Mechanism-based Suppression of Dideoxynucleotide Resistance by K65R Human Immunodeficiency Virus Reverse Transcriptase Using an α-Boranophosphate Nucleoside Analogue*

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The amino acid change K65R in human immunodeficiency virus type 1-reverse transcriptase (RT) confers viral resistance to various 2',3'-dideoxynucleoside drugs in vitro. Using pre-steady state kinetic methods, we found that K65R-reverse transcriptase is 3.2-14-fold resistant to 2',3'-dideoxynucleotides in vitro relative to wild-type reverse transcriptase, in agreement with resistance levels observed in vivo. A decreased catalytic rate constant \( k_{\text{pol}} \) mostly accounts for the lower incorporation efficiency observed for 2',3'-dideoxynucleotides. Examination of the crystal structure of the RT-DNA:dNTP complex suggested that both the charge at position 65 and the 3'-OH of the incoming nucleotide act in synergy during the creation of the phosphodiester bond, resulting in a more pronounced decreased catalytic rate constant for 2',3'-dideoxynucleotides than for dNTPs. This type of intramolecular activation of the leaving phosphate by the 3'-OH group appears to be conserved in several nucleotide phosphotransferases. These data were used to design dideoxynucleotide analogues targeting K65R RT specifically. α-Boranophosphate ddATP was found to be a 2-fold better substrate than dATP and inhibited DNA synthesis by K65R RT 153-fold better than ddATP. This complete suppression of drug resistance at the nucleotide level could serve for other reverse transcriptases for which drug resistance is achieved at the catalytic step.

Nucleoside analogues are a major class of molecules active against the human immunodeficiency virus (HIV).1 Their viral target is reverse transcriptase (RT), an essential RNA- and DNA-dependent DNA polymerase encoded by the viral pol gene (1). Nucleoside analogues must be phosphorylated to nucleoside 5'-triphosphate by cellular kinases in the infected cell to become efficient and specific viral DNA synthesis inhibitors. Most of the analogues do not possess a 3'-OH. Hence, when incorporated selectively into viral DNA by RT, DNA chain termination occurs and is responsible for the antiviral effect.

During anti-retroviral therapies, the emergence of drug-resistant viruses limits the efficiency of nucleoside drugs, and this limitation represents a major cause of failure to control a HIV infection. Most drug-resistant viruses isolated from nucleoside analogue-treated patients harbor mutations in the viral pol gene (2). Remarkably, the set of resistance mutations selected during antiretroviral therapy is specific to the nucleoside drug. When 3'-azido-3'-deoxythymidine (AZT) is given as the sole drug, several patterns of amino acid changes involving M41L, D67N, K70R, L210W, T215F or Y, and K219Q give rise to up to a 200-fold AZT-resistant virus (3–5). When 2',3'-dideoxynucleotides (ddNs) are given to patients, ddN-resistance emerges and is mainly correlated with single amino acid substitutions such as L74V or K65R (2, 5–7).

The K65R mutation is found infrequently on viral isolates from patients treated with ddI and ddC (8, 9). However, its clinical relevance is increasing since K65R has been identified in a significant number of viral isolates resistant to three recent antiretroviral drugs (R)-9-(2-phosphonylmethoxypropyl)adenine, (−)β-D-dioxolaneguanosine, and Abacavir (1592U89) (2, 10–15). K65R is thus a multidrug resistance substitution, and understanding the mechanism by which K65R confers drug resistance might help designing new drugs either eliciting no resistance or active against drug-resistant viruses.

K65R RT has been extensively studied in vivo and in vitro, but a detailed molecular mechanism by which this substitution confers ddNTP resistance is still lacking. K65R RT has been reported to perform an equally (12) or more processive (16) DNA synthesis than wild-type RT, although the relationship between processivity and drug resistance is elusive. K65R RT shows resistance to ddNTPs relative to wild-type RT in steady-state kinetic assays by an unknown mechanism (6, 17). The crystal structure of an RT-DNA:dNTP complex has revealed the catalytic mechanism of nucleotide incorporation into DNA (18), but many questions remain concerning the role of K65R in drug resistance. Indeed, it can be seen in the crystal structure that the lysine 65 side chain contacts an oxygen atom on the γ-phosphate of the nucleotide poised for catalysis at the reverse transcriptase active site. The relation of this contact to drug resistance is puzzling since the γ-phosphate is too far (>7.5 Å) from the 3'-ribose position to explain satisfactorily how Arg might discriminate the 3'-deoxy moiety of an incoming ddNTP. Mu...
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Pre-steady State Kinetics of Nucleotide Incorporation into DNA—Pre-steady state kinetics were performed using dATP, dCTP, dTTP, ddATP, ddCTP, and α-BH₂-ddATP in conjunction with wild-type and K65R RT as described (20). The formation of product (P) over time was fitted with a burst equation,

