Characterization of amino acids Arg, Ser and Thr at position 70 within HIV-1 reverse transcriptase

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Objectives: The amino acid position 70 in HIV-1 reverse transcriptase (RT) plays an important role in nucleoside RT inhibitor (NRTI) resistance. K70R is part of the thymidine analog mutations, but also other amino acid changes have been associated with NRTI resistance, such as K70E and K70G. In this study, we investigated the in vivo selection of the HIV-1 RT mutations K70S and K70T and their in vitro effect on drug resistance and replication capacity.

Methods: Recombinant viruses with RT mutations were generated to measure the in vitro drug susceptibility and replication capacity. Bayesian network analysis and three-dimensional modeling were performed to understand the selection and impact of the RT70 mutations.

Results: K70S and K70T were found at a low frequency in RTI-experienced HIV-1 patients (0.10% and 0.20%). Baeyesian network learning identified no direct association with the in vivo exposure to any specific RTI. However, direct associations of K70S with mutations within the Q151M-complex and of K70T with K65R were observed. In vitro phenotypic testing revealed only minor effects of K70R/S/T as single mutations, associated with Q151M and within the context of the Q151M-complex.

Discussion: These results suggest that the selection of K70S/T and their phenotypic impact are influenced by the presence of other mutations in RT. However, the low impact on in vitro phenotype here observed, alongside with the low in vivo prevalence, the exclusive direct association with known major RTI mutations and the unknown correlation with in vivo response, do not yet necessitate the inclusion of K70S/T in drug resistance interpretation systems.

Keywords: HIV-1, Resistance, Mutation, Reverse transcriptase

Introduction

Nucleoside reverse transcriptase (RT) inhibitors (NRTIs) are analogs of deoxyribonucleosides that lack the 3'-hydroxyl group and cause chain termination during reverse transcription. NRTIs often constitute the backbone of combination antiretroviral therapy (cART) in HIV-1 patients. Despite the success of cART in reducing morbidity and mortality, its long-term activity can be compromised because of drug resistance development. Resistance to NRTIs generally occurs either by the excision or by the exclusion/discrimination mechanism. The first mechanism consists of an enhanced ATP-mediated excision of the inhibitor at the dNTP binding site, as initially observed for the thymidine analog zidovudine (AZT) and typically caused by thymidine associated mutations (TAMs). The TAMs confer cross-resistance to most NRTIs and are associated with hypersusceptibility towards foscarnet (PFA). Second, resistance by exclusion consists of the selective discrimination against NRTIs but still allows incorporation of dNTPs, as described for several mutations and the multi-NRTI drug resistance pathway marked by Q151M.
Position 70 in RT plays an important role in NRTI resistance development. Different mutations at this position have been linked to resistance: K70R as part of the TAM pathway enhances ATP-mediated excision, and K70E induces resistance by lowering maximum rate of inhibitor incorporation by RT and antagonizes the TAM-mediated nucleotide excision. K70Q in the background of the multi-NRTI Q151M mutation pathway results in a decreased binding affinity of the inhibitor. Previously, we identified a HIV-1 patient in which K70S emerged within the context of the Q151M mutation pathway. This mutation was substituted for K70T in subsequent drug-free in vitro cultivation experiments. In this study, we investigated the in vivo selection of K70S and K70T and their impact on replication capacity and RTI drug susceptibility: as single mutations, associated with Q151M and within the context of the Q151M-complex.

Materials and Methods

Patient information and in vivo drug resistance profile

The identified patient received several sequential mono- and bitherapies during the observation period (Fig. 1). The polymorphic mutations A98S, I135T and G196E were observed in a sample that was isolated in 1992, after a short course of AZT and interferon-α (IFN-α) that was followed by a long episode of IFN-α monotherapy. In 1992, zalcitabine (DDC) was initiated and was subsequently replaced by didanosine (DDI). At that time, the mutations S68G and Q151M could be detected, together with two additional polymorphic mutations (V35I and I178M). In 1993, the patient received a short episode of AZT-DDI bitherapy which was soon interrupted and followed by short courses of DDC and stavudine (D4T) monotherapy. Three extra mutations from the multi-nucleoside Q151M resistance pathway (V75I, F77L and F116Y) and one extra polymorphism (I202V) were developed in the sample that was isolated 2 weeks after the start of D4T therapy. Immediately before the initiation of the non-nucleoside reverse transcriptase inhibitor (NNRTI) loviride (α-APA), a fourth isolate was available from which the sequence was retrospectively determined, revealing the disappearance of V35I and the presence of the mixture A62AV. During the selective pressure of loviride and lamivudine (3TC), this mixture disappeared again and the NNRTI mutation K103N together with the 3TC mutation M184V and a yet uncharacterized K70S mutation were detected. The K70S mutation was substituted for K70T in subsequent drug-free in vitro cultivation experiments.

