

# Biochemical characterization of a multi-drug resistant HIV-1 subtype AG reverse transcriptase: antagonism of AZT discrimination and excision pathways and sensitivity to RNase H inhibitors

Anna Schneider<sup>1</sup>, Angela Corona<sup>2</sup>, Imke Spöring<sup>3</sup>, Mareike Jordan<sup>1</sup>, Bernd Buchholz<sup>4</sup>, Elias Maccioni<sup>2</sup>, Roberto Di Santo<sup>5</sup>, Jochen Bodem<sup>3</sup>, Enzo Tramontano<sup>2</sup> and Birgitta M. Wöhrl<sup>1,\*</sup>

<sup>1</sup>Universität Bayreuth, Lehrstuhl Biopolymere und Forschungszentrum für Bio-Makromoleküle, Universitätsstrasse 30, 95447 Bayreuth, Germany, <sup>2</sup>Department of Life and Environmental Sciences, University of Cagliari, Cittadella Universitaria di Monserrato, SS 554, 09042, Monserrato, Cagliari, Italy, <sup>3</sup>Julius-Maximilians-Universität Würzburg, Institut für Virologie und Immunbiologie, Versbacher Strasse 7, 97078 Würzburg, Germany, <sup>4</sup>Universität Heidelberg, Medizinische Fakultät Mannheim, Klinik für Kinder- und Jugendmedizin, Theodor-Kutzer-Ufer 1–3, 68167 Mannheim, Germany and <sup>5</sup>Dipartimento di Chimica e Tecnologie del Farmaco, Istituto Pasteur-Fondazione Cenci Bolognetti, "Sapienza" Università di Roma, Rome, I-00185, Italy

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## ABSTRACT

We analyzed a multi-drug resistant (MR) HIV-1 reverse transcriptase (RT), subcloned from a patient-derived subtype CRF02\_AG, harboring 45 amino acid exchanges, amongst them four thymidine analog mutations (TAMs) relevant for high-level AZT (azidothymidine) resistance by AZTMP excision (M41L, D67N, T215Y, K219E) as well as four substitutions of the AZTTP discrimination pathway (A62V, V75I, F116Y and Q151M). In addition, K65R, known to antagonize AZTMP excision in HIV-1 subtype B was present. Although MR-RT harbored the most significant amino acid exchanges T215Y and Q151M of each pathway, it exclusively used AZTTP discrimination, indicating that the two mechanisms are mutually exclusive and that the Q151M pathway is obviously preferred since it confers resistance to most nucleoside inhibitors. A derivative was created, additionally harboring the TAM K70R and the reversions M151Q as well as R65K since K65R antagonizes excision. MR-R65K-K70R-M151Q was competent of AZTMP excision, whereas other combinations thereof with only one or two exchanges still promoted discrimination. To tackle the multi-drug resistance problem, we tested if the MR-RTs could still be inhibited by RNase H inhibitors. All MR-RTs exhib-

ited similar sensitivity toward RNase H inhibitors belonging to different inhibitor classes, indicating the importance of developing RNase H inhibitors further as anti-HIV drugs.

## INTRODUCTION

Patients infected with human immunodeficiency virus (HIV) are usually treated with a combination therapy of three or more antiretroviral drugs that belong to different inhibitor classes. However, the outcome of such a highly active antiretroviral therapy (HAART) depends on the sensitivity of the virus to the drugs as well as on the drug adherence of the patient. Lack of compliance often results in the occurrence of drug resistant virus and the need for other antiviral treatment regimens. Among the resistance associated mutations, thymidine analog mutations (TAMs) are of great importance due to the administration of zidovudine (azidothymidine, AZT) and/or stavudine (d4T) as the nucleoside reverse transcriptase inhibitor (NRTI) substances of HAART. Most importantly, TAMs also generate cross-resistance to other NRTIs (1–3).

Two different mechanisms confer HIV resistance against AZT. The mutant AZT-resistant reverse transcriptase (RT) can either selectively excise the already incorporated AZT monophosphate (AZTMP) in the presence of ATP, thus creating an AZT-P<sub>4</sub>-A dinucleotide (1–4) or it can discriminate between the NRTI triphosphate and the corresponding dNTP. While HIV type 1 (HIV-1) preferentially uses the ex-

\*To whom correspondence should be addressed. Tel: +49 921 55 3542; Email: birgitta.woehrl@uni-bayreuth.de

cision pathway, the predominant resistance mechanism of HIV-2 is discrimination (5,6).

Excision of the incorporated inhibitor is due to five primary resistance substitutions (M41L, D67N, K70R, T215F/Y and K219Q/E) also called TAMs because they emerge upon treatment with the thymidine analogs AZT and stavudine (d4T). The major TAM T215Y results in  $\pi$ - $\pi$  stacking of the aromatic rings of ATP and Tyr and it is thus essential for AZTMP excision (4). In HIV-1 subtype B a sixth TAM, L210W, often occurs together with M41L and T215Y and contributes substantially to high-level AZT resistance (7,8). While AZT and d4T are good substrates for the excision reaction, cytidine analogues, e.g. zalcitabine (ddC) or lamivudine (3TC), are removed rather inefficiently (2,9). In HIV-2, AZT discrimination is characterized by the mutations A62V, V75I, F77I, F116Y and Q151M. Among these, Q151M is the most important mutation. Thus the mutation pattern is also called Q151M multi-drug resistance (MDR) complex (6,10).

Q151M alone or the Q151M MDR complex also emerge in HIV-1 upon treatment with inhibitors that are poor substrates for the excision reaction, since Q151M confers multi-NRTI resistance to most NRTIs and nucleotide RT inhibitors (NtRTIs), except tenofovir disoproxil fumarate (TDF) (11,12). Q151M is usually the first mutation to appear followed by at least two additional amino acid exchanges of the Q151M MDR complex (13). Q151M has been detected in HIV-1 upon combination chemotherapy with AZT plus didanosine (ddI) or ddC. About 5% of patients treated with NRTIs acquire this mutation. Similar to HIV-2, Q151M in HIV-1 appears to impede the incorporation of AZTTP rather than enhancing the excision of incorporated AZTMP (6,10,11,14–17).

Furthermore, treatment with d4T appears to be directly associated with Q151M and in addition K65R (15). Both amino acid exchanges result in slower incorporation rates for NRTIs relative to the corresponding natural dNTPs (18–21). While Q151M and K65R are positively associated to each other, the occurrence of K65R antagonizes nucleotide excision caused by TAMs since it interferes with ATP binding, necessary for NRTI excision (21–23). The reduced rate of excision is most pronounced for AZT. However, transient kinetic analyses showed that the combination of TAMs and K65R also decreases the ability of the RT to discriminate against NRTIs. Thus, in the context of TAMs, K65R leads to a counteraction of excision and discrimination, resulting in AZT susceptibility (19,23). Structural analyses of a K65R RT indicate that the guanidinium planes of K65R and the conserved residue R72 are stacked, thereby forming a molecular platform which restricts rotation of both residues. Consequently, the adaptability of the polymerase active site is restricted, which impairs both substrate incorporation and NRTI excision (21,24,25).

Here, we report the biochemical characterization of the recombinant RT enzyme of a patient-derived, multi-drug resistant (MR) HIV-1 subtype AG circulating recombinant form CRF02\_AG (26). Subtype AG is responsible for about 5% of HIV-1 cases in Europe (27). Before isolation of the MR-RT the patient was treated over a time span of 12 years, beginning in 1997, with various combinations of NRTIs and non-nucleoside RT inhibitors (NNRTIs), i.e. AZT, d4T,

abacavir (ABC), ddI, lamivudine (3TC) and the NNRTI efavirenz (EFV) (26). Amongst the 45 amino acid exchanges in the MR-RT compared to the CRF02\_AG WT RT, TAMs for the excision pathway as well as discrimination mutations from the Q151M MDR complex, and in addition K65R are present. Moreover, MR-RT harbors the G190E mutation conferring resistance against NNRTIs, whereas several of the other exchanges have been identified as compensatory mutations in subtype B RT which help to improve the replication capacity of the virus (18,28).

Although in HIV-1 subtype B treatment with AZT results in the occurrence of TAMs, resistance to most other NRTIs, e.g. ABC, ddI and TDF is achieved via the discrimination pathway, which also confers resistance to AZT and thus might lead to the disappearance of TAMs. We therefore compared the MR-RT with the corresponding subtype CRF02\_AG WT RT and investigated whether the patient-derived MR-RT exhibits AZT resistance and whether both AZT resistance mechanisms are still functional, indicating an additive effect. We also characterized the polymerization behavior and fidelity of the wild-type (WT) and MR-RT. Although all drugs administered against RT targeted the polymerase domain, there are seven amino acid substitutions localized in the ribonuclease H (RNase H) domain. Therefore, we determined the RNase H activities and analyzed the inhibitory effect of several not yet approved RNase H inhibitors (RHIs) effective against subtype B RT. Our results imply that RHIs might be useful in the treatment of multi-drug resistant HIV strains.

## MATERIALS AND METHODS

### Cloning, expression and protein purification

RT encoding sequences from a patient derived plasma sample were amplified by RT-PCR using mouse leukemia virus RT and Phusion DNA Polymerase (NEB, Germany). RT-PCR products were gel purified and cloned into TopoXL vectors (Invitrogen-Thermo Fisher, USA). Nucleotide sequences of 10 insert containing single clones were determined, and all encoded the same resistance associated mutations as the genotypic pool sequences. Cloning and sequencing was repeated from a second patient derived plasma sample to ensure the sequence quality and to exclude recombination during the RT reaction. The coding regions of the CRF02\_AG WT RT subunits p66 and p51 were amplified from pBD6–15 (Genbank Acc. No. AY271690) (29). The amplified coding regions of WT and MR-RT (GenBank Acc. No. KT581450) were cloned into the pACYCDuet<sup>TM</sup>-1 vector (Novagen, Darmstadt, Germany), using restriction enzyme cleavage sites NcoI and BamHI for p51 or BglII and XhoI for p66, respectively. Co-expression was performed in *Escherichia coli* strain BL21 Star (DE3) (Invitrogen-ThermoFisher, USA) in Luria broth at 37°C in the presence of chloramphenicol (34  $\mu$ g/ml). After induction with 500  $\mu$ M isopropyl-thiogalactoside (IPTG) the temperature was reduced to 25°C and protein overexpression was performed overnight. The heterodimers contained a 6 $\times$  His tag at the N-terminus of the p51 subunit. The p66/6 $\times$ His-p51 heterodimers of WT and MR-RT were purified via Ni-affinity chromatography (HisTrap, GE Healthcare, Munich, Germany) followed by heparin

affinity chromatography (HiTrap heparin, GE Healthcare, Munich, Germany), which allowed the removal of 6×His-p51 homodimers. Judged by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and Coomassie staining the purified proteins contained approximately equal amounts of p66 and p51.