\[ P = A(1 - \exp(-k_{app} t)) + k_{cat} \]  

(Eq. 1)

where A is the amplitude of the burst, \( k_{app} \) is the apparent kinetic constant of formation of the phosphodiester bond, and \( k_{cat} \) is the kinetic constant of the steady-state, linear phase. The dependence of \( k_{app} \) on dNTP concentration is described by the hyperbolic equation,

\[ k_{app} = k_{pol}(dNTP)/(K_{d} + (dNTP)) \]  

(Eq. 2)

where \( K_{d} \) and \( k_{pol} \) are the equilibrium constant and the catalytic rate constant of the dNTP for RT, respectively. \( K_{d} \) and \( k_{pol} \) were determined from curve-fitting using Kaleidagraph (Synergy Software, PA).

Three-dimensional Computer Modeling of RT—The coordinates of RT in complex with DNA and nucleotide were obtained from the Brookhaven Protein Data base (PDB). The PDB accession number is 1RTD. The crystal structure models were displayed using TURBO and MOLESCRIPT programs (25, 26).

RESULTS

The K65R substitution in RT is responsible for viral resistance to a variety of ddN. In the crystal structure of RT in complex with DNA and nucleotide, the ε-NH₂ group of lysine 65 contacts the γ-phosphate of the incoming nucleotide. This phosphate is part of the PP₃ product of the polymerization reaction. Because PP₃- or ATP-mediated repair of analogue-terminated DNA chains are mechanisms of drug resistance (24, 27), the K65R substitution might enhance the pyrophosphorolytic reaction and thus RT-mediated repair of ddNMP-terminated DNA. On the other hand, K65R RT has been reported to discriminate against ddNTPs better than wild-type RT using steady-state kinetic assays (17, 19). This suggests that the K65R substitution is able to interact either directly or indirectly with the 3'-OH of the incoming nucleotide, and that resistance proceeds by enhanced discrimination against ddNTPs rather than repair of ddNMP-terminated DNA. As a first step to determine which of these mechanisms might be involved in K65R RT-mediated ddNTP resistance, the crystal structure of a ternary complex made of RT, a DNA primer/template, and a nucleotide was examined.

The 3'-OH Hydrogen of the Incoming Nucleotide Is Critical for Efficient Catalysis—Examination of the crystal structure of the RT-DNA-ddNTP ternary complex (18) shows that the 3'-OH of the incoming nucleotide makes an intramolecular hydrogen bond with one nonbridging oxygen of the β-phosphate (Fig. 1). In this manner, the nucleotide is held in the active site so as to facilitate the in-line attack of the 3'-OH of the primer onto the α-phosphate of the incoming nucleotide. Apart from lysine 65, arginine 72, the main chain nitrogen of alanine 114, and the magnesium ions stabilize the triphosphate moiety. The intramolecular hydrogen bond does not exist when a ddNTP is incorporated, and this lack of interaction may explain why ddNTPs are less efficient substrates for incorporation than dNTPs. This catalytically important hydrogen bond has been inferred in both the bacteriophage T7 DNA polymerase/DNA/ddNTP and the rat β-polymerase DNA/ddNTP ternary complexes (28, 29). Interestingly, a similar intramolecular hydrogen bond activating phosphoryltransfer also exists in nucleotide diphosphate kinase (23). In this case, the 3'-OH of the phosphorylated nucleoside diphosphate hydrogens to O-6 bridging the β-phosphate and activates it, explaining why ddNDPs are 10⁴-fold less efficient substrates than dNDPs.

Because this catalytically important hydrogen bond occurs between the 3'-OH and the β-phosphate belonging to the PP₃ moiety of the nucleotide, this hydrogen bond might also be critical during pyrophosphorolysis, the reverse reaction of DNA.