Cells and compounds

Human embryo kidney cells (293T) were purchased from the ATCC (LGC Standard, Teddington, UK) and cultivated in DMEM (Life Technologies, Gent, Belgium) containing 10% fetal calf serum (FCS) (Perbio Science, Erembodegem, Belgium), 20 μg/ml gentamicin (Life Technologies) and 75 mM NaHCO3 (Life Technologies). U87.CD4 cells transfected with CXCR4 and CCR59 were cultured in DMEM supplemented with 15% FCS, 75 mM NaHCO3, 0.2 mg/ml geneticin (Life Technologies), 20 μg/ml gentamicin and 1 μg/ml puromycin (Sigma-Aldrich, Bornem, Belgium). Subcultivation was performed every 2–3 days by digestion with trypsin/EDTA (Life Technologies). MT-4 cells10 were grown in RPMI 1640 (Life Technologies) supplemented with 10% FCS, 75 mM NaHCO3, 2 mM glutamine (Life Technologies) and 20 μg/ml gentamicin. All cell cultures were maintained at 37°C in a humidified CO2-controlled atmosphere.

Zidovudine (AZT), stavudine (D4T), didanosine (DDI), lamivudine (3TC), abacavir (ABC) and efavirenz (EFV) were obtained through the AIDS Research and Reference Reagent Program (Division of AIDS, NIAID, NIH). Tenofovir (TDF), raltegravir (RAL) and AMD3100 were kind gifts of Gilead Sciences (Foster City, CA, USA), Merck & Co (Rahway, NJ, USA) and Anormed (Langley, BC, Canada), respectively. Foscarnet (PFA) was purchased from Sigma-Aldrich (Diegem, Belgium).

Plasmids

p83-2 containing the 5′-part of the HIV-1 subtype B strain NL4.3 (GenBank accession no. AF324493) was used as starting material for site directed mutagenesis. This vector was obtained through the AIDS Research and Reference Reagent Program (Division of AIDS, NIAID, NIH) from Dr Ronald Desrosiers.11

The molecular clone used to generate recombinant viruses was pNL4.3-ART-EGFP (enhanced green fluorescent protein). It displays a deletion between
RT codon 1 and 560 and contains the gene encoding an EGFP between env and nef without affecting the expression of any HIV gene.12

**Site directed mutagenesis**

The RT region was amplified from p83-2 using PfuTurbo (Stratagene, Amsterdam, The Netherlands) and primers KVL098 and KVL099.12 The RT amplicons were cloned into pCR-XL-TOPO plasmids, using the TOPO® XL PCR Cloning Kit (Life Technologies) and *Escherichia coli* DH5α-T1 competent cells (Life Technologies). Clones were randomly picked and cultured. Subsequently, plasmid DNA was isolated using QIAprep Spin Miniprep Kit (QIAGen, Venlo, The Netherlands) and the RT region was confirmed by sequencing.12 This plasmid was used for the site directed mutagenesis experiments.

Mutations at position 62, 68, 70, 75, 77, 116, and 151 were introduced by extending primers containing the desired mutations through thermocycling with PfuTurbo (Stratagene), followed by digestion of the template with DpnI (Fermentas, St. Leon-Rot, Germany) and subsequent transformation in *E. coli* DH5α-T1 competent cells. The following site-directed-mutants (SDM) were constructed: K70R, K70S, K70T, Q151M, Q151M + K70S, Q151M + K70T, A62V/S68G/V75I/F77L/F116Y/Q151M (Q151M-complex), Q151M-complex + K70S and Q151M-complex + K70T. Plasmid DNA was extracted using QIAprep Spin Miniprep Kit (QIAGen). The presence of the desired mutation and the absence of additional mutations in the original backbone were confirmed by sequencing the entire RT region as previously described.12

