The Molecular Mechanism of Multidrug Resistance by the Q151M Human Immunodeficiency Virus Type 1 Reverse Transcriptase and Its Suppression Using α-Boranophosphate Nucleotide Analogues*

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Nucleoside analogues are currently used to treat human immunodeficiency virus infections. The appearance of up to five substitutions (A62V, V75I, F77L, F116Y, and Q151M) in the viral reverse transcriptase promotes resistance to these drugs, and reduces efficiency of the antiretroviral chemotherapy. Using pre-steady state kinetics, we show that Q151M and A62V/N75I/F77L/F116Y/Q151M substitutions confer to reverse transcriptase (RT) the ability to discriminate an analogue relative to its natural counterpart, and have no effect on repair of the analogue-terminated DNA primer. Discrimination results from a selective decrease of the catalytic rate constant $k_{\text{cat}}$; 18-fold (from 7 to 0.3 s$^{-1}$), 13-fold (from 1.9 to 0.14 s$^{-1}$), and 12-fold (from 13 to 1 s$^{-1}$) in the case of ddATP, ddCTP, and 3'-azido-3'-deoxycytidine 5'-triphosphate (AZTTP), respectively. The binding affinities of the triphosphate analogues for RT remain unchanged. Molecular modeling explains drug resistance by a selective loss of electrostatic interactions between the analogue and RT. Resistance was overcome using α-boranophosphate nucleotide analogues. Using A62V/N75I/F77L/F116Y/Q151M substitutions on RT, $k_{\text{cat}}$ increases up to 70- and 13-fold using α-boranophosphate-ddATP and α-boranophosphate AZTTP, respectively. These results highlight the general capacity of such analogues to circumvent multidrug resistance when RT-mediated nucleotide resistance originates from the selective decrease of the catalytic rate constant $k_{\text{cat}}$.

The human immunodeficiency virus (HIV) infects more than 40 million individuals in the world. 3'-Azido-3'-deoxycytidine (AZT, zidovudine) was the first antiretroviral drug to receive approval from the FDA in 1987 to treat HIV-1-infected patients. AZT is a nucleoside analogue acting on viral replication. It is metabolically activated by cellular kinases of the host cell to its corresponding triphosphate form AZTTP before reaching its target, reverse transcriptase (RT). RT is an essential viral DNA polymerase responsible for viral DNA synthesis. AZTTP is a poor substrate for cellular DNA polymerases, but is incorporated into the nascent viral DNA strand by RT with the same efficiency as its natural nucleotide counterpart dTTP. Because AZT lacks a 3'-hydroxyl group (3'-OH) on its ribose moiety, AZTMP is incorporated into DNA and viral DNA synthesis is terminated.

The prolonged use of AZT as the sole drug in the clinic has resulted in the emergence of AZT-resistant viruