cross-resistance was observed between NcRTI-1 and currently used RT inhibitors, apart from limited cross-resistance with 3TC/FTC.
Analysis of the genotype of > 1700 of these viruses showed that the combination of active site mutations M184V + Y115F correlated most with resistance to NcRTI-1 (FC = 75). Analysis also indicated that the K65R mutation is associated with hypersusceptibility to NcRTI-1 and that it reverses the reduced susceptibility caused by M184V. These findings were confirmed in SDM strains. This reciprocity between the K65R and M184V mutation is unparalleled among RT inhibitors. When replicating wild-type HIV-1 in the presence of NcRTI-1, M184V + Y115F were selected. In the presence of both NcRTI-1 and tenofovir, NcRTI-1 prevents the selection of K65R.

INTRODUCTION

- Conversion of the HIV RNA genome into DNA by viral reverse transcriptase (RT) is a key step in the early stages of the HIV-1 life cycle.
- Two classes of RT inhibitors are currently approved for antiretroviral therapy:
  - Nucleos(t)ide RT Inhibitors (N(t)RTIs): abacavir (ABC), didanosine, emtricitabine (FTC), lamivudine (3TC), stavudine, tenofovir disoproxil fumarate (TDF), zalcitabine, and zidovudine (ZDV)
  - Non-Nucleoside RT Inhibitors (NNRTIs): delavirdine, efavirenz (EFV), and nevirapine (NVP)
- A series of compounds that belong to a novel class of HIV RT inhibitors has been described[1,2]. They differ from N(t)RTIs by chemical structure, absence of chain terminating properties, and lack of phosphorylation requirement, and from NNRTIs in terms of mechanism of action and binding pocket. Since they reversibly bind to the RT active site in competition with natural dNTP substrates (Figure 1), we refer to this class as Nucleotide-competing RT Inhibitors (NcRTIs).
- NcRTI-1, the prototype compound, has an EC_{50} for HIV-1 IIIB (wild type) of 30 nM
- To get a better view on how mutations associated with resistance to current RT inhibitors impact the NcRTI class, the susceptibility of over 6000 recent clinical isolates for NcRTI-1 was determined.

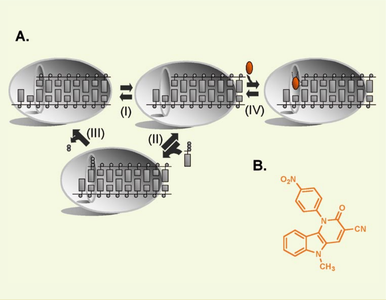

Figure 1:
A. Model of the mechanism of action of NcRTIs. I: After binding the template/primer, RT (large sphere) translocates to clear the active site, sliding one base upstream along the template/primer. II: Hence, RT is able to bind the next incoming dNTP. III: This is followed by polymerization and release of PPi. IV: NcRTI (orange) reversibly binds the active site of RT via interactions with the enzyme as well as with the primer-end that needs to be a pyrimidine (dashed lines). II and IV: In binding the active site, NcRTIs compete with the next incoming nucleotide.
B. Chemical structure of NcRTI-1, a prototype compound of this chemical series.

METHODS

- Recombinant HIV-1 viruses, derived from clinical samples, were constructed by cotransfection of MT4 cells with sample derived viral protease and RT coding sequences and an HIV-1 HXB2-derived proviral clone deleted in the protease and RT coding region[3].
- Site-directed mutant RT coding sequences were generated from a pGEM vector containing the HIV-1 clone HXB2 protease and RT coding sequence by using a QuikChange site-directed mutagenesis kit, and HPLC-purified primers. Plasmids were sequenced to confirm that they contained the desired mutations. Mutant viruses were created by recombination of the mutant protease-RT sequence with a protease-RT deleted HIV-1 HXB2 proviral clone[3].
- To *in vitro* select viral strains resistant to RT inhibitors, MT4-LTR-EGFP cells were infected in the presence of the inhibitor (at 2 or 3 times EC_{50}). Cultures were passaged every 3 to 4 days at the same concentration of inhibitor, until full virus breakthrough. At that time, virus was harvested and used for a new round of selection at a higher compound concentration. At each breakthrough, the harvested virus was genotyped to identify acquired mutations.

RESULTS

	3TC	ZDV	TDF	ABC	NVP	EFV	NcRTI-1
3TC	1.0	0.28	0.20	0.60	0.33	0.28	0.56
ZDV		1.0	0.64	0.58	0.30	0.29	0.17
TDF			1.0	0.45	0.29	0.27	0.06
ABC				1.0	0.34	0.33	0.32
NVP					1.0	0.82	0.20
EFV						1.0	0.16
NcRTI-1							1.0

Pearson correlation coefficient: < 0.4 | 0.4 - 0.7 | > 0.7

Table 1: Resistance correlation between different RT inhibitors is shown as the correlation coefficient calculated by linear regression of a cross-correlation plot.