**Generation of recombinant viruses**

RT recombinant viruses containing the EGFP gene were generated as previously described.12 Briefly, the SDMs were amplified with the Platinum PfX DNA Polymerase (Life Technologies) and primers KVL098 and KVL099. The day before transfection 700 000 293T cells were subcultivated in small culture flasks containing 5 ml DMEM for 293T cells. Two micrograms of purified PCR product (QIAquick PCR Purification Kit, QIAGen) and 10 μg of purified XbaI-digested pNL4-3-ART-EGFP (PuriLink HiPure Plasmid Filter Purification Kit, Life Technologies) were co-transfected in these 293T cells using the standard calcium-phosphate method. After overnight incubation, medium was refreshed and 48 hours later, the supernatant was transferred to freshly plated U87.CD4.CXCR4.CCR5 cells. Cell cultures were monitored for EGFP expression using fluorescence microscopy. Virus supernatant was harvested by low speed centrifugation and stored at −80°C for further use. The RT sequence of the recombinant viruses was confirmed.

**Drug susceptibility testing**

Drug susceptibility testing of the recombinant viruses was performed as previously described.12 Briefly, 20 000 U87.CD4.CCR5.CXCR4 cells were infected with the viruses in the presence of triplicate 5-fold serial dilutions of the drugs (AZT, ABC, D4T, TDF, DDI, 3TC, ETV, PFA and RAL) within a 96-well tray. For AMD3100, infection was not carried out until half an hour after the addition of the compound to allow for interaction between AMD3100 with CXCR4. Twenty-four hours post-infection, the percentage of EGFP-expressing cells was determined by flow cytometry. EC50 was calculated according to Reed and Muench for wild-type and recombinant viruses in triplicate.13 The mean fold change (FC) and the standard deviation (SD) were obtained from three independent triplicate experiments. Two-sided one-sample *t*-test, two-sample *t*-tests, Wilcoxon-signed rank test or Mann–Whitney test were performed as appropriate. The statistical significance was set at *P*<0.05. All data were analyzed using GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego, CA, USA).

**Analysis of replication capacity**

For the analysis of replication capacity, one million exponentially growing MT-4 cells were infected with recombinant virus (pre-diluted to achieve 0.1% of EGFP-positive cells after 72 hours) in a total volume of 500 μl. The testing was done in triplicate for each virus in 24-well plates. After 4 hours, the remaining virus was washed away with PBS and cells were cultured in 2 ml of RPMI at 37°C. Seventy-two hours after infection, the amount of cells in each triplicate was normalized to 750 000 cells/ml and the removed cells were fixed with 2% paraformaldehyde. This procedure was repeated every 24 hours for the following 72 hours (i.e. 96, 120, and 144 hours). The percentage of EGFP-expressing cells was determined by flow cytometry. The amount of infected cells was normalized to the percentage of infected cells at 72 hours (day 0). The curves were fitted exponentially and the resulting *k*-values (*y* = *e*^*kt*) were used as a measure of replication capacity.14 Two-sided two-sample *t*-tests (*P*<0.05) were performed using GraphPad Prism version 5.01 for Windows (GraphPad Software).

**Three-dimensional (3D) modeling**

The crystal structures used for the generation of molecular models (1RTD and 3KLE) were downloaded from the Protein Data Bank (PDB). For 1RTD, the nucleotide was removed and mutations at position 70 were introduced using Chimera.15 The structure for AZTTP was created using CS ChemOffice (CambridgeSoft Corporation), and saved as a mol2 file for import into AutoDock.
Tools 1.5.4. RT structures with mutations at position 70 and AZTTP were prepared for docking using AutoDock Tools 1.5.4.: all hydrogens were added. Gasteiger partial charges were assigned to the protein and the small molecule, non-polar hydrogens were merged with carbon atoms and AD4 atom types were assigned. Flexible residue files for K65, K70R/T, and R72 were generated in AutoDock Tools, as well as rigid receptor files for the remaining residues in the protein structures. Box coordinates were positioned over the polymerase active site. AZTTP was docked to mutant RT using AutoDoc Vina, with an exhaustiveness of 100. Energy-minimized residue poses were overlaid using the SuperPose protein superposition server.