EXPERIMENTAL PROCEDURES

Proteins, Reagents, Expression, and Purification of Enzymes—The wild-type RT gene construct p66RTB has been described (21). This plasmid was further modified and used to construct the mutant RT gene at codon 65 (K65R) using synthetic oligonucleotides and a strategy described in Ref. 21. All constructs were verified by restriction enzyme analysis and nucleotide sequencing. All recombinant RTs were expressed and purified as p66/p51 heterodimers, and active sites titrated as described (21). DNA oligonucleotides were obtained from Life Technologies. Oligonucleotides were 5'-³²P-labeled using T4 polynucleotide kinase (New England Biolabs). All ³²P-labeled nucleotides, adenosine 5'-triphosphate, 2'-deoxy- and 2',3'-dideoxynucleoside 5'-triphosphates (dNTPs and ddNTPs) were purchased from Pharmacia.

Hydrophosphorolysis of Analogue-terminated DNA by RT—The pyrophosphorolytic repair assay has been described (24). Briefly, the primer/template system (50 nM) used for both pyrophosphorolysis and ATP-mediated repair assays was a 5'-³²P-labeled 21-mer DNA primer (5'-A-TACCTTTAACCATATGATATC-3') annealed to a 35-mer (5'-NNNNNNNNNNGATACATAGTATGGTAAAGTAT-3') DNA template. The varying template "X" specifies a single nucleotide insertion site for the nucleotide analogue under study, four bases away from the 3' end of the primer. N represents any of the four nucleotides different from X. DNA polymerization was initiated by the addition of wild-type or K65R RT (100 nM) and nucleotides (5 µM of the analogue, 5'-triphosphate, and 25 µM each of 3 dNTPs at the exclusion of the natural counterpart of the nucleotide analogue) for 15 min at 37 °C in RT buffer. The repair reaction was started by adding the natural counterpart of the nucleotide analogue to reach a final concentration of 25 µM in the presence of either pyrophosphate (PP₃) or ATP. In this manner, the repair reaction is performed in the presence of the next correct nucleotide binding on top of the terminated primer, under conditions approximating those found in the infected cell. Aliquots were withdrawn during the time course of the reaction, and products analyzed by denaturing gel electrophoresis. The % repair of blocked primer is the ratio ×100 of extension products larger than 25 nucleotides over those larger than 24 nucleotides (23, 24).

tional analysis of lysine 65 and molecular modeling of this substitution in the RT active site have suggested that K65R alters the binding affinity of the ddNTP substrate (19). However, since dNTPs and ddNTPs are identical at their γ-phosphate position, it remains unclear how selectivity for 3'-OH is achieved.

Pre-steady state kinetics is the method of choice to measure the substrate efficiency of a nucleotide analogue for RT. The efficiency of incorporation of a nucleotide into DNA is given by the ratio \( k_{app}/K_{cat} \), being the rate constant for the creation of the phosphodiester bond and \( K_{cat} \) the equilibrium binding (or affinity) constant of the nucleotide for RT (20). Discrimination of a ddNTP relative to its natural counterpart dNTP is reflected by the efficiency of incorporation of the ddNMP into DNA relative to that of the natural substrate dNMP. Although this has been done for various ddNTPs and wild-type RT, such constants have not been determined for K65R RT.

In this paper, we have made use of pre-steady state kinetics to determine the affinity constant \( K_{d} \) as well as the catalytic constant \( k_{pol} \) of formation of the phosphodiester bond for several dNTP and ddNTP substrates by either wild-type or K65R RT. We show that the presence of an intramolecular hydrogen bond between the 3'-OH and one oxygen atom of the β-phosphate of the incoming nucleotide is critical for an efficient catalytic step. This intramolecular interaction represents the missing link to explain how ddNTPs are discriminated up to 14-fold better by K65R RT than wild-type RT. We show that these data can be used to design nucleotide analogues that efficiently suppress drug resistance at the enzyme level.
polymerization. Indeed, when the DNA chain is terminated with a ddNMP, no hydrogen bond would be formed between the incoming PPi and the 3'-ddNMP-terminated DNA. We hypothesized that this lack of interaction should affect either PPi binding or the pyrophosphorolysis reaction rate, and might relate K65R with pyrophosphorolysis and drug resistance.