### Mutagenesis

Site-directed mutagenesis was performed according to the Quick Change mutagenesis protocol (Agilent Technologies, Santa Clara, CA, USA). After introduction of the required mutation into the p66 gene using plasmid pACYCDuet-1-p66, the mutated gene served as a template for p51 gene amplification. The mutated p51 gene was then cloned into the corresponding pACYCDuet-1-p66, resulting in a plasmid encoding the mutated p66 and p51 sequences.

### 5' end-labeling of primers

5' <sup>32</sup>P-end-labeling of the P<sub>30</sub> primer (P<sub>30</sub>) (5'-GCTGTAATGGCGTCCCTGTTCGGGCGCCTC), as well as the corresponding AZTMP terminated P<sub>30</sub> primer (P<sub>30</sub>-AZTMP) or the DNA primer P<sub>17</sub> (5'-GTAAACGACGGCCATG) (biomers.net; Ulm, Germany) was done as described previously (30).

### Polymerase fidelity measurements

Fidelity assays were performed using the 5' [<sup>32</sup>P]-end-labeled P<sub>30</sub> DNA primer which was hybridized to one of the four 50mer DNA templates T<sub>50</sub>dX with different sequences in the single-stranded regions (T<sub>50</sub>dA 5'-GCTGTGGCCGTCTCTTGTAGAGGCGCCGAACAGGGACGCCATTACAGC), (T<sub>50</sub>dC 5'-GGTGTGGAA AATGTGATGGCGAGGCGCCGAACAGGGACGCCATTACAGC), (T<sub>50</sub>dG 5'-ACTTTACAAAATCTCATCCGGAGGCGCCCGAACAGGGACGCCATTACAGC), (T<sub>50</sub>dT 5'-GCGACGGAAAAGCCCAGGCTGAGGGCGCCCGAACAGGGACGCCATTACAGC) (biomers.net; Ulm, Germany). A total of 20 nM of each <sup>32</sup>P-P<sub>30</sub>/T<sub>50</sub>dX substrate were pre-incubated in reaction buffer [50 mM Tris/HCl, pH 8.0, 80 mM KCl, 6 mM MgCl<sub>2</sub>, 0.5 mM dithiothreitol (DTT)] for 2 min at 37°C with 0.08 U of pyrophosphatase (Sigma Aldrich Chemie GmbH, Taufkirchen, Germany) and 1.25 mM of the first mismatched or matched dNTP as a control. Reactions were started by the addition of 1.25 μM RT enzyme and stopped after 10 min at 37°C with an equal volume of urea loading buffer [0.1% bromophenol blue, 0.1% xylene cyanol, 8 M urea, 89 mM Tris/HCl pH 8.3, 89 mM boracic acid, 25 mM ethylenediaminetetraacetate (EDTA)]. Reaction products were separated on a denaturing 10% polyacrylamide/8 M urea gel, visualized by phosphoimaging (Dürr Medical CR 35 Bio; Bietigheim-Bissingen, Germany) and quantified densitometrically with the software AIDA Image Analyzer V450 (raytest, Staubenhardt, Germany). Since all products longer than P<sub>31</sub> comprise the first correct dTTP incorporation they were also included in quantification of the primer extensions as described previously (31).

### DNA-dependent DNA polymerase activity assay

A total of 100 pmol of 5' [<sup>32</sup>P]-end-labeled 17-mer DNA primer (30) were annealed to single stranded M13mp18 DNA (New England BioLabs, Frankfurt, Germany). Primer elongation was performed in a total volume of 10 μl of reaction buffer (see above) in the presence of 30 nM [<sup>32</sup>P]-P<sub>17</sub>/M13mp18 and 200 μM dNTPs. After the addition of 83 nM RT the reaction was carried out for 1, 3 and 6 min at 37°C and stopped by adding 10 μl urea loading buffer. Polymerization products were separated and visualized as described above.

### Quantitative polymerization assay

RT polymerase activities were measured with 80 μM dTTP, 12 nM of RT enzyme and 100 ng/ml poly(rA)/oligo(dT)<sub>16</sub> in a total volume of 25 μl according to the instructions of the EnzChek RT assay kit (Invitrogen Life Technologies, Darmstadt, Germany). Reactions were started by the addition of the RT variant, incubated for 10 min at 37°C and stopped with 15 mM EDTA. Elongation products were detected by the addition of PicoGreen and measured with a Synergy 2 microplate reader at 502/523 nm using the reader software Gen5 2.0 (BioTek, Winooski, Vermont, USA). For quantification a standard curve was generated with double stranded lambda DNA (100–1 ng/ml dsDNA) (EnzChek, Invitrogen Life Technologies, Darmstadt, Germany). The specific activities of the polymerization reactions are given in Units (U), where 1 U corresponds to the incorporation of 1 nmol TMP by 1 mg of enzyme within 10 min at 37°C.

### Fluorescence equilibrium titrations

To determine the dissociation constant ( $K_D$ ) a 6-carboxyfluorescein (6-FAM) labeled 22/34-mer DNA/DNA substrate (primer 5'-6FAM-GGCGTCCCTGTTCGGGCGCCT, template 5'-CGGTCTTTGTAAAGGCGCCCGAACAGGGACGCC) (Metabion GmbH, Planegg-Martinsried, Germany) was used. Fluorescence equilibrium titrations were performed on a Fluorolog-Tau-3 spectrofluorometer (HORIBA JobinYvon GmbH, Unterhaching, Germany) at 25°C with 8 nM substrate and increasing amounts of enzyme (excitation 484 nm, emission 517 nm, slit width 3 nm).  $K_D$ -values were calculated via non-linear curve fitting of the normalized fluorescence data to a two component binding equation:

$$F = F_S + \left\{ (F_{ES} - F_S) \cdot \frac{(K_D + [E] + [S] - \sqrt{(K_D + [E] + [S])^2 - (4 \cdot [S] \cdot [E])})}{2 \cdot [S]} \right\}$$

$F_S$  and  $F_{ES}$ , fluorescence of the free and enzyme bound substrate;  $F$ , observed fluorescence value,  $[E]$ , enzyme concentration;  $[S]$ , substrate concentration,  $K_D$ , dissociation constant.

### AZTMP excision

The P<sub>30</sub> DNA primer was [<sup>32</sup>P]-end-labeled, terminated with AZTTP and purified as described (31). [<sup>32</sup>P]-P<sub>30</sub>-AZTMP was hybridized to a T<sub>50</sub> template DNA (5'-GCTGTGGAAAATCTCATGCAGAGGCGCC

CGAACAGGGACGCCATTACAGC) (30). A total of 20 nM P<sub>30</sub>-AZTMP/T<sub>50</sub>, 0.01 U/μl of pyrophosphatase (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) and 5 mM ATP (Jena Bioscience, Jena, Germany) were pre-incubated for 5 min at 37°C in reaction buffer in a total volume of 20 μl. AZTMP excision reactions were started by the addition of 600 nM enzyme, carried out for 30 min at 37°C and stopped by adding 20 μl of urea loading buffer (31,32). Excision products and educts were separated via denaturing gel electrophoresis (8% polyacrylamide/8 M urea gels) and analyzed as described above.

#### AZTTP discrimination

The 5' [<sup>32</sup>P]-end-labeled P<sub>30</sub> primer was hybridized to the T<sub>50</sub> template. In a total volume of 25 μl, 100 nM of [<sup>32</sup>P]-P<sub>30</sub>/T<sub>50</sub> substrate, 1 μM AZTTP or TTP, respectively, and 0.01 U/μl pyrophosphatase (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) were pre-incubated for 5 min at 37°C. Reactions were started by the addition of 200 nM enzyme, incubated for another 30 s and stopped by adding 20 μl urea loading buffer. Products and educts were separated on a denaturing 10% polyacrylamide/8 M urea gel and visualized and quantified as described above.

#### Determination of $K_M$ and $v_{max}$

Reactions were performed according to the discrimination assay in presence of increasing AZTTP concentrations from 1.5 nM to 50 μM. Reaction products were separated by denaturing gel electrophoresis, and data were fitted using the fitting program GraFit 5.0.12 (Erithacus Software limited, West Sussex, UK) and the Michaelis–Menten equation and as previously described (31).

#### RNase H activity determination and inhibitor testing

HIV RT-associated RNase H activity assays were performed as described (33,34). Briefly, RT associated RNase H activity was measured in 100 μl reaction volume containing 50 mM Tris/HCl, pH 7.8; 6 mM MgCl<sub>2</sub>; 1 mM DTT; 80 mM KCl; 0.25 μM RNA/DNA hybrid (5'-GAUCUGAGCCUGGGAGCU-FLUORESCIN-3', 5'-DABCYL-AGCTCCCAGGCTCAGATC-3'). The reaction mixture was incubated for 1 h at 37°C, then stopped by the addition of 50 μl EDTA 0.5 M, pH 8.0. Products were quantified with a Victor 3 plate reader (Perkin Elmer) at 490/528 nm. Different amounts of enzyme were used according to a linear range of a dose-response curve: 3.7 nM WT (subtype AG), 3.7 nM MR, 11.1 nM MR-70R, 11.1 nM MR-65K-70R, 33.3 nM MR-RT-70R-151M, 11.1 nM MR-RT-65K-70R-151Q and 33.3 nM HIV-1 subtype B AZT<sub>res-B</sub> RT. RHI testing was performed as described previously (35). All experiments were done at least three times.

#### Qualitative DNA polymerase-independent RNase H activity

Qualitative RNase H activity was determined as reported previously (36). Cleavage of a non-specific substrate was performed with a 29

nucleotides (nt) Cy5-labeled RNA (5'- Cy5-AGUCUGAGCCUGGGAGCAGCCUGGGAGCU-3' hybridized to a 29 nt DNA 3'-TCAGACTCGGACCCCTCGTCGGACCCTCGA-5'). The reaction was initiated by adding 1 μl of 100 mM MgCl<sub>2</sub> to 9 μl of reaction mixture containing 50 mM Tris-HCl, pH8.0, 80 mM KCl, 2 mM DTT, 200 nM substrate and 3.7, 11.1 or 33.3 nM of enzyme. The sample was incubated at 37°C for 10 min and the reaction was quenched with 10 μl of loading buffer (8M Urea in TBE buffer). Hydrolysis products were separated by denaturing polyacrylamide gel electrophoresis and visualized by fluorescence imaging (ChemIDoc, BioRad, Hercules, CA, USA)

#### Kinetic efficiency of the DNA polymerase-independent RNase H

Kinetic analysis of the DNA-polymerase independent RNase H activity was performed according to Lineaweaver–Burk plots with the Sigmaplot10 software. Velocity ( $v$ ) was expressed as fmoles/min.