Bayesian network learning
A Bayesian network (BN) is a probabilistic model that describes statistical independencies between variables, with dependencies visualized in a directed acyclic graph. Following a previously described methodology, clinical data were pooled from the HIV Drug Resistance Database (Stanford University, CA, USA), the University Hospital Leuven (Belgium) and Hospital Egaz Moniz (Lisbon, Portugal). The analysis included viral isolates spanning the RT region that were sampled from HIV-1 patients receiving cART containing NRTIs ± NNRTIs, with at most 1 sequence selected from each therapy line of a patient. The robustness of the network was assessed with a non-parametric bootstrap using 100 replicates.

Results
In vivo emergence of mutations at position 70 in RT
A search within a dataset of 15,845 sequences from 12,968 RTI-experienced HIV-1 patients revealed a frequency of 19.1% K70R, 0.1% K70S and 0.2% K70T. BNs were constructed to investigate interactions between mutations at position 70, known (N)NRTI resistance mutations and therapy experience. The consensus network is shown in Fig. 2. BN analysis revealed a direct positive association of the non-polymorphic mutations within the Q151M-complex (V75I, F77L, F116Y, and Q151M) with K70S but only with a low bootstrap value. The mutation K70T was directly associated with K65R. No direct connection with a specific NRTI was observed for K70S/T.

Drug susceptibility of RT mutants
To investigate the effect of K70S and K70T mutations on drug susceptibility, the mutations were introduced into the NL4.3 backbone as single mutants or in combination with either Q151M or the Q151M-complex. The single TAM mutation K70R was included in the experiments as a positive control. Susceptibility to several NRTIs (AZT, D4T, DDI, 3TC, ABC, TDF) was tested in a single cycle assay using U87.CD4.CXCR4.CCR5 cells (Fig. 3). As NRTI mutants are often characterized with hypersusceptibility to PFA, this drug was also included. The NNRTI EFV, the integrase inhibitor RAL and the CXCR4 antagonist AMD3100 were included as control drugs.

For the single mutants K70R, K70S and K70T only minor changes in drug susceptibility towards NRTIs were observed (Fig. 3A). Although the biological relevance is uncertain, statistically significant reduced susceptibilities were observed for K70R towards AZT (1.72 ×) and for K70T towards 3TC (1.34 ×). Surprisingly, statistically significantly increased susceptibilities were observed for K70S and K70T towards EFV (0.50 × and 0.55 ×).

Q151M resulted in statistically significant low-level resistance against AZT and DDI (2.1 × and 3.84 ×, respectively) (Fig. 3B). The susceptibility levels towards all NRTIs decreased for the entire Q151M-complex (FC ranging between 2.2 × and 26.4 ×) (Fig. 3C). The addition of K70S/T to Q151M did not result into statistically significant changes in drug susceptibility, except for a slightly lower susceptibility against 3TC for Q151M + K70T (shift from 1.23 × to 2.35 ×) (Fig. 3B). In the context of Q151M-complex, the addition of K70S/T resulted into a slightly higher susceptibility to AZT (shift from 26.26 × to 17.79 × and 21.35 ×, respectively) and PFA (shift from 0.9 × to 0.5 × and 0.4 ×, respectively) and conferred a slight reduction in the susceptibility to EFV (shift from 1.72 × to 2.91 × and 3.00 ×, respectively). The addition of K70S/T to Q151M-complex did not change significantly the susceptibility to other drugs.

The fold-changes towards the control drugs RAL and AMD3100 ranged between 0.73 × and 1.98 ×, of which some values were statistically significantly different from wild-type.

Replication capacity of the RT mutants
To investigate the impact of the described mutations and mutational patterns on the replication capacity, growth kinetic experiments were performed in a drug-free environment. Results for the different viruses are shown in Fig. 4. A statistically significant improved replication capacity was observed for K70S (k-value of 2.56 versus 2.14 for wild-type NL4.3). The six mutants with Q151M and Q151M-complex, displayed a replication capacity significantly impaired compared to wild-type (k-values between 0.61 and 0.78). The addition of K70S and K70T did not significantly impact the replication capacity of Q151M and Q151M-complex.

3D modeling of the RT mutants
To gain insight into the phenotypic impact of mutations at position 70 towards AZT and PFA, 3D models were constructed (only effects of K70R/T...
are visualized in Fig. 5). The positions of K65, K70 and R72 were analyzed in a crystal structure of a ternary complex RT-dsDNA-AZTppppA' (Fig. 5A). The side chains of K70S/T are too short to interact with bound ATP. Moreover, K70T would abrogate the stabilizing effect of arginine on ATP binding. In an attempt to assess the roles of K70R and K70S/T in changing susceptibility to PFA, we modeled these mutations using the structure of a ternary RT-DNA-dNTP complex (Fig. 5B and C).
models predict that K70T results in a shift of R72 whereby the positioning of K65, K70T and R72 stabilize the interaction with bound PFA. This conformation also stabilizes the b and c phosphates of docked AZT-TP. Modeling the interaction of K70R and R72 reveals that K70R can stack against R72, which alters contacts with the phosphates of an incoming nucleotide, and by extension, with PFA.

Discussion
In a clinical sample obtained from an HIV-1 patient we observed the acquisition of K70S in the presence of the multi-NRTI Q151M mutation pathway.7 In subsequent drug-free in vitro cultivation experiments this mutation evolved to K70T.8 At the onset of this in vitro study, only K70R and K70E were already reported as mutations associated with resistance to specific NRTIs.

Bayesian Network learning was applied on a public dataset combined with data from Leuven and Lisbon. K70S and K70T had a low prevalence in RTI-experienced HIV-1 patients (0.1–0.2%), in contrast to K70R that had a prevalence of 19.1%. K70S and K70T were not directly associated with a particular drug, indicating that they are probably secondary mutations to the Q151M mutation pathway and K65R, respectively. The direct association of K70S with multi-NRTI resistance mutations was in agreement with its in vivo selection in our patient.7 The results from the BN learning largely confirmed the current scientific consensus: e.g. direct associations between NVP and EFV and specific NNRTI mutations (e.g. NVP and Y181C and V106A/I; EFV and Y188L), 3TC/FTC and M184IV, ABC and TAM2 and Y115F, AZT/D4T and TAM1, and TDF and K65R.

Some previously characterized associations at RT position 70 were also captured by the Bayesian Network approach (Table 1). A direct association was detected between K70R, TAM and exposure to AZT or D4T. The in vivo selection of K70E and K70Q by TDF-containing therapy was also mapped in the network. Additionally, arcs were observed between M184IV and K70E/Q, potentially due to the fact that 3TC and FTC are often part of the selecting regimens.

Although the mutations K70S and K70T were selected in a background already containing NRTI
resistance mutations, their impact as single mutation was also investigated to define their individual contribution to NRTI resistance. This revealed an opposite effect in resistance against AZT and PFA for K70R and K70S/T, respectively. Arginine slightly decreased, whereas serine and threonine slightly increased susceptibility levels for these drugs. This effect in drug susceptibility was also observed within the context of the Q151M-complex. Although changes in drug susceptibility were observed with statistical significance, neither arginine, serine, nor threonine caused extensive changes in susceptibility to other NRTIs when other drug resistance mutations were absent. The addition of K70S/T to Q151M and to the Q151M-complex did not substantially influence the NRTI susceptibility profile, except for a minor reduction in susceptibility towards 3TC for Q151M + K70T. The results of previous studies showed that the individual contribution of mutations at RT position 70 to in vitro drug resistance were minor but that the contribution towards 3TC and FTC in vitro drug resistance was often more substantial than to other NRTIs (Table 1).

Mutation K70R induces a change of the positioning of R72, leading to altered contacts with the phosphates of an incoming nucleotide and with PFA, which could not be restored by the addition of K70S or K70T.

In general, resistance to NRTIs can either involve excision of the incorporated nucleotide analog, or discrimination against the inhibitor at the level of binding and/or incorporation. Recent structural data suggested that K70R facilitates binding of ATP that acts as a pyrophosphate donor in the excision of incorporated AZT. Enzyme kinetic studies have shown that K70E can diminish rates of excision of AZT. In this case, repulsion between K70E and the phosphates of ATP could diminish binding of ATP. In the context of the Q151M-complex, K70Q is responsible for resistance to TDF by changes in the hydrogen bond binding pattern in the polymerase active site of RT.

Modeling studies were performed to gain insight into the phenotypic impact of K70R and K70S/T towards AZT and PFA. Superposition of the models suggested that changes at position 70 can affect the positioning of K65 and R72 that both interact with the phosphate moieties of a bound nucleotide. Mutation K70R induces a change of the positioning of R72, leading to altered contacts with the phosphates of an incoming nucleotide and with PFA, which could partly explain the reduction of in vitro drug susceptibility to AZT and PFA. In contrast, 70S/T can form a hydrogen bond with the side chain of K65. This rearrangement along with the lack of stacking ‘unfreezes’ R72, and contacts between its side chain and PFA appear to be possible under these conditions. Although further analyses are required to

Figure 5  Possible effects of substitutions at position 70 in HIV-1 RT. (A) Positions of K65, K70R and R72 in the crystal structure of a ternary RT-dsDNA-AZTppppA’ complex (PDB code 3KLE) are shown in green. Mutation of K70T would abrogate the stabilizing effect of Arg on ATP binding. (B) Positions for K70, K65 and R72 in the crystal structure of a ternary RT-dsDNA-dNTP complex (PDB code 1RTD) are shown in green. Mg²⁺ ions are green spheres. Residue poses for the flexible docking of AZT-TP to K70T RT and K70R RT are shown in cyan and magenta, respectively. The AZT-TP pose shown is that seen for the docking simulation using the K70R structure generated from 1RTD. Modeled interactions between K65, K70T and R72 result in a shift of R72 into a position that can stabilize the β and γ phosphates of a docked AZT-TP molecule. The same effect can be expected for bound PFA. (C) Stacking interactions between the guanidiniums of 70R and R72 diminish contacts with the phosphates of AZT, resulting in a rearrangement of these groups. The coloring scheme and the AZT-TP pose used are the same as in panel B.
Table 1  Associations of amino acid changes at RT position 70 with drug exposure and drug susceptibilities