Pyrophosphorolysis of ddNMP-terminated DNA by WT and K65R RT—This hypothesis was tested using a primer terminated by thymine nucleotide analogues presenting different 3'-groups. Four thymine analogues were used in this experiment: AZTTP, 3'-NH2-dTTP, d4TTP, and ddTTP. Pyrophosphorolysis or ATP-mediated repair of either analogue-terminated DNA was examined using a gel-based assay as described previously (24). In this assay, DNA synthesis by RT is terminated at a specific template position by incorporation of a 3'-modified thymidine analogue in the absence of dTTP. Then, dTTP and either ATP or PPi are added to the reaction, excision of the terminator occurs, and synthesis resumes because of the presence of dTTP. The appearance of extension products is diagnostic of the ability of RT to repair the terminated DNA chain using ATP or PPi. The results are presented in Fig. 2.

Wild-type RT is able to repair AZTMP-terminated DNA with best efficiency when compared with other terminated DNAs (Fig. 2A). Using 150 μM PPi, the repair efficiency of AZTMP-terminated DNA is ~4-fold better than that observed using 3.2 mM ATP, the latter having almost no effect on other terminated DNAs (not shown). The efficiency of PPi-mediated repair follows the decreasing order AZTTP > d4TTP > 3'-NH2-dTTP > ddTTP, indicating that the nature of the 3'-ribose is important for the repair reaction. In agreement with results obtained by others, the ddNMP-terminated DNA chain is poorly repaired by RT relative to AZTMP-terminated DNA (27, 30). Using K65R RT, again 3.2 mM ATP do not promote significant repair of ddNMP-terminated DNA when compared with PPi (not shown). Using either K65R RT or wild-type RT, AZTMP-terminated DNA is repaired better than dCMP or ddAMP-terminated DNA using 150 μM PPi (Fig. 2B). Unlike in the case of AZTMP-terminated DNA, ddNMP-terminated DNA repair seems to level off during the course of the repair reaction. This suggests that either the repair reaction reaches an equilibrium between repair and synthesis during the course of the experiment, or most of the RT-ddNMP-terminated DNA complex is under a form which is catalytically noncompetent for the repair reaction. As described in the case of AZT resistance, the latter form could be a dead-end complex made of RT, the terminated DNA, and the next correct nucleotide binding to the primer, as described under “Experimental Procedures.”

Polymerization products were visualized and quantitated using photostimulatable plates and a FujiImager. The % of repaired primer was expressed as a function of time. A, unblocking the analogue-terminated primer using PPi, and wild-type RT. Time courses for repair of primer terminated by AZTMP, 3'-NH2-dTMP, d4TMP and ddTMP are indicated as –N3 ( ), –N3 ( ), –d4 ( ), or –ddT ( ), respectively. B, unblocking the analogue-terminated primer using PPi, and either wild-type RT (closed black symbols) or K65R RT (open white symbols). Time courses for the repair of primer terminated by AZTMP, dCMP, or ddAMP are indicated as –N3, –dC, or –dA, respectively.
and was examined using wild-type and K65R RT comparatively.

**Discrimination of Dideoxynucleotides against Deoxynucleotides by Wild-type RT**—Discrimination of a ddNTP relative to a dNTP can be measured using pre-steady state kinetics of incorporation of a single nucleotide into DNA by RT (20, 31). In these experiments, the binding affinity $K_d$ of a nucleotide for RT can be determined together with the rate constant $k_{pol}$ of phosphodiester bond formation. The ratio $k_{pol}/K_d$ is defined as the efficiency of nucleotide incorporation into DNA, and allows comparison of incorporation efficiencies between any nucleotide substrates. Such measurements were first performed for adenine and cytidine nucleotides, and the results are presented in Table I.

Wild-type RT discriminates ddATP and ddCTP relative to dATP and dCTP, respectively. Using dATP and ddATP, the discrimination originates less from a loss of affinity than from a decrease in the catalytic rate constant. Indeed, $k_{pol}$ decreases 7-fold from 50.16 s$^{-1}$ to 7.47 s$^{-1}$ while $k_{pol}$ decreases 7.7-fold from 50.2 s$^{-1}$ to 6.89 s$^{-1}$. The overall incorporation efficiency $k_{pol}/K_d$ of ddATP drops 35-fold from 6.7 s$^{-1}$ to 0.19 s$^{-1}$ upon the absence of a 3′-OH group in the adenine nucleotide. Although less pronounced, the results are similar when ddCTP is compared with dCTP. The absence of a 3′-OH group in the cytidine nucleotide increases the affinity constant 2-fold (from 7.9 to 14.25 μM) while the catalytic constant decreases 2.8-fold (7.3 to 2.6 s$^{-1}$). The overall incorporation efficiency $k_{pol}/K_d$ decreases only 5-fold from 0.93 s$^{-1}$ to 0.18 s$^{-1}$ upon the absence of a 3′-OH group in the cytidine nucleotide. We note that the catalytic rate constant $k_{pol}$ (dCTP) at 50.2 s$^{-1}$ is almost 7-fold higher than $k_{pol}$ (dCTP) at 7.3 s$^{-1}$, a result in agreement with others (20, 31). We conclude that wild-type RT exhibits a "natural" resistance to ddATP and ddCTP. Wild-type RT is able to discriminate 5- and 5-fold ddATP and ddCTP relative to their natural counterparts dATP and dCTP, respectively.

**Discrimination of Dideoxynucleotides against Deoxynucleotides by K65R RT**—The same experiments were conducted using K65R RT. As shown in Table I, this variant RT discriminates ddATP and ddCTP relative to dATP and dCTP in a more pronounced fashion than wild-type RT, respectively. Using dATP and ddATP, the discrimination again originates less from a decreased affinity than from a decrease in the catalytic rate constant. $K_d$ increases 6.9-fold from 6.9 to 47.5 μM while $k_{pol}$ decreases 16.4-fold from 11.6 s$^{-1}$ to 0.7 s$^{-1}$. This yields an overall 112-fold decrease in incorporation efficiency $k_{pol}/K_d$ from 1.69 s$^{-1}$ to 0.015 s$^{-1}$ upon the absence of a 3′-OH group in the adenine nucleotide. For cytidine nucleotides, both a 9.2-fold increase in $K_d$ and a 8-fold decrease in $k_{pol}$ yield a 74-fold drop in the efficiency of incorporation $k_{pol}/K_d$ from 0.96 to 0.013 s$^{-1}$. We conclude that K65R RT exhibits its resistance to both ddATP and ddCTP as judged by the 112- and 74-fold discrimination of ddATP and ddCTP relative to dATP and dCTP, respectively.

**Resistance against Dideoxynucleotides by K65R RT Relative to Wild-type RT**—Resistance to ddNTPs by a variant RT is defined as the ratio between discrimination observed using variant RT over discrimination observed for wild-type RT. For adenine nucleotides, K65R RT discriminates 112-fold against ddATP whereas wild-type RT discriminates only 35-fold against ddATP. Therefore, K65R RT is 3.2-fold resistant to ddATP. When cytidine nucleotides are considered, a discrimination of 74-fold by K65R RT is compared with a 5-fold discrimination by wild-type RT, yielding a 14-fold resistance to ddCTP by K65R RT. We note that in the case of adenine nucleotides, K65R RT acquires resistance to ddATP at the expense of general dNTP incorporation efficiency. Indeed, $K_d$ (dATP) remains unchanged and $k_{pol}$ alone accounts for the decrease efficiency as judged by a decrease of the $k_{pol}/K_d$ (dATP) value from 6.7 to 1.7 s$^{-1}$ μM$^{-1}$. This is also the case for dCTP where a drop in $k_{pol}$ from 7.9 to 2.8 s$^{-1}$ is observed, but the decrease in $k_{pol}$ was compensated by an actual increase in affinity of dCTP for the K65R RT relative to wild-type RT. This compensatory effect keeps the efficiency of incorporation around 0.9 s$^{-1}$. To determine whether this difference in efficiency of incorporation was purely or pyrimidine specific, the same pre-steady state kinetics experiments were conducted using dTTP and either wild-type or K65R RT. The results are shown in Table I. The K65R substitution increases the affinity of RT for dTTP about 2-fold, i.e. $K_d$ (dTTP) drops from 17.5 to 9.7 μM. Interestingly, the same catalytic determinants of the phosphodiester bond formation lie in one intramolecular bond into the incoming nucleotide and not exclusively in the enzyme. We reasoned that this intrinsic property of the nucleotide could be used to design analogues having an enhanced incorporation rate into DNA. In this manner, a chemical modification on the nucleotide that would affect positively the catalytic rate constant might be of greatest interest for K65R RT since ddNTP resistance originates from a decreased rate constant relative to wild-type RT. Several nucleotide analogues carrying a chemical modification onto the α-phosphate have been described (23, 32). Among them, α-BH$_3$-dNTPs have been found to be about 2-fold better substrates for RT than their unmodified dNTP counterparts. When present in the $R_a$-configuration of either AZTTP or dATP, the borono (BH$_3$) group enhances 2-3-fold DNA chain termination by wild-type and K65R RT (23). The Pro-$R_a$ position is shown in Fig. 1. Since the borono incorporation properties were mainly due to an enhanced catalytic step, it was of interest to check whether this would also be the case using α-BH$_3$-ddNTP and K65R RT. Therefore, $R_a$-α-BH$_3$-ddATP was synthesized, purified, and used in pre-steady state experiments as described above. The results are presented in Table II, and the most relevant are presented graphically in Fig. 3.

Using wild-type RT, the presence of the BH$_3$ group enhances about 4-fold the incorporation properties of ddATP into DNA.

**Table I**

| Wild-type RT | K65R RT |
|-------------|---------|
|             | dATP    | ddATP   | dCTP    | ddCTP   | dTTP    | ddTTP   | dATP    | ddATP   | dCTP    | ddCTP   | dTTP    | ddTTP   |
| $K_d$ (μM$^a$) | 7.47    | 33.8    | 7.88    | 14.25   | 17.5$^b$| 6.89    | 47.54   | 2.84    | 26.1    | 9.7     |         |         |
| $k_{pol}$ (s$^{-1}$) | 50.16   | 6.49    | 7.33    | 2.59    | 13.2$^b$| 11.63   | 0.71    | 2.72    | 0.34    | 3.3     |         |         |
| $k_{pol}/K_d$ (s$^{-1}$ μM$^{-1}$) | 6.71    | 0.192   | 0.93    | 0.182   | 0.75$^b$| 1.69    | 0.015   | 0.96    | 0.013   | 0.34    |         |         |

$^a$ $K_d$ and $k_{pol}$ were determined as described under "Experimental Procedures." Standard deviations were <20%.

$^b$ Value from Selmi et al. (24).
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### Table II

| Kinetic constants for BH$_3$-ddATP | Selectivity$^a$ for BH$_3$-ddATP over | Wild-type | K65R RT |
|-----------------------------------|--------------------------------------|-----------|----------|
| $K_0$ (μM)$^b$                   |                                      | 29.9      | 6.5      |
| $k_{pol}$ ($s^{-1}$)              |                                      | 22.9      | 14.9     |
| $k_{pol}/K_0$ ($s^{-1} \cdot \mu M^{-1}$) |                                      | 0.75      | 2.3      |

$^a$ Values from Table I were used to determine the enhancement values relative to dATP or ddATP, written as “Selectivity for BH$_3$-ddATP over dATP” or “Selectivity for BH$_3$-ddATP over ddATP.” For $K_0$ values, the selectivity is the ratio of $K_0$ (dATP)/$K_0$ (BH$_3$-ddATP) or $K_0$ (ddATP)/$K_0$ (BH$_3$-ddATP). For $k_{pol}$ values, the selectivity is the ratio $k_{pol}$ (BH$_3$-ddATP)/$k_{pol}$ (dATP) or $k_{pol}$ (BH$_3$-ddATP)/$k_{pol}$ (ddATP). For $k_{pol}/K_0$ values, the selectivity is the ratio $k_{pol}/K_0$ for BH$_3$-ddATP over either $k_{pol}/K_0$ for dATP or $k_{pol}/K_0$ for ddATP.

$^b$ $K_0$ and $k_{pol}$ were determined as described under “Experimental Procedures.” Standard deviations were <20%.

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**DISCUSSION**

RT-mediated drug resistance is a common problem found during antiretroviral therapies, and much effort is devoted toward the characterization of drug-resistant mechanisms. Recently, several of these mechanisms have been elucidated at the molecular level. In all cases, the variant (or “drug-resistant”) RT has acquired or enhanced biochemical properties regarding either discrimination of the nucleotide analogue or repair of the analogue-terminated DNA chain (5, 33).