- The susceptibility of over 6000 recent clinical HIV-1 isolates for NcRTI-1 and different N(t)RTIs and NNRTIs was assessed:
  - 82% of the profiled clinical isolates remained susceptible for NcRTI-1 (fold change in EC_{50} (FC) < 4), 15.7% of the population showed a reduced susceptibility (FC 4-10) while only 2.3% showed resistance (FC > 10).
  - No cross-resistance was observed between NcRTI-1 and the NNRTIs EFV or NVP, neither with the N(t)RTIs ZDV, TDF, and ABC. Only with the N(t)RTIs 3TC and FTC, limited cross-resistance could be detected (Pearson Correlation Coefficient = 0.56) (Table 1).

- Analysis of the genotype of a random set of more than 1700 of the 6000 tested clinical isolates (Figure 2) showed that:
  - The combination of mutations M184V + Y115F correlated most with reduced susceptibility. Given that Met184 and Tyr115 are part of the HIV RT active site, this finding underscores the mechanistic model for NcRTIs.
  - Another compelling pattern that emerged from the study of clinical isolates is the K65R-associated hypersusceptibility of NcRTI-1. Moreover, the K65R mutation reverses the M184V-induced resistance of HIV-1 for NcRTI-1.
  - NcRTI-1 activity is not affected by the presence of NNRTI resistance mutations, nor by the presence of the major N(t)RTI induced, multi-drug resistant mutation patterns: the thymidine-associated mutations (TAMs), the T69insertion complex, and the Q151M complex. (data not shown)

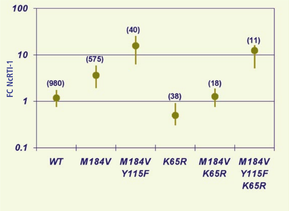

Figure 2: A random set of over 1700 clinical HIV-1 isolates was categorized into 6 groups according to their genotype. For each group the median FC for NcRTI-1, the interquartile range, and the number of viruses is shown. WT indicates the group of viruses without mutations at position 184, 115 or 65.

- Findings with clinical isolates were confirmed in site-directed mutant (SDM) strains (Table 2):
  - While the single mutations M184V and Y115F showed a moderate FC of 5.0 and 7.9, respectively, the combination resulted in a FC of 75.
  - The presence of the K65R point mutation causes an increased susceptibility of HIV-1 for NcRTI-1 (FC = 0.46), and reverses M184V-induced reduction of NcRTI sensitivity (FC from 5.0 to 0.89).
  - NcRTI-1 remained active on a wide variety of strains containing NNRTI resistance associated mutations.
  - The major N(t)RTI induced, multi-drug resistant mutation patterns (TAMs, T69insertion complex and Q151M complex) do not affect NcRTI-1activity.

	NcRTI-1	3TC	ZDV	TDF	NVP
Y115F M184V	75 (2.45-200)	> 100 (3)	0.5 (0.1-45-1)	0.9 (0.1-0.5-1.2)	2.3 (2.3-2.7)
M184V	5.0 (18. 3.8-6.5)	> 100 (19)	1.0 (24. 0.61-1.2)	0.8 (18. 0.44-1)	2.3 (16. 1.2-3.1)
Y115F	7.9 (2. 6.3-14)	0.7 (4. 0.73-1.1)	0.9 (8. 0.81-1.1)	1.0 (8. 0.85-1.4)	2.9 (4. 2.3-5.3)
K065R	0.5 (5. 0.29-0.57)	> 20 (3)	2.0 (4. 1.9-2.3)	6.0 (1)	3.4 (5. 2.4-3.7)
K065R M184V	0.9 (6. 0.85-0.93)	> 100 (7)	1.1 (8. 0.87-1.3)	2.3 (8. 1.7-2.7)	1.2 (7. 1.1-1.5)
M041L D067N K070R T215Y	3.0 (5. 1.9-3.6)	8.1 (4. 7.44-4)	67 (8. 22-70)	3.3 (5. 1.8-4.3)	2.8 (4. 2.5-5)
T069S ins069-070S-S L210W T215Y	0.9 (3. 0.84-1.1)	9.5 (2. 7.2-13)	53 (4. 36-68)	2.3 (2. 2.2-2.5)	1.1 (3. 0.85-1.5)
A62V V75I F77L F116Y Q151M	1.0 (5. 0.88 - 1.2)	ND	> 100 (5)	3.7 (5. 3.4 - 6.7)	ND
Y181C	2.3 (15. 1.3-5.4)	1.1 (62. 0.79-1.7)	0.6 (164. 0.4-1.1)	0.7 (105. 0.52-1)	> 100 (70)
K103N	1.9 (3. 1.3-2)	1.1 (32. 0.86-1.5)	1.1 (111. 0.76-1.7)	0.6 (35. 0.5-0.85)	50 (8. 29-126)
K103N Y181C	2.1 (147. 1.3-2.9)	0.9 (508. 0.71-3.2)	0.7 (538. 0.32-1.2)	0.5 (209. 0.29-0.78)	> 100 (6)
F227C L234I	2.0 (5. 1.5-2.2)	1.1 (32. 0.94-1.4)	0.3 (118. 0.2-0.43)	0.6 (61. 0.39-0.78)	31 (9. 11-39)
Y188L	1.7 (4. 1.6-1.8)	1.9 (51. 1.3-2.6)	0.7 (115. 0.42-1.1)	0.7 (67. 0.4-0.93)	> 100 (6)
L100I K103N	2.6 (5. 1.9-3.2)	0.5 (46. 0.3-0.68)	0.3 (147. 0.22-0.46)	0.3 (51. 0.24-0.44)	> 100 (7)
K101E K103N	2.1 (5. 1.5-2.1)	1.3 (235. 0.94-1.8)	1.5 (302. 0.87-2.3)	0.5 (58. 0.4-0.75)	> 100 (6)
HIV-2 (ROD)	14 (10. 10-17)	0.6 (39. 0.46-0.96)	0.1 (38. 0.051-0.12)	1.0 (10. 0.81-1.7)	> 100 (4)