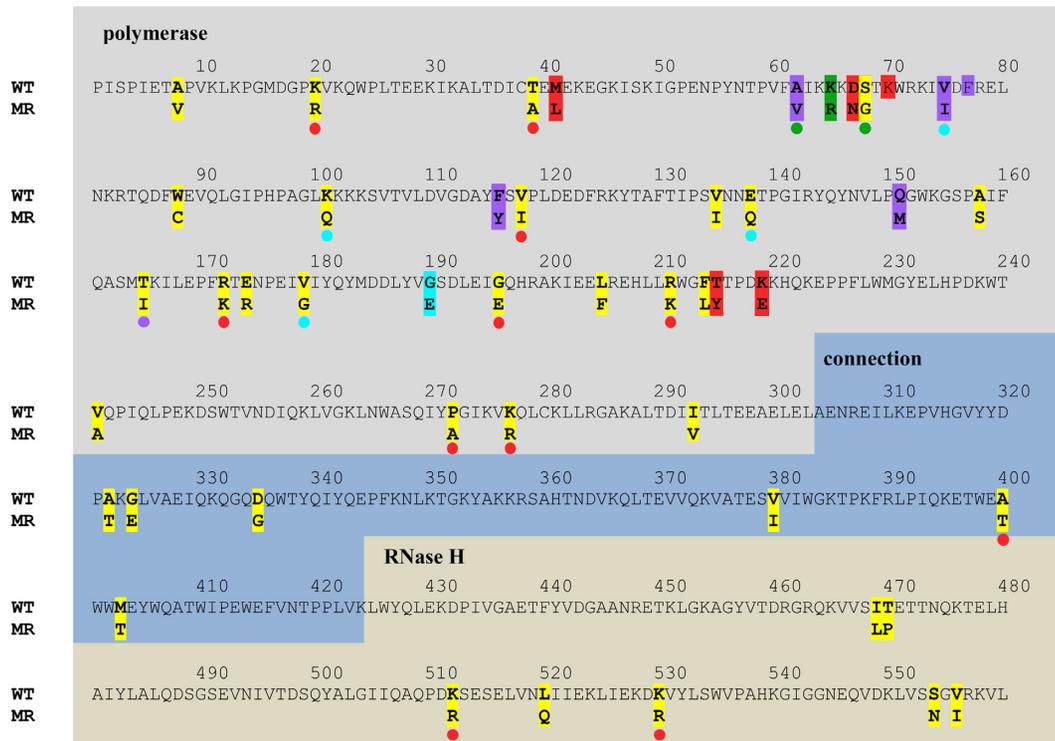
## RESULTS AND DISCUSSION

#### Temporal evolution of drug resistance mutations

The MR HIV-1 CRF02\_AG patient isolate from July 2008 harbored 45 amino acid exchanges in the RT as compared to the molecular CRF02\_AG WT clone pBD15–6 (Figure 1) (29). The first genotypic analyses were performed in March 2000 (Supplementary Table S1). Up to that point the patient had been subjected to therapies including the NRTIs 3TC, AZT, ddI, d4T (Supplementary Table S2). AZT resistant HIV-1 strains typically possess TAMs to remove incorporated NRTIs as AZTMP or d4T, whereas the Q151M discrimination pathway usually occurs upon administration of other NRTIs like 3TC and ddI (6,10,11,14). The accessory substitutions of the Q151M MDR complex help retain the replication capacity or improve the discrimination efficiency (11).

Sequencing of the patient isolate in March 2000 revealed the presence of four TAMs (M41L, D67N, K70R, T215Y) as well as two mutations of the Q151M discrimination pathway (F116Y and Q151M), whereas the K65R mutation was still missing (Supplementary Table S1). While the K70R mutation was never discovered in later genotypic analyses, K65R was first detected in June 2002. d4T was the only NRTI applied from November 2000 to June 2002. K65R confers resistance to d4T, TDF and possibly to 3TC, ddI and ABC, but not to AZT (37–40). The presence of K65R in combination with multiple TAMs is particularly interesting. In HIV-1 subtype B, strains harboring K65R together with multiple TAMs were only detected with a frequency of <0.1%. What is more, K65R has never been found in combination with T215F/Y, indicating that, in contrast to subtype AG, in subtype B K65R and the T215F/Y pathway are not compatible (22). Furthermore, the accessory discrimination mutations A62V and V75I emerged, but F77I has never been found (Supplementary Table S1).

In May 2003 the accessory TAM K219E occurred (Supplementary Table S1) although at that point in time the



**Figure 1.** Amino acid sequence alignment of subtype CRF02\_AG WT and MR-RT. The positions of the amino acid substitutions in the MR-RT are highlighted as colored boxes. Red boxes: TAMs; purple boxes: Q151M MDR pathway; cyan box: EFV resistance mutation; green box: K65R mutation; yellow boxes: all other mutations. Compensatory mutations mentioned in the text that are associated with the major NRTI and NNRTI resistance mutations described for subtype B are labeled with dots in the same color as the main mutations. The sequence alignment was performed using the program Lalign (74). The polymerase domain is shown in gray, the connection subdomain in blue, and the RNase H domain in light brown.

two NRTIs 3TC and TDF, which are not readily excised, were used (Supplementary Table S2). TAM L210W, which in subtype B occurs occasionally in combination with other TAMs, depending on the NRTIs used (8), could never be detected. However, L210W has not been identified frequently in the CRF02\_AG subtype. This might at least in part be attributed to the fact that in subtype B only one transversion is needed to obtain the codon for Trp (TTG > TGG), while in CRF02\_AG two point mutations are required (CTG > TGG) (41).

Concerning the AZT resistance pattern, the mutated genotype remained almost stable since May 2003 (Supplementary Table S1). Finally, in July 2008 four out of six TAMs relevant for highly efficient AZTMP excision (M41L, D67N, T215Y and K219E, missing K70R and L210W) as well as four out of five discrimination mutations (A62V, V75I, F116Y and Q151M, lacking F77I) were present (26,42).

### Characteristics of the multi-drug resistant RT

In addition to TAMs and discrimination mutations, MR-RT possesses several mutations that are also present in drug resistant subtype B RT, and are associated with resistance to NRTIs and NNRTIs (Figure 1). These mutations have compensatory effects and often lead to an improved replication capacity of the virus, e.g. by restoring the polymerization efficiency of the RT. However, for many of them the molecular function has not been investigated in detail (18).

Sequence comparisons of HIV-1 positive naïve and HAART treated patients revealed a correlation between K20R and 3TC resistance and an association with TAMs, while T39A appears to be associated with previous AZT and d4T treatment and with the development of TAMs (43). A62V and S68G restore the impaired nucleotide incorporation of the K65R mutation, thus improving the replication capacity of the virus (44). Similarly, the V75I exchange is compensatory for G190E, which emerges upon NNRTI treatment. G190E confers a more than 50-fold reduced sensitivity toward EFV but results in low polymerization activity (28,43). According to the HIV Drug Resistance Database (<http://hivdb.stanford.edu/>), K101Q, E138Q and exchanges at position V179 are also detected in patients treated with EFV. However, in subtype B the MR-RT exchange V179 to Gly (V179G) has not been found (<http://hivdb.stanford.edu/>). The P236L mutation characteristic for delavirdine (DLV) resistance is absent in MR-RT (<http://hivdb.stanford.edu/>). V118I occurs in combination with multiple TAMs (45). The polymorphism R172K impairs AZTMP excision (46), while T165I is associated with Q151M (47). G196E is also detected in NRTI resistant viruses (48). The polymorphisms R211K and L214F contribute to AZT resistance (49), however, in MR-RT the reversal F214L was detected. The thumb subdomain residues 255–286 of HIV-1 RT are important for proper DNA binding (50). Polymorphisms in the thumb region at positions 272, 277 and 286 have been shown to be as-

sociated with TAMs and therapy failure. Pro272, Arg277 and Thr286 are selected during NRTI combinations like d4T/ddI, d4T/3TC and abacavir (ABC), which have been used in this study (51). In MR-RT only K277R was found in combination with P272A, while at position 286 a Gly is present in WT and MR-RT (Figure 1).

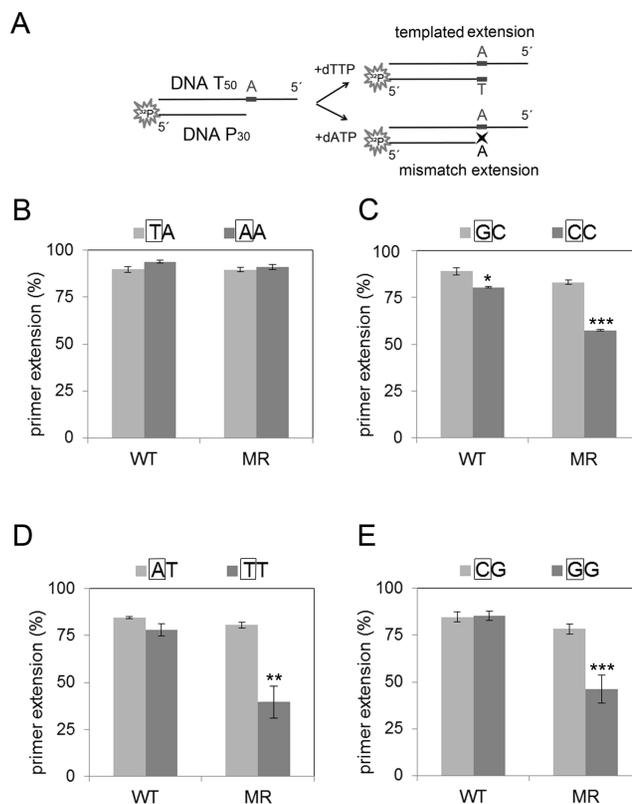
In subtype B several mutations in the connection subdomain (i.e. E312Q, G335C/D, N348I, A360I/V, V365I and A376S) have been identified that significantly contribute to AZT resistance by reducing RNase H activity (52). Interestingly, none of these mutations is present in MR-RT. However, the A400T mutation as well as two of the mutations existing in the RNase H domain (K512R, K530R) have been shown to occur in subtype B during NRTI treatment (Figure 1) (53,54) (see below).