One mechanism of AZT resistance has been recently proposed (27). It involves excision of the terminal AZTMP by AZT-resistant RT in a reaction chemically similar to pyrophosphorolysis, the reversal of the polymerization reaction. The γ-phosphate of ATP attacks the terminal AZTMP phosphodiester bond to restore a free 3′-OH end and release AppppAZT. The presence of specific AZT-resistant mutations in RT has two effects: it decreases the formation of a dead-end complex made of RT, the terminated DNA chain, and the next correct nucleotide, and it favors the catalytic step of the pyrophosphorolytic-like unblocking mechanism (27). Hence, AZTMP is not discriminated at the AZT-resistant RT active site relative to its natural counterpart dTTP, but AZT resistance originates from a post-incorporative repair (or unblocking) mechanism.

In wild-type RT, lysine 65 interacts with the γ-phosphate of the incoming nucleotide. The β- and γ-phosphates of the latter become the leaving PP$_i$ after creation of the phosphodiester bond. Hence, once DNA synthesis has been terminated by insertion of a ddNMP analogue, the first event of pyrophosphorolysis is the binding of PP$_i$ to lysine 65. Since this is a repair reaction, the K65R substitution might promote prime unblocking though enhanced pyrophosphorolysis by K65R RT relative to wild-type RT. Our results show that not only the efficiency of repair of ddNMP-terminated DNAs by wild-type and drug-resistant RT is low relative to that of AZTMP-terminated DNA chains but also pyrophosphorolysis is not significantly higher using K65R RT relative to wild-type RT. Therefore, the K65R RT-mediated ddNTP-resistant mechanism does not involve a repair mechanism driven by either PP$_i$, or ATP, such as in the case of AZT resistance. In fact, K65R has been described as an AZT-resistant suppressor substitution (10), suggesting that ddN resistance and AZT resistance do not share a common, enhanced “pyrophosphorolytic-like” mechanism. It would be of interest to determine if the K65R substitution would actually decrease the catalytic rate constant of ATP-mediated unblocking of the AZTMP-terminated primer when present in RT bearing the M41L/D67N/K70R/L210W/T215FY/K219Q AZT-resistant substitutions.

Interestingly, the 3′-end of the terminated primer is critical for efficient pyrophosphorolysis, suggesting that the chemical nature of the 3′-group of the terminated DNA is either part of the PP$_i$-binding site or is involved in catalysis of phosphodiester bond breakage. For a given ddNTP, wild-type pyrophosphorolysis level is low due to the absence of the hydrogen bond between the 3′-group and β-phosphate. This fact is due to the intrinsic nature of the nucleotide that cannot be compensated by an amino acid substitution in RT. Therefore, it is tempting to speculate that during ddN therapies, the selection of amino acid substitutions increasing ddNTP discrimination is favored over those enhancing pyrophosphorolysis.

The importance of the 3′-OH of the incoming nucleotide in catalysis has been demonstrated for Taq and bacteriophage T7 DNA polymerases (28, 34, 35). In the ternary complex made of bacteriophage T7 DNA polymerase, DNA, and a nucleotide, it was shown that the hydroxyl group of tyrosine 526 could compensate for the absence of the 3′-OH of the incoming nucleotide. Although DNA polymerase share a common phosphoryl...
transfer mechanism, the nucleotide-binding site of DNA polymerases of the type I family (Escherichia coli DNA polymerase I, Taq DNA polymerase, and bacteriophage T7 DNA polymerase) is made of an helix (helix O in E. coli DNA polymerase I) whereas it consists of two antiparallel β-strands in reverse transcrip-tants (β3 and β4 in HIV-RT). Therefore, our work shows that this intramolecular hydrogen bond in the incoming nucleotide is also important for catalysis by HIV-1 RT. Furthermore, the presence of hydrogen bonds in the vicinity of the 3′-OH and the β-phosphate seems to correlate with the pyrophosphorolytic activity of polymerases. Bacteriophage T7 DNA polymerase has a high pyrophosphorolysis activity and tyrosine 526 can compensate for the lack of 3′-OH, whereas other polymerases (Taq, RT) lacking stabilization of the catalytic complex by a residue spatially equivalent to tyrosine 526 show a much lower pyrophosphorolysis activity. Taken together, these data suggest that the 3′-end of the primer helps defining the PPi-binding site on the ternary complex made of polymerase, terminated DNA, and PPi.