<table>
<thead>
<tr>
<th>AA change</th>
<th>In vitro or in vivo drug exposure</th>
<th>In vitro drug resistance (fold-change)</th>
<th>Remarks</th>
<th>References</th>
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<tr>
<td>K70R</td>
<td>Typical TAM mutation</td>
<td>PFA, 3TC, AZT (1 x)</td>
<td>Results are from SDM. Mathiesen et al. (2007)(^21)</td>
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<td>AZT (3.2 x), 3TC (1.8 x), D4T (1.7 x), ABC (1.6 x), DDI (1.4 x), TDF (1 x)</td>
<td>Results are from SDM. Mathiesen et al. (2007)(^21)</td>
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<td>K70E</td>
<td>In vitro selection with PMPA</td>
<td>TDF (8 x), 3TC (7 x), DDC (1 x), D4T (1 x), DDI (1 x), PFA (0.6 x), AZT (0.5 x)</td>
<td>Results are from SDM. Hachiya et al. (2011)(^6)</td>
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<td></td>
<td>3TC (3.5 x), ABC (2.8 x), TDF (2.5 x), AZT (1.7 x)</td>
<td>Results are from SDM. Cherrington et al. (1996)(^22)</td>
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<td>TDF (29 x), FTC (41 x), DDI (1.6 x), ABC (1.3 x), D4T (0.4 x), AZT (&lt;0.3 x)</td>
<td>Results are from biochemical characterization of SDM. Sluis-Cremer et al. (2007)(^5)</td>
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<td>3TC (11 x), D4T (6.2 x), ABC (3.7 x), DDI (1.5 x), AZT (0.9 x)</td>
<td>Results are from clinical isolates containing K70E + M184V as only NRTI mutations. Bradshaw et al. (2007)(^23)</td>
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<td>K70G</td>
<td>In vivo emergence during TDF+FTC cART</td>
<td>TDF (1.7 x), DDI (1.6 x), AZT (1.5 x)</td>
<td>Results are from SDM. Within the context of Q151M-complex, K70G only introduced statistically significant changes to D4T (4.6 x higher). Hachiya et al. (2011)(^6)</td>
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<td></td>
<td>3TC (4 x), PFA (2 x), AZT (0.2 x)</td>
<td>Results are from SDM. Mathiesen et al. (2007)(^21)</td>
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<td>In vivo emergence during prolonged TDF+FTC cART</td>
<td>3TC (85 x), FTC (63 x), DDI (5.3 x), ABC (3.2 x), TDF (1.5 x), D4T (0.9 x), AZT (&lt;0.8 x)</td>
<td>Results are from clinical isolates containing K70G + M184V as only NRTI mutations. Bradshaw et al. (2007)(^23)</td>
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<td>3TC (19 x), D4T (6.7 x), ABC (3.7 x), DDI (1.5 x), AZT (0.9 x)</td>
<td>Results are from SDM. Within the context of Q151M-complex, K70G only introduced statistically significant changes to D4T (4.6 x higher). Hachiya et al. (2011)(^6)</td>
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<td>K70Q</td>
<td>In vivo emergence in the background of Q151M-complex during prolonged TDF+EFV cART</td>
<td>DDI (5 x), 3TC (3.3 x), ABC (1.9 x), AZT (1.5 x), TDF (1.5 x), D4T (1.2 x)</td>
<td>Results are from SDM. Within the context of Q151M-complex, K70Q only introduced statistically significant changes to TDF (5 x higher), D4T (4.4 x higher) and DDI (2.4 x higher). Hachiya et al. (2011)(^6)</td>
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<td>K70N</td>
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<td>ABC (1.8 x), 3TC (1.6 x), DDI (1.5 x), D4T (1.3 x), AZT (1.2 x), TDF (1 x)</td>
<td>Results are from SDM. Within the context of Q151M-complex, K70N only introduced statistically significant changes to DDI (2.4 higher). Hachiya et al. (2011)(^6)</td>
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<td>K70T</td>
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<td>DDI (3.7 x), 3TC (3.1 x), ABC (1.4 x), TDF (1 x), AZT (0.9 x), D4T (0.8 x)</td>
<td>Results are from SDM. Hachiya et al. (2011)(^6)</td>
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<td>K70Δ</td>
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<td>FTC (4.4 x), 3TC (2.8 x), ABC (1 x), DDI (1 x), D4T (1 x), TDF (0.8 x), AZT (0.2 x)</td>
<td>Results are from SDM. Within the context of Q151M, K70Δ increased resistance to FTC/3TC (shift from 1.4–1.9 x to 7.8–3.5 x). Hu et al. (2007)(^24)</td>
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Note: AZT, zidovudine; D4T, stavudine; DDI, didanosine; 3TC, lamivudine; ABC, abacavir; TDF, tenofovir; PFA, foscarin; PMPA, adeovir; EFV, efavirenz. SDM, site-directed mutant. Δ, deletion. AA, amino acid change. cART, combination antiretroviral therapy. NRTI, nucleoside reverse transcriptase inhibitor. cART, combination antiretroviral therapy. Fold-changes > 1 x are displayed in bold. Fold-changes < 1 x are displayed in italic.
determine the molecular mechanisms by which amino acid changes at position 70 affect NRTI susceptibility, this model helps to explain the (increased) susceptibility to PFA of K70S/T. Additionally, the side chains of serine and threonine are too short to facilitate binding of ATP which is required for the excision of AZT and this might explain the (increased) susceptibility towards AZT and the preferential selection of K70S/T within the background of Q151M instead of TAMs.

The K70S/T mutations also slightly interfered with NNRTI resistance. K70S and K70T alone induced an increase in susceptibility towards EFV but in the context of Q151M-complex they reduced the susceptibility towards EFV. K70 is one of the residues within the RT finger domain that folds over the incoming dNTP or analog. How these amino acid changes can impact the susceptibility to EFV is unclear as K70 is not part of the NNRTI binding pocket. However, the selection of NRTI resistance mutations by NNRTI28,29 and the interference of NRTI resistance mutations with in vitro NNRTI resistance have been reported before.30,31 The role of K70 in in vivo NNRTI resistance could be supported by the observation that K70E/G/N/Q/S/T (not K70R) were 1.5 to 5 times more prevalent in NNRTI-experienced patients than in NRTI-experienced patients who were still NNRTI-naïve.32

Collectively, our results and data from literature showed that particular amino acid changes at position 70 could make small contributions to RTI drug susceptibility and that their in vivo selection and in vitro impact could be influenced by drug exposure and viral genetic background. However, the in vivo clinical significance of these effects remains elusive.

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