### Fidelity

Several of the 45 amino acid exchanges, e.g. TAMs and the Q151M complex, can be directly attributed to resistance mutations. However, the enzyme also harbors compensatory exchanges, e.g. S68G which has been shown to improve the replicative capacity (Figure 1) (18). WT HIV-1 subtype B RT exhibits a rather low fidelity of replication, which on the one hand is advantageous during antiretroviral therapy using NRTIs, on the other hand this can also lead to rapid mutational changes resulting in resistance mutations against the drugs applied. Discrimination of NRTIs in resistant RTs and thus increased selectivity for the correct dNTP is often correlated with a higher replication fidelity of the RT (55).

Compared to the CRF02\_AG WT RT, MR-RT harbors several substitutions that have been implicated with an increase in fidelity, i.e. K65R, V75I and Q151M (21,56,57). Since subtype CRF02\_AG WT RT exhibited a ca. 2-fold higher polymerase activity than MR-RT (Table 1), we chose a fidelity assay in which effects of differences in catalytic activities could be excluded. Thus only the incorporation of the first matched or mismatched nucleotide was analyzed (Figure 2). Despite the limitations of this assay, the data indicate a higher fidelity of the MR-RT when non-templated dCTP, dGTP or dTTP was offered. CRF02\_AG WT RT incorporated those mismatched dNTPs almost as efficiently as the correct dNTP, whereas significant differences could be observed with the MR-RT, e.g. AT match versus TT mismatch results in 80.6% versus 39.5% incorporation (Supplementary Table S3). However, no differences could be detected for the TA match and AA mismatch, neither for the WT enzyme (89% match versus 93.9% mismatch) nor for the MR-RT (89.7% match versus 91.2% mismatch incorporation).

HIV-1 RT preferentially inserts dATP mismatches in favor of other nucleotides (58,59). This appears to be an intrinsic property of the RT during reverse transcription, although the biochemical background for this predisposition is unknown (60). Our data suggest an increased fidelity of the MR-RT which might contribute to the discrimination of NRTIs versus the corresponding correct dNTP.



**Figure 2.** Fidelity of subtype CRF02\_AG WT and MR-RT. Primer extension reactions were performed with 20 nM P/T DNA, 0.08 U of pyrophosphatase, 1.25  $\mu$ M of enzyme and 1.25 mM of the templated nucleotide (dTTP) or 1.25 mM of the mismatched nucleotide (dATP) at 37°C for 10 min. (A) Schematic representation of a primer extension reaction using T<sub>50</sub>dA as template. An end-labeled [<sup>32</sup>P]-P<sub>30</sub>/T<sub>50</sub>dA DNA/DNA substrate was used to perform a templated extension with dTTP or a mismatch extension in the presence of dATP. Extensions using the other templates were performed accordingly. Percentage of primer extension products after templated (light gray) and mismatched (dark gray) extensions of the P<sub>30</sub> primer hybridized to the template T<sub>50</sub>dA (AA mismatch) (B), T<sub>50</sub>dC (CC mismatch) (C), T<sub>50</sub>dT (TT mismatch) (D), T<sub>50</sub>dG (GG mismatch) (E) by WT and MR-RT. Reaction products were separated on a 10% sequencing gel and quantified. Each diagram depicts the mean values and standard deviations (black bars) of three independent incorporation experiments. For quantification of the extended products, the total amount of labeled DNA per lane was set to 100%. *P*-values  $\leq 0.05$  represent statistically significant differences to the WT protein (\**P*-value  $\leq 0.05$ ; \*\**P*-value  $\leq 0.01$ ; \*\*\**P*-value  $\leq 0.001$ ).

### Polymerization properties

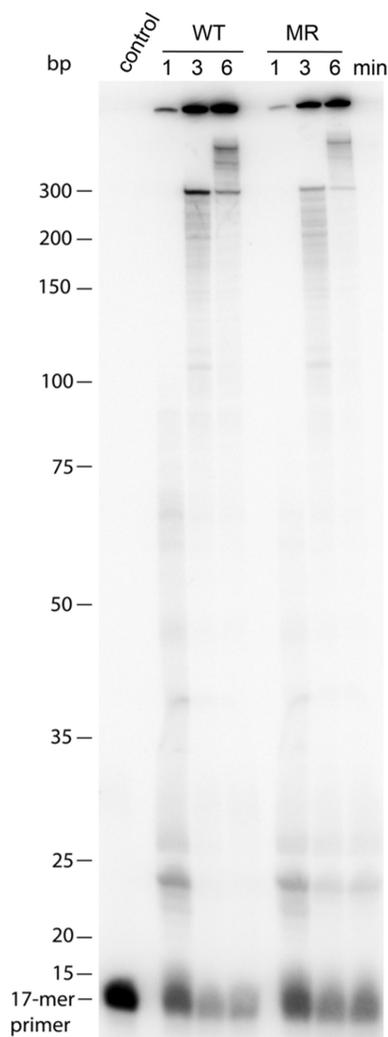
To better understand the substitutions' impact on the properties of MR-RT we further analyzed its polymerization behavior. Compared to the CRF02\_AG WT RT, extension of a DNA primer on the heteropolymeric single stranded M13 substrate indicates a lower polymerization activity of the MR-RT, since less primer is elongated (Figure 3). This is also reflected in the reduced specific activities (SPACs) determined on poly(rA)/oligo(dT) and the 3-fold higher dissociation constant (*K<sub>D</sub>*) obtained for MR-RT in equilibrium titration experiments by measuring the fluorescence of a fluorescent labeled DNA/DNA primer/template (P/T) (Table 1).

**Table 1.** Binding affinity for dsDNA and specific polymerization activities

	$K_D$ (nM)	SPAC <sup>1</sup> (U/ $\mu$ g <sub>enzyme</sub> )
WT <sup>2</sup>	9.0 $\pm$ 0.6	6.3 $\pm$ 0.3
MR	28.7 $\pm$ 2.0	3.2 $\pm$ 0.2
MR-70R	62.0 $\pm$ 3.8	3.1 $\pm$ 0.2
MR-65K-70R	50.7 $\pm$ 3.3	3.7 $\pm$ 0.5
MR-70R-151Q	73.1 $\pm$ 3.7	3.2 $\pm$ 0.4
MR-65K-70R-151Q	50.2 $\pm$ 3.0	3.1 $\pm$ 0.2
AZT <sub>res-B</sub>	5.4 $\pm$ 0.4	2.9 $\pm$ 0.1

<sup>1</sup>specific activity, 1 U catalyzes 1 nmol of incorporated TMP within 10 min at 37°C.

<sup>2</sup>WT = subtype CRF02\_AG



**Figure 3.** DNA-dependent DNA polymerase activity of subtype CRF02\_AG WT and MR-RTs. Reactions were carried out at 37°C for the times indicated on top of the gel with 30 nM [<sup>32</sup>P]-P<sub>17</sub>/M13mp18, 200  $\mu$ M of each dNTP and 83 nM WT or MR-RT, or without RT (control) in a reaction volume of 10  $\mu$ l. Extension products were analyzed by denaturing gel electrophoresis on a 10% sequencing gel and visualized by a phosphoimaging device. DNA size markers are shown on the left.

### AZT resistance

Therapy of the patient started by administering AZT and 3TC (Supplementary Table S1) (26). The acquisition of

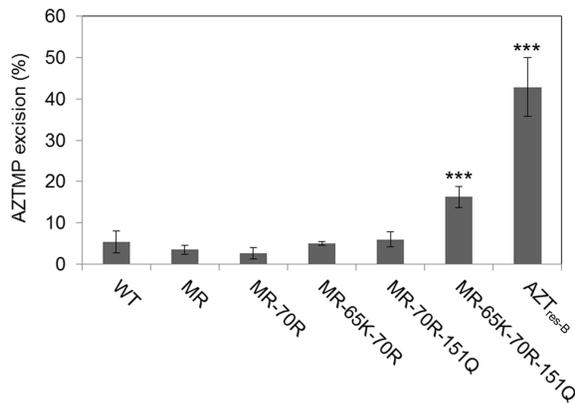
TAMs confers high-level resistance to AZT but not to 3TC. This might be the reason for the selection of the discrimination substitutions since according to the HIV Drug Resistance Database (<http://hivdb.stanford.edu/>) Q151M and the Q151M complex support low to moderate resistance to 3TC and efficient discrimination of various other NRTIs which have also been used during ongoing therapy (Supplementary Table S2) (26). In addition, the enzyme also carries the codon K65R which was suggested to antagonize AZT excision (22,23,61). However, the presence of the most relevant substitutions of the two AZT pathways, i.e. T215Y and Q151M, in addition to K65R raised the question as to which pathway could still be used by MR-RT.

AZT excision and discrimination experiments with WT and MR-RT showed that MR-RT is capable of AZTTP discrimination but not excision. To determine which amino acids are essential for AZTTP excision in the context of the MR-RT, we created several variants with amino acid substitutions that should further AZTMP removal. First, the K70R mutation, which is the missing fifth TAM residue promoting excision, was introduced (MR-70R). A second variant was constructed which in addition harbors the reversal R65K (MR-65K-70R), to abrogate negative effects of K65R on AZT excision. In a third variant, the pivotal discrimination substitution Q151M was reversed to WT and combined with K70R (MR-RT-70R-151M) to further support the excision reaction. Finally, MR-RT-65K-70R-151Q harbors all three codons that together should promote AZTMP excision and abrogate discrimination. An AZT-resistant HIV-1 subtype B RT (AZT<sub>res-B</sub>) encoding only the five TAMs, but no other resistance mutations was included as a positive control for AZTTP excision (62).

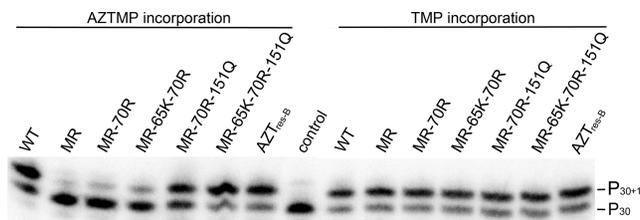
Analysis of the SPACs with a poly(rA)/oligo(dT) substrate on a DNA P/T showed that the polymerization activities of the MR-RT derivatives reached values of ca. 3.1–3.7 U. Similarly, determination of the binding affinities revealed comparable  $K_D$ -values between ca. 28 and 73 nM, indicating that the substitutions had not significant effect on these properties of the enzymes (Table 1). Thus the enzymes could be used for excision and discrimination assays.