The absence of enhanced repair activity of the terminated primer lead us to study ddNTP discrimination by K65R RT. When discrimination is involved in RT-mediated resistance to nucleotides, amino acid substitutions near or in the RT active site confer to RT the ability to use the nucleotide analogue with a lower efficiency than its natural dNTP counterpart. In theory, this lower efficiency of incorporation can originate from either a lower affinity of the nucleotide analogue for RT ($K_d$ effect) or a slower rate of incorporation of the nucleotide analogue into DNA ($k_{pol}$ effect). These constants are usually measured using pre-steady state kinetics of incorporation of a single nucleotide into DNA. An example of this type of resistance mechanism is provided by the M184V substitution in RT, which confers a 1000-fold resistance to 3TC in vivo and in vitro (2). Purified RT exhibits altered 3TCTP selection properties. While the affinity of 3TCTP for M184V RT is unchanged relative to that for wild-type RT, the M184V substitution decreases 23–36-fold the rate of 3TCMP incorporation into DNA (36). This substitution was so far the sole example of RT-mediated HIV-1 drug resistance due to nucleotide discrimination provided by a slower catalytic step rate. Our data show that K65R RT-mediated deoxyribose is due to a similar decrease in the catalytic rate. However, the M184V substitution is located close to the primer 3′-end and impedes correct alignment of the reactive centers during catalysis (18, 36), whereas K65R is located >7.5 Å away from the 3′-OH of the primer. Although nucleotide discrimination arises from either M184V or K65R, the molecular mechanisms are quite different. The K65R substitution serves to amplify the negative effects due to the lack of hydrogen bond between the 3′-OH and the β-phosphate of the incoming ddNTP.

Dideoxynucleotides substituted in the α-phosphate were used to probe our model, α-BH$_3$-nucleotide analogues have been found to increase the catalytic rate of phosphodiester bond formation (23). This is particularly true for K65R RT where the dramatic 153-fold improvement of this incorporation efficiency allowed ddNTP resistance by this multidrug-resistant RT to be totally suppressed. Thus, α-BH$_3$-dATP provides the first example of a mechanism-based nucleotide analogue able to target a multidrug-resistant RT specifically and restore full analogue sensitivity. In addition, such catalytic rate enhancement suggests that the chemical step of phosphodiester bond formation for ddAMP incorporation into DNA is rate-limiting, not a conformational change preceding catalysis (20).

What is the actual chemical mechanism for the rate enhancement? We would like to propose that the negative charge introduced by the BH$_3$ group at the Pro-$R_h$ position (Fig. 1) becomes the driving force for PPi release, the presence of the BH$_3$ group is repulsive for the newly created negative charge on the α,β-phosphate bridging oxygen, and thus promotes departure of the PPi molecule. In this manner, bond scission becomes independent from amino acid side chains of Arg$^{72}$ and Lys$^{65}$, which are crucial for charge neutralization with unmodified nucleotide substrates. This proposal is consistent with the fact that the R72A substitution does not affect incorporation of α-BH$_3$-dTTP, α-BH$_3$-AZTTP, nor α-BH$_3$-dATP analogues, but leads to a severely impaired RT (>200-fold) using natural dNTPs (23). Thus, our results demonstrate that Arg$^{72}$ and Lys$^{65}$ have no role in activating the α-phosphate for the nucleophilic attack, a task entirely performed by the catalytic acidic residues and magnesium ions.

The type of analogue-based drug-resistant suppression described here might be of general interest in the case of other variant RTs for which nucleotide analogue resistance involves a decreased catalytic rate constant value, such as M184V RT and 3TCTP resistance. The boronophosphate modification is a general chemical rate-enhancer that might also prove useful for another yet uncharacterized variant RT, such as Q151M RT, that might use a similar resistance mechanism as well as for other nucleotide analogues such as (R)-9-(2-phosphonylmethoxypropyl)adenine-5′-diphosphate, (−)-β-dioloxanoguanosine-5′-triphosphate, and Abacavir-5′-triphosphate for which K65R is a clinically relevant resistance substitution.

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