Table 2: HIV-1 strains with mutations in the RT gene were generated by site-directed mutagenesis. The susceptibility for NcRTI-1 and other RT inhibitors is shown. Number of measurements and interquartile ranges are indicated between brackets (n; Q1-Q3).

- For several RT inhibitors, alone or in combination, the mutations selected when replicating HIV-1 IIIB in their presence, were determined (Table 3):
  - When NcRTI-1 was present as sole inhibitor, HIV-1 IIIB acquired mutations M184V and Y115F, in line with tests on clinical isolates and SDMs (Figure 3).
  - When 3TC or FTC was combined with TDF, the K65R mutation was selected, in accordance with previous publications[4].
  - Combining NcRTI-1 with TDF, did not result in selection of the K65R mutation, even after prolonged exposure (190 days). Instead, the virus acquired the K70E or K70N mutation.

Figure 3: *In vitro* selection experiment with NcRTI-1 starting from HIV-1 IIIB. Each dot represents virus breakthrough at the indicated NcRTI-1 concentration. Mutational patterns found in the harvested virus population are shown. X-axis indicates time of selection (days).

Compound(s)	Passage	Conc (µM)	Selected RT Mutations
3TC	10	128	**M184V**
3TC	4	8	**M184I**
FTC	6	4	**M184I**
FTC	5	4	**M184V**
FTC	6	4	**M184I**
TDF	10	40	V179I **K065R**
TDF	7	40	**K065R**
TDF	23	80	**K065R**
NcRTI-1	10	2.4	**Y115F M184V**
TDF/3TC	12	20/16	**K065R** E248K
TDF/3TC	9	10/8	**K065R**
TDF/FTC	9	20/2	**K065R**
TDF/FTC	13	20/2	K070E
TDF/FTC	10	10/1	**K065R**
TDF/NcRTI-1	57	40/0.6	M041L K070N L193I
TDF/NcRTI-1	55	40/0.6	M041L K070E Y181C N348I
TDF/NcRTI-1	21	20/0.3	K070E
TDF/EFV	17	40/0.008	**K065R K103N**

Table 3: *In vitro* selection experiments were performed by replicating HIV-1 IIIB in the presence of one or two RT inhibitors. The table indicates the number of passages, the final compound concentration and the mutations present in the harvested virus population.

CONCLUSIONS

- Mutational patterns associated with reduced and increased susceptibility to NcRTIs are different from patterns associated with currently used RT inhibitors. Only limited cross-resistance with 3TC could be detected.
- The combination of RT-active site mutations M184V + Y115F correlated most significantly with resistance to NcRTI-1 (FC = 75x). This combination is also selected *in vitro* when replicating HIV-1 IIIB in the presence of increasing concentrations of NcRTI-1.
- Unlike 3TC or FTC where M184V causes complete resistance (FC > 100), the effect of M184V on NcRTI-1 susceptibility is limited (FC = 5.0).
- The K65R mutation causes hypersusceptibility to NcRTI-1. In addition, presence of K65R reverses the M184V-induced reduction of NcRTI-1 sensitivity. This reciprocity between the K65R and M184V mutation is unparalleled among RT inhibitors.
- When combined with TDF, NcRTI-1 prevents the selection of K65R by TDF. In these experiments, viruses acquired K70E, a mutation previously suggested as an alternative pathway of TDF resistance[5].
- The *in vitro* resistance data hint that the novel NcRTI class of RT inhibitors will be a valuable addition to the current N(t)RTI backbone in antiretroviral therapy.

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