AZTMP removal assays with an AZTMP terminated primer/template (P/T) substrate in the presence of 5 mM ATP demonstrated that only the triple variant MR-65K-70R-151Q exhibited significant AZTMP excision activity, although it was still lower than that of the subtype B control RT AZT<sub>res-B</sub> (Figure 4).

To determine whether the RTs were capable of AZTTP discrimination, 1  $\mu$ M of either AZTTP or TTP was of-



**Figure 4.** AZTMP excision. AZTMP excision reactions with the CRF02\_AG WT and MR RTs were performed with 20 nM of a 5' [<sup>32</sup>P] end-labeled and AZTMP terminated [<sup>32</sup>P]-P<sub>30</sub>-AZTMP/T<sub>50</sub> substrate in the presence of 5 mM ATP, 0.01 U/μl of pyrophosphatase and 600 nM RT as indicated. Reactions were started by RT addition and stopped after 5 min at 37°C. After separation of the reaction products and educt by denaturing gel electrophoresis, the bands were quantified densitometrically. The total amount of AZTMP-terminated primer per lane was set to 100% to calculate the percentage of excised AZTMP. Each diagram depicts the mean values and standard deviations (black bars) of three independent experiments. *P*-values ≤ 0.05 represent statistically significant differences to the WT protein (\*\*\**P*-value ≤ 0.001).



**Figure 5.** Discrimination between AZTTP and TTP during nucleotide incorporation. For comparison of AZTMP versus TMP incorporation by CRF02\_AG WT and MR RTs, and AZT<sub>res-B</sub> RT, 100 nM of labeled [<sup>32</sup>P]-P<sub>30</sub>/T<sub>50</sub> substrate and 1 μM of AZTTP or TTP were pre-incubated with 0.01 U/μl of pyrophosphatase for 5 min at 37°C. Reactions were started by the addition of 200 nM of RT as indicated and performed for 30 s at 37°C. Control, reaction without enzyme. Educts and products were separated by denaturing gel electrophoresis on a 10% sequencing gel.

ferred for incorporation (Figure 5). While no significant differences were detectable for the incorporation of TTP, MR-RT as well as MR-70R and MR-65K-70R exhibited very low incorporation activities with AZTTP, indicating discrimination capability. Only when the reversal M151Q of the major discrimination codon was present (MR-70R-151Q, MR65K-70R-151Q, AZT<sub>res-B</sub>), discrimination between TTP and AZTTP was strongly impaired. The *K<sub>M</sub>* for AZTTP of MR-RT compared with the two variants comprising the reverse codon M151Q, i.e. MR-70R-151Q and MR-65K-70R-151Q, was 12-fold or 33-fold lower, respectively (Table 2). This clearly confirms the ability of the MR-RT for AZTTP discrimination in spite of the presence of all five TAMs. This is also illustrated by a nearly 300-fold higher *K<sub>M</sub>*-value for AZTTP of the MR-RT as compared to the WT (Table 2).

Obviously, the Q151M pathway developed due to the administration of drugs that could not be efficiently eliminated

by the excision pathway. Although analyses with subtype B RT indicated that K65R and the TAM T215F/Y are not compatible, i.e. they were not found in combination (22), we could show that the MR-RT which harbors both residues concomitantly exhibits polymerase and RNase H function (see below) and resulted in a replicating virus that could be isolated from the patient.

However, it was not sufficient for AZTMP excision to occur even if all five TAMs were present (MR-70R), or if in addition K65R (MR-65K-70R) or Q151M (MR-70R-151Q) were reversed (Figure 4). Interestingly, probably due to the presence of the resistance mutation K65R, MR-70R-151Q was neither able of efficient AZTTP discrimination nor AZTMP excision. Only the combination of both reversals of K65R and Q151M in the presence of all TAMs (MR-65K-70R-151Q) resulted in AZTMP excision (Figure 4), albeit less efficient than with AZT<sub>res-B</sub> RT. This might be due to several other mutations not directly involved in AZT resistance, e.g. the polymorphism R172K or the exchange G190E, which are both present in MR-RT but not in the WT. R172K was shown to decrease DNA binding affinity and to suppress the excision mechanism by TAMs (46). Furthermore, R172K might contribute to the observed lower DNA binding affinities of the MR-RT variants. Although G190E is a NNRTI resistance mutation (63), it is often detected with V75I of the Q151M complex. G190E severely impairs polymerization activities due to repositioning of the primer, which in turn is antagonistic to excision (28,64). Therefore, the presence of these mutations could explain why MR-65K-70R-151Q is still less efficient in excising AZTMP than the AZT<sub>res-B</sub> RT.

Our data indicate that the two pathways are mutually exclusive and that although four TAMs including T215Y are present, only AZTTP discrimination by the Q151M complex is functional, thus enabling the enzyme to exhibit high level resistance against various NRTIs that have been administered during therapy.

### RNase H activities

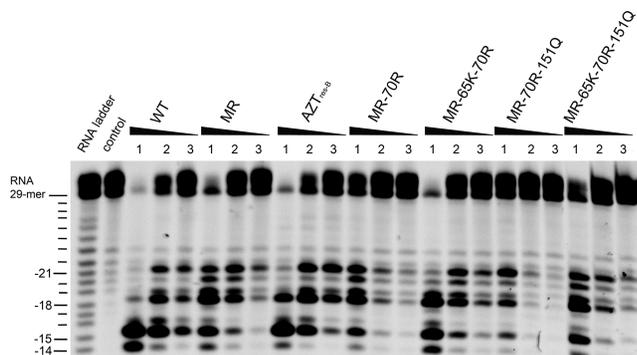
Although the patient received only RT inhibitors targeted against the polymerase domain, compared to the subtype CRF02\_AG WT 11 amino acid substitutions were detected in the connection subdomain (A322T, G324E, D335G, V380I, A400T, M403T) and in the RNase H domain (I469L, T470P, K512R, L520Q, K530R, S554N, V556I) (Figure 1). In HIV-1 subtype B two of the mutations, A400T and K512R, have been correlated with the occurrence of TAMs in a dose-dependent manner, being more frequent as the number of TAMs increased (53). The frequency of K530R is also increased in NRTI treated persons (12.1% versus 1.1% in untreated isolates) (54). A400T has been associated with increased AZTMP excision in subtypes B and CRF01\_AE (65). In the context of TAMs, subtype CRF01\_AE appeared to be less susceptible to AZT than subtype B, which was suggested to be due to reduced RNase H activities and improper positioning of the primer/template in the RNase H domain (65).

Improper positioning of the substrate might also lead to differences in the cleavage sites. To determine whether the enzymes exhibited altered RNase H activities we per-

**Table 2.**  $K_M$ ,  $v_{max}$  and  $K_M/v_{max}$  values for AZTMP-incorporation

	$K_M$ (nM)	$v_{max}$ (nM/min)	$v_{max}/K_M$ (1/min) · 10 <sup>-3</sup>
WT <sup>1</sup>	29.2 ± 9.8	11.8 ± 1	404.1 ± 139.9
MR	3585.5 ± 312.5	11.8 ± 0.4	3.3 ± 0.3
MR-70R-151Q	296.1 ± 19.6	12.8 ± 0.2	43.2 ± 2.9
MR-65K-70R-151Q	107.4 ± 15.4	13.2 ± 0.4	122.9 ± 18.0

<sup>1</sup>WT = subtype CRF02\_AG

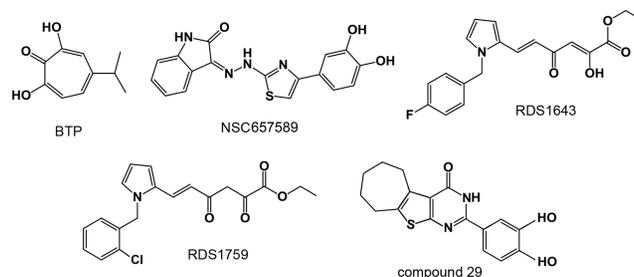


**Figure 6.** Qualitative RNase H assay. Autoradiogram of a typical RNase H cleavage experiment. RNase H reactions with 100 nM 5' labeled Cy5-RNA<sub>29</sub>/DNA<sub>29</sub> hybrid and RT variants at concentrations of (i) 33.3 nM, (ii) 11.1 nM and (iii) 3.7 nM were performed for 10 min at 37°C. RNA ladder, Cy5-RNA<sub>29</sub>/DNA<sub>29</sub> hybrid boiled, without RT, control, reaction without RT. RNA size markers are shown on the left.

formed RNase H activity assays with a fluorescent labeled RNA<sub>29</sub>/DNA<sub>29</sub> using WT and MR-RT variants as well as AZT<sub>res-B</sub> RT to uncover possible differences in RNase H activities between subtype B and CRF02\_AG (Figure 6). The RT variants appeared to be less active than the WT on the substrate used, while the cleavage pattern was not significantly affected. Therefore, to better characterize the mutant RT-associated RNase H function we measured their catalytic efficiencies by determining  $K_M$ - and  $k_{cat}$ -values (Table 3). We found that the MR-RTs' RNase H activities are significantly impaired as compared to the WT enzyme. In particular, although in some cases the RT variants showed  $K_M$ -values lower than the WT RT, all enzymes exhibited a significantly lower  $k_{cat}$ . Overall, MR-RT revealed a kinetic efficiency ( $k_{cat}/K_M$ ) comparable to WT RT, while the insertion of the K70R codon resulted in a significantly lower RNase H enzyme efficiency (Table 3). An RNA<sub>39</sub>/DNA<sub>29</sub> substrate harboring the HIV-1 polypurine tract (PPT) sequence was correctly cleaved by WT and MR-RT and its derivatives (data not shown), indicating the variants can still create the correct PPT RNA primer necessary for plus strand DNA synthesis during reverse transcription. However, the lower specific RNase H activity and strongly reduced catalytic efficiency of MR-70R could be one of the reasons why this combination, harboring all five TAMs was not found in the patient.

### RNase H inhibitor testing

The occurrence of MR viruses diminishes virus drug susceptibility and undermines HAART, allowing MR viruses to replicate with high viral loads. Similar to the CRF02\_AG



**Figure 7.** Chemical structures of HIV-1 subtype B RNase H inhibitors

isolate described here, such viruses often harbor resistance mutations against a variety of NRTIs, NNRTIs as well as protease inhibitors (26). HIV-1 RNase H is an attractive additional target for drug discovery and development, since it is essential for reverse transcription during retroviral replication. Several classes of HIV-1 RHIs have been developed, which inhibit the RNase H of HIV-1 subtype B (66–69). Thus we tested known inhibitors representing different inhibitor classes (Figure 7):

- (i) a natural product isolated from *Thuja plicata* 2,7-dihydroxy-4-isopropyl-cyclohepta-2,4,6-triene ( $\beta$ -Thujaplicinol, BTP), that binds the two divalent  $Mg^{2+}$  ions in the RNase H catalytic site (36);
- (ii) (Z)-3-(2-(4-(3,4-dihydroxyphenyl)thiazol-2-yl)hydrazono)indolin-2-one (NSC657589) which is able to inhibit HIV-1 RT-associated RNA dependent DNA polymerase (RDDP) as well as RNase H activities, possibly by binding to a region between the polymerase catalytic aspartate triad (Asp110, Asp185, Asp186) and the NNRTI pocket, hence contiguous to the NNRTI pocket but different from it (70);
- (iii) the active site diketo acid derivative 6-[1-(4-fluorophenyl)methyl-1H-pyrrol-2-yl]-2,4-dioxo-5-hexenoic acid ethyl ester (RDS1643) (71), the diketo ester derivative E-ethyl-6-(1-(2-chlorobenzyl)-1H-pyrrol-2-yl)-2,4-dioxohex-5-enoate (RDS1759) (72);
- (iv) 2-(3,4-dihydroxyphenyl)-6,7,8,9-tetrahydro-3H-cyclohepta[4,5]thieno[2,3-d]pyrimidin-4(5H)-one (compound 29). Biochemical experiments suggest that it binds at the HIV-1 RT p66/p51 interface (73).

Our data show that the RNase H activities of all RT enzymes can be inhibited by the different classes of inhibitors at low micromolar or even nanomolar concentrations in the case of BTP (Table 4), confirming that targeting RNase H is a valid approach especially in the context of already com-

**Table 3.** Kinetic analysis of RNase H activities

	Specific RNase H activity <sup>1</sup> (U)	$K_M$ (nM)	$k_{cat}$ (min <sup>-1</sup> )	Efficiency $k_{cat}/K_M$ (min <sup>-1</sup> · nM <sup>-1</sup> )
WT <sup>2</sup>	14	114 ± 21	66 ± 9	0.48 ± 0.06
MR	53	46 ± 7	18.7 ± 1.8	0.40 ± 0.02
MR-70R	130	87 ± 14	7.8 ± 1.4	0.09 ± 0.001
MR-70R-65K	79	90 ± 17	12.9 ± 1.8	0.14 ± 0.003
MR-70R-151Q	159	236 ± 62	6.4 ± 0.2	0.028 ± 0.008
MR-70R-65K-151Q	74	80 ± 7	12.6 ± 2.3	0.15 ± 0.015

<sup>1</sup>pmol of enzyme needed for 1 nmol/h of product.

<sup>2</sup>WT = subtype CRF02\_AG.

**Table 4.** Inhibition of HIV-1 subtype AG RTs by RNase H inhibitors

	RNase H IC <sub>50</sub> (μM) <sup>1</sup>				
	BTP	RDS1759	RDS1643	NSC657589	Compound 29
WT <sup>2</sup>	0.31 ± 0.02	19.5 ± 3.3	24.5 ± 4.3	1.79 ± 0.61	2.51 ± 0.14
MR	0.88 ± 0.07	18.6 ± 5.6	21.4 ± 6.9	1.74 ± 0.23	1.04 ± 0.45
MR-70R	0.34 ± 0.01	18.5 ± 4.4	16.6 ± 5.4	1.40 ± 0.23	1.35 ± 1.09
MR-70R-65K	0.81 ± 0.28	23.2 ± 3.3	17.3 ± 5.2	2.40 ± 0.17	1.70 ± 0.03
MR-70R-151Q	0.43 ± 0.17	28.1 ± 1.6	25.3 ± 6.7	2.64 ± 0.33	1.60 ± 0.90
MR-70R-65K-151Q	0.41 ± 0.25	21.5 ± 3.0	22.3 ± 1.3	3.42 ± 0.11	1.66 ± 0.32
AZT <sub>res-B</sub>	0.34 ± 0.01	69.5 ± 14.7	83 ± 20	13.8 ± 2.8	9.2 ± 2.5

<sup>1</sup>compound concentration able to inhibit 50% of RNase H enzymatic activity.

<sup>2</sup>WT = subtype CRF02\_AG.

promised sensitivity to the majority of currently approved drugs.

## CONCLUSIONS

Characterization of MR-RT and its derivatives demonstrates that the AZT discrimination pathway antagonizes the excision mechanism, even if the TAMs relevant for excision are present. Analyses with subtype B RT indicated that K65R and the TAM T215F/Y are not compatible in the absence of the Q151M complex and were rarely found if Q151M or Q151M and at least two additional mutations of the complex were present. Thus, a strong functional antagonism of K65R and T215F/Y in the presence of two or more additional TAMs may exist (22). We show here, that subtype AG MR-RT which harbors K65R, T215F and three additional TAMs concomitantly is still functional as a polymerase and RNase H. Moreover, in the presence of the Q151M complex K65R does not lead to AZT sensitivity. In the MR-RT, AZT resistance appears to be retained via the discrimination pathway. Only if both codons 65 and 151 of the discrimination pathway were restored to the WT residues (i.e. K65, M151), excision could occur, indicating that both exchanges are essential to prevent excision. Our data show, that the two AZT resistance pathways are mutually exclusive and not additive. Obviously, establishment of the M151Q complex is advantageous for the virus during varying treatment regimens with changing NRTIs, since it is efficient against multiple NRTIs. Our data might have important consequences for genotypic resistance interpretation algorithms, e.g. the HIV Drug Resistance Database (<http://hivdb.stanford.edu/>), which give high AZT resistance mutation scores for Q151M occurring in coexistence with the TAMs T215F and M41L. However,

we can show here, that these mutations do not create an additive impact on AZT resistance.

Moreover, MR-RT also exhibits a much higher fidelity than the WT RT, which further supports discrimination against NRTIs. Although several mutations are present in the RNase H domain, our results with various RHIs demonstrate their inhibitory potential, implying that further development, improvement and application of RHIs could be a potent tool to combat multi-drug resistant HIV strains.

## ACCESSION NUMBER

GenBank Acc. No. KT581450.

## SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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*Conflict of interest statement.* None declared.

## REFERENCES

- Meyer, P.R., Matsuura, S.E., So, A.G. and Scott, W.A. (1998) Unblocking of chain-terminated primer by HIV-1 reverse transcriptase through a nucleotide-dependent mechanism. *Proc. Natl. Acad. Sci. U.S.A.*, **95**, 13471–13476.
- Meyer, P.R., Matsuura, S.E., Mian, A.M., So, A.G. and Scott, W.A. (1999) A mechanism of AZT resistance: an increase in nucleotide-dependent primer unblocking by mutant HIV-1 reverse transcriptase. *Mol. Cell*, **4**, 35–43.
- Boyer, P.L., Sarafianos, S.G., Arnold, E. and Hughes, S.H. (2001) Selective excision of AZTMP by drug-resistant human immunodeficiency virus reverse transcriptase. *J. Virol.*, **75**, 4832–4842.
- Tu, X., Das, K., Han, Q., Bauman, J.D., Clark, A.D. Jr, Hou, X., Frenkel, Y.V., Gaffney, B.L., Jones, R.A., Boyer, P.L. *et al.* (2010) Structural basis of HIV-1 resistance to AZT by excision. *Nat. Struct. Mol. Biol.*, **17**, 1202–1209.
- van der Ende, M.E., Guillon, C., Boers, P.H., Ly, T.D., Gruters, R.A., Osterhaus, A.D. and Schutten, M. (2000) Antiviral resistance of biologic HIV-2 clones obtained from individuals on nucleoside reverse transcriptase inhibitor therapy. *J. Acquir. Immune Defic. Syndr.*, **25**, 11–18.
- Boyer, P.L., Sarafianos, S.G., Clark, P.K., Arnold, E. and Hughes, S.H. (2006) Why do HIV-1 and HIV-2 use different pathways to develop AZT resistance? *PLoS Pathog.*, **2**, e10.
- Harrigan, P.R., Kinghorn, I., Bloor, S., Kemp, S.D., Najera, I., Kohli, A. and Larder, B.A. (1996) Significance of amino acid variation at human immunodeficiency virus type 1 reverse transcriptase residue 210 for zidovudine susceptibility. *J. Virol.*, **70**, 5930–5934.
- Hooker, D.J., Tachedjian, G., Solomon, A.E., Gurusinghe, A.D., Land, S., Birch, C., Anderson, J.L., Roy, B.M., Arnold, E. and Deacon, N.J. (1996) An in vivo mutation from leucine to tryptophan at position 210 in human immunodeficiency virus type 1 reverse transcriptase contributes to high-level resistance to 3'-azido-3'-deoxythymidine. *J. Virol.*, **70**, 8010–8018.
- Naeger, L.K., Margot, N.A. and Miller, M.D. (2002) ATP-dependent removal of nucleoside reverse transcriptase inhibitors by human immunodeficiency virus type 1 reverse transcriptase. *Antimicrob. Agents Chemother.*, **46**, 2179–2184.
- Boyer, P.L., Clark, P.K. and Hughes, S.H. (2012) HIV-1 and HIV-2 reverse transcriptases: different mechanisms of resistance to nucleoside reverse transcriptase inhibitors. *J. Virol.*, **86**, 5885–5894.
- Shirasaka, T., Kavlick, M.F., Ueno, T., Gao, W., Kojima, E., Alcaide, M.L., Choekijichai, S., Roy, B.M., Arnold, E., Yarchoan, R. *et al.* (1995) Emergence of human immunodeficiency virus type 1 variants with resistance to multiple dideoxynucleosides in patients receiving therapy with dideoxynucleosides. *Proc. Natl. Acad. Soc. U.S.A.*, **92**, 2398–2402.
- Harada, S., Hazra, R., Tamiya, S., Zeichner, S.L. and Mitsuya, H. (2007) Emergence of human immunodeficiency virus type 1 variants containing the Q151M complex in children receiving long-term antiretroviral chemotherapy. *Antiviral Res.*, **75**, 159–166.
- Shafer, R.W. and Schapiro, J.M. (2008) HIV-1 drug resistance mutations: an updated framework for the second decade of HAART. *AIDS Rev.*, **10**, 67–84.
- Sarafianos, S.G., Das, K., Hughes, S.H. and Arnold, E. (2004) Taking aim at a moving target: designing drugs to inhibit drug-resistant HIV-1 reverse transcriptases. *Curr. Opin. Struct. Biol.*, **14**, 716–730.
- Nouhin, J., Madec, Y., Ngo-Giang-Huong, N., Ferradini, L. and Nerrienet, E. (2013) Increased risk of Q151M and K65R mutations in patients failing stavudine-containing first-line antiretroviral therapy in Cambodia. *PLoS One*, **8**, e73744.
- Iversen, A.K., Shafer, R.W., Wehrly, K., Winters, M.A., Mullins, J.I., Chesebro, B. and Merigan, T.C. (1996) Multidrug-resistant human immunodeficiency virus type 1 strains resulting from combination antiretroviral therapy. *J. Virol.*, **70**, 1086–1090.
- Shafer, R.W., Iversen, A.K., Winters, M.A., Aguiniga, E., Katzenstein, D.A. and Merigan, T.C. (1995) Drug resistance and heterogeneous long-term virologic responses of human immunodeficiency virus type 1-infected subjects to zidovudine and didanosine combination therapy. the AIDS clinical trials group 143 virology team. *J. Infect. Dis.*, **172**, 70–78.
- Menendez-Arias, L. (2013) Molecular basis of human immunodeficiency virus type 1 drug resistance: Overview and recent developments. *Antiviral Res.*, **98**, 93–120.
- White, K.L., Margot, N.A., Ly, J.K., Chen, J.M., Ray, A.S., Pavelko, M., Wang, R., McDermott, M., Swaminathan, S. and Miller, M.D. (2005) A combination of decreased NRTI incorporation and decreased excision determines the resistance profile of HIV-1 K65R RT. *AIDS*, **19**, 1751–1760.
- White, K.L., Chen, J.M., Feng, J.Y., Margot, N.A., Ly, J.K., Ray, A.S., MacArthur, H.L., McDermott, M.J., Swaminathan, S. and Miller, M.D. (2006) The K65R reverse transcriptase mutation in HIV-1 reverses the excision phenotype of zidovudine resistance mutations. *Antivir. Ther.*, **11**, 155–163.
- Das, K., Bandwar, R.P., White, K.L., Feng, J.Y., Sarafianos, S.G., Tuske, S., Tu, X., Clark, A.D. Jr, Boyer, P.L., Hou, X. *et al.* (2009) Structural basis for the role of the K65R mutation in HIV-1 reverse transcriptase polymerization, excision antagonism, and tenofovir resistance. *J. Biol. Chem.*, **284**, 35092–35100.
- Parikh, U.M., Barnas, D.C., Faruki, H. and Mellors, J.W. (2006) Antagonism between the HIV-1 reverse-transcriptase mutation K65R and thymidine-analogue mutations at the genomic level. *J. Infect. Dis.*, **194**, 651–660.
- Parikh, U.M., Zelina, S., Sluis-Cremer, N. and Mellors, J.W. (2007) Molecular mechanisms of bidirectional antagonism between K65R and thymidine analog mutations in HIV-1 reverse transcriptase. *AIDS*, **21**, 1405–1414.
- Deval, J., White, K.L., Miller, M.D., Parkin, N.T., Courcambek, J., Halfon, P., Selmi, B., Boretto, J. and Canard, B. (2004) Mechanistic basis for reduced viral and enzymatic fitness of HIV-1 reverse transcriptase containing both K65R and M184V mutations. *J. Biol. Chem.*, **279**, 509–516.
- Sluis-Cremer, N., Sheen, C.W., Zelina, S., Torres, P.S., Parikh, U.M. and Mellors, J.W. (2007) Molecular mechanism by which the K70E mutation in human immunodeficiency virus type 1 reverse transcriptase confers resistance to nucleoside reverse transcriptase inhibitors. *Antimicrob. Agents Chemother.*, **51**, 48–53.
- Kozisek, M., Henke, S., Sasková, K.G., Jacobs, G.B., Schuch, A., Buchholz, B., Müller, V., Kräusslich, H.G., Rezáková, P., Konvalinka, J. *et al.* (2012) Mutations in HIV-1 gag and pol compensate for the loss of viral fitness caused by a highly mutated protease. *Antimicrob. Agents Chemother.*, **56**, 4320–4330.
- Abecasis, A.B., Wensing, A.M., Paraskevis, D., Vercauteren, J., Theys, K., Van de Vijver, D.A., Albert, J., Asjo, B., Balotta, C., Beshkov, D. *et al.* (2013) HIV-1 subtype distribution and its demographic determinants in newly diagnosed patients in Europe suggest highly compartmentalized epidemics. *Retrovirology*, **10**, 7.
- Boyer, P.L., Gao, H.Q. and Hughes, S.H. (1998) A mutation at position 190 of human immunodeficiency virus type 1 reverse transcriptase interacts with mutations at positions 74 and 75 via the template primer. *Antimicrob. Agents Chemother.*, **42**, 447–452.
- Tebit, D.M., Zekeng, L., Kaptué, L., Kräusslich, H.G. and Herchenröder, O. (2003) Construction and characterisation of a full-length infectious molecular clone from a fast replicating, X4-tropic HIV-1 CRF02\_AG primary isolate. *Virology*, **313**, 645–652.
- Hartl, M.J., Kretzschmar, B., Frohn, A., Nowrouzi, A., Rethwilm, A. and Wöhr, B.M. (2008) AZT resistance of simian foamy virus reverse transcriptase is based on the excision of AZTMP in the presence of ATP. *Nucleic Acids Res.*, **36**, 1009–1016.
- Schneider, A., Schweimer, K., Rösch, P. and Wöhr, B.M. (2015) AZT resistance alters enzymatic properties and creates an ATP binding site in SFVmac reverse transcriptase. *Retrovirology*, **12**, 21.
- Hartl, M.J., Wöhr, B.M., Rösch, P. and Schweimer, K. (2008) The solution structure of the simian foamy virus protease reveals a monomeric protein. *J. Mol. Biol.*, **381**, 141–149.
- Corona, A., Schneider, A., Schweimer, K., Rösch, P., Wöhr, B.M. and Tramontano, E. (2014) Inhibition of foamy virus reverse transcriptase by human immunodeficiency virus type 1 RNase H inhibitors. *Antimicrob. Agents Chemother.*, **58**, 4086–4093.
- Esposito, F., Sanna, C., Del Vecchio, C., Cannas, V., Venditti, A., Corona, A., Bianco, A., Serrilli, A.M., Guarcini, L., Parolin, C. *et al.* (2013) *Hypericum hircinum* L. components as new single-molecule inhibitors of both HIV-1 reverse transcriptase-associated DNA polymerase and ribonuclease H activities. *Pathog. Dis.*, **68**, 116–124.

35. Esposito, F., Corona, A., Zinzula, L., Kharlamova, T. and Tramontano, E. (2012) New anthraquinone derivatives as inhibitors of the HIV-1 reverse transcriptase-associated ribonuclease H function. *Chemotherapy*, **58**, 299–307.
36. Chung, S., Himmel, D.M., Jiang, J.K., Wojtak, K., Bauman, J.D., Rausch, J.W., Wilson, J.A., Beutler, J.A., Thomas, C.J., Arnold, E. *et al.* (2011) Synthesis, activity, and structural analysis of novel alpha-hydroxytropolone inhibitors of human immunodeficiency virus reverse transcriptase-associated ribonuclease H. *J. Med. Chem.*, **54**, 4462–4473.
37. Stone, C., Ait-Khaled, M., Craig, C., Griffin, P. and Tisdale, M. (2004) Human immunodeficiency virus type 1 reverse transcriptase mutation selection during in vitro exposure to tenofovir alone or combined with abacavir or lamivudine. *Antimicrob. Agents Chemother.*, **48**, 1413–1415.
38. Margot, N.A., Lu, B., Cheng, A., Miller, M.D. and Study 903 Team (2006) Resistance development over 144 weeks in treatment-naive patients receiving tenofovir disoproxil fumarate or stavudine with lamivudine and efavirenz in study 903. *HIV Med.*, **7**, 442–450.
39. Garcia-Lerma, J.G., MacInnes, H., Bennett, D., Reid, P., Nidtha, S., Weinstock, H., Kaplan, J.E. and Heneine, W. (2003) A novel genetic pathway of human immunodeficiency virus type 1 resistance to stavudine mediated by the K65R mutation. *J. Virol.*, **77**, 5685–5693.
40. Sungkanuparph, S., Manosuthi, W., Kiertiburanakul, S., Saekang, N., Pairoj, W. and Chantratita, W. (2008) Prevalence and risk factors for developing K65R mutations among HIV-1 infected patients who fail an initial regimen of fixed-dose combination of stavudine, lamivudine, and nevirapine. *J. Clin. Virol.*, **41**, 310–313.
41. Santoro, M.M., Alteri, C., Ronga, L., Flandre, P., Fabeni, L., Mercurio, F., D'Arrigo, R., Gori, C., Palamara, G., Bertoli, A. *et al.* (2012) Comparative analysis of drug resistance among B and the most prevalent non-B HIV type 1 subtypes (C, F, and CRF02\_AG) in Italy. *AIDS Res. Hum. Retroviruses*, **28**, 1285–1293.
42. Marcelin, A.G., Delaugerre, C., Wirdein, M., Viegas, P., Simon, A., Katlama, C. and Calvez, V. (2004) Thymidine analogue reverse transcriptase inhibitors resistance mutations profiles and association to other nucleoside reverse transcriptase inhibitors resistance mutations observed in the context of virological failure. *J. Med. Virol.*, **72**, 162–165.
43. Saracino, A., Monno, L., Scudeller, L., Cibelli, D.C., Tartaglia, A., Punzi, G., Torti, C., Lo Caputo, S., Mazzotta, F., Scotto, G. *et al.* (2006) Impact of unreported HIV-1 reverse transcriptase mutations on phenotypic resistance to nucleoside and non-nucleoside inhibitors. *J. Med. Virol.*, **78**, 9–17.
44. Svarovskaia, E.S., Feng, J.Y., Margot, N.A., Myrick, F., Goodman, D., Ly, J.K., White, K.L., Kuty, N., Wang, R., Borroto-Esoda, K. *et al.* (2008) The A62V and S68G mutations in HIV-1 reverse transcriptase partially restore the replication defect associated with the K65R mutation. *J. Acquir. Immune Defic. Syndr.*, **48**, 428–436.
45. Zaccarelli, M., Tozzi, V., Lorenzini, P., Forbici, F., Narciso, P., Ceccherini-Silberstein, F., Trotta, M.P., Bertoli, A., Liuzzi, G., Marconi, P. *et al.* (2007) The V118I mutation as a marker of advanced HIV infection and disease progression. *Antivir. Ther.*, **12**, 163–168.
46. Hachiya, A., Marchand, B., Kirby, K.A., Michailidis, E., Tu, X., Palczewski, K., Ong, Y.T., Li, Z., Griffin, D.T., Schuckmann, M.M. *et al.* (2012) HIV-1 reverse transcriptase (RT) polymorphism 172K suppresses the effect of clinically relevant drug resistance mutations to both nucleoside and non-nucleoside RT inhibitors. *J. Biol. Chem.*, **287**, 29988–29999.
47. Scherrer, A.U., von Wyl, V., Gotte, M., Klimkait, T., Cellerai, C., Yerly, S., Boni, J., Held, L., Ledergerber, B., Gunthard, H.F. *et al.* (2012) Polymorphic mutations associated with the emergence of the multinucleoside/tide resistance mutations 69 insertion and Q151M. *J. Acquir. Immune Defic. Syndr.*, **59**, 105–112.
48. Svicher, V., Sing, T., Santoro, M.M., Forbici, F., Rodriguez-Barrios, F., Bertoli, A., Beerenwinkel, N., Bellocchi, M.C., Gago, F., d'Arminio Monforte, A. *et al.* (2006) Involvement of novel human immunodeficiency virus type 1 reverse transcriptase mutations in the regulation of resistance to nucleoside inhibitors. *J. Virol.*, **80**, 7186–7198.
49. Stürmer, M., Staszewski, S., Doerr, H.W., Larder, B., Bloor, S. and Hertogs, K. (2003) Correlation of phenotypic zidovudine resistance with mutational patterns in the reverse transcriptase of human immunodeficiency virus type 1: interpretation of established mutations and characterization of new polymorphisms at codons 208, 211, and 214. *Antimicrob. Agents Chemother.*, **47**, 54–61.
50. Huang, H.F., Chopra, R., Verdine, G.V. and Harrison, S.C. (1998) Structure of a covalently trapped catalytic complex of HIV-1 reverse transcriptase: Implications for drug design. *Science*, **282**, 1669–1675.
51. Betancor, G., Puertas, M.C., Nevot, M., Garriga, C., Martinez, M.A., Martinez-Picado, J. and Menendez-Arias, L. (2010) Mechanisms involved in the selection of HIV-1 reverse transcriptase thumb subdomain polymorphisms associated with nucleoside analogue therapy failure. *Antimicrob. Agents Chemother.*, **54**, 4799–4811.
52. Nikolenko, G.N., Delviks-Frankenberry, K.A., Palmer, S., Maldarelli, F., Fivash, M.J. Jr, Coffin, J.M. and Pathak, V.K. (2007) Mutations in the connection domain of HIV-1 reverse transcriptase increase 3'-azido-3'-deoxythymidine resistance. *Proc. Natl. Acad. Sci. U.S.A.*, **104**, 317–322.
53. Lengrubner, R.B., Delviks-Frankenberry, K.A., Nikolenko, G.N., Baumann, J., Santos, A.F., Pathak, V.K. and Soares, M.A. (2011) Phenotypic characterization of drug resistance-associated mutations in HIV-1 RT connection and RNase H domains and their correlation with thymidine analogue mutations. *J. Antimicrob. Chemother.*, **66**, 702–708.
54. Santos, A.F., Lengrubner, R.B., Soares, E.A., Jere, A., Sprinz, E., Martinez, A.M., Silveira, J., Sion, F.S., Pathak, V.K. and Soares, M.A. (2008) Conservation patterns of HIV-1 RT connection and RNase H domains: Identification of new mutations in NRTI-treated patients. *PLoS One*, **3**, e1781.
55. Lloyd, S.B., Kent, S.J. and Winnall, W.R. (2014) The high cost of fidelity. *AIDS Res. Hum. Retroviruses*, **30**, 8–16.
56. Matamoros, T., Kim, B. and Menendez-Arias, L. (2008) Mechanistic insights into the role of Val75 of HIV-1 reverse transcriptase in misinsertion and mispair extension fidelity of DNA synthesis. *J. Mol. Biol.*, **375**, 1234–1248.
57. Rezende, L.F., Curr, K., Ueno, T., Mitsuya, H. and Prasad, V.R. (1998) The impact of multidideoxynucleoside resistance-conferring mutations in human immunodeficiency virus type 1 reverse transcriptase on polymerase fidelity and error specificity. *J. Virol.*, **72**, 2890–2895.
58. Abram, M.E., Ferris, A.L., Shao, W., Alvord, W.G. and Hughes, S.H. (2010) Nature, position, and frequency of mutations made in a single cycle of HIV-1 replication. *J. Virol.*, **84**, 9864–9878.
59. Abram, M.E., Ferris, A.L., Das, K., Quinoñes, O., Shao, W., Tuske, S., Alvord, W.G., Arnold, E. and Hughes, S.H. (2014) Mutations in HIV-1 reverse transcriptase affect the errors made in a single cycle of viral replication. *J. Virol.*, **88**, 7589–7601.
60. Müller, V. and Bonhoeffer, S. (2005) Guanine-adenine bias: a general property of retroviruses that is unrelated to host-induced hypermutation. *Trends Genet.*, **21**, 264–268.
61. Parikh, U.M., Bachelier, L., Koontz, D. and Mellors, J.W. (2006) The K65R mutation in human immunodeficiency virus type 1 reverse transcriptase exhibits bidirectional phenotypic antagonism with thymidine analog mutations. *J. Virol.*, **80**, 4971–4977.
62. Krebs, R., Immendorfer, U., Thrall, S.H., Wöhrl, B.M. and Goody, R.S. (1997) Single-step kinetics of HIV-1 reverse transcriptase mutants responsible for virus resistance to nucleoside inhibitors zidovudine and 3-TC. *Biochemistry*, **36**, 10292–10300.
63. Huang, W., Gamarnik, A., Limoli, K., Petropoulos, C.J. and Whitcomb, J.M. (2003) Amino acid substitutions at position 190 of human immunodeficiency virus type 1 reverse transcriptase increase susceptibility to delavirdine and impair virus replication. *J. Virol.*, **77**, 1512–1523.
64. Kleim, J.P., Rosner, M., Winkler, I., Paessens, A., Kirsch, R., Hsiou, Y., Arnold, E. and Riess, G. (1996) Selective pressure of a quinoxaline nonnucleoside inhibitor of human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) on HIV-1 replication results in the emergence of nucleoside RT-inhibitor-specific (RT Leu-74->Val or Ile and Val-75->Leu or Ile) HIV-1 mutants. *Proc. Natl. Acad. Sci. U.S.A.*, **93**, 34–38.
65. Delviks-Frankenberry, K.A., Nikolenko, G.N., Maldarelli, F., Hase, S., Takebe, Y. and Pathak, V.K. (2009) Subtype-specific differences in the human immunodeficiency virus type 1 reverse transcriptase connection subdomain of CRF01\_AE are associated with higher levels of resistance to 3'-azido-3'-deoxythymidine. *J. Virol.*, **83**, 8502–8513.

66. Corona,A., Masaoka,T., Tocco,G., Tramontano,E. and Le Grice,S.F. (2013) Active site and allosteric inhibitors of the ribonuclease H activity of HIV reverse transcriptase. *Future Med. Chem.*, **5**, 2127–2139.
67. Tramontano,E. (2006) HIV-1 RNase H: Recent progress in an exciting, yet little explored, drug target. *Mini Rev. Med. Chem.*, **6**, 727–737.
68. Tramontano,E. and Di Santo,R. (2010) HIV-1 RT-associated RNase H function inhibitors: recent advances in drug development. *Curr. Med. Chem.*, **17**, 2837–2853.
69. Distinto,S., Maccioni,E., Meleddu,R., Corona,A., Alcaro,S. and Tramontano,E. (2013) Molecular aspects of the RT/drug interactions. perspective of dual inhibitors. *Curr. Pharm. Des.*, **19**, 1850–1859.
70. Distinto,S., Esposito,F., Kirchmair,J., Cardia,M.C., Gaspari,M., Maccioni,E., Alcaro,S., Markt,P., Wolber,G., Zinzula,L. *et al.* (2012) Identification of HIV-1 reverse transcriptase dual inhibitors by a combined shape-, 2D-fingerprint- and pharmacophore-based virtual screening approach. *Eur. J. Med. Chem.*, **50**, 216–229.
71. Tramontano,E., Esposito,F., Badas,R., Di Santo,R., Costi,R. and La Colla,P. (2005) 6-[1-(4-fluorophenyl)methyl-1H-pyrrol-2-yl]-2, 4-dioxo-5-hexenoic acid ethyl ester a novel diketo acid derivative which selectively inhibits the HIV-1 viral replication in cell culture and the ribonuclease H activity in vitro. *Antiviral Res.*, **65**, 117–124.
72. Corona,A., Di Leva,F.S., Thierry,S., Pescatori,L., Cuzzucoli Crucitti,G., Subra,F., Delelis,O., Esposito,F., Rigogliuso,G., Costi,R. *et al.* (2014) Identification of highly conserved residues involved in inhibition of HIV-1 RNase H function by diketo acid derivatives. *Antimicrob. Agents Chemother.*, **58**, 6101–6110.
73. Masaoka,T., Chung,S., Caboni,P., Rausch,J.W., Wilson,J.A., Taskent-Sezgin,H., Beutler,J.A., Tocco,G. and Le Grice,S.F. (2013) Exploiting drug-resistant enzymes as tools to identify thienopyrimidinone inhibitors of human immunodeficiency virus reverse transcriptase-associated ribonuclease H. *J. Med. Chem.*, **56**, 5436–5445.
74. Huang,X. and Miller,W. (1991) A time-efficient, linear-space local similarity algorithm. *Adv. Appl. Math.*, **12**, 337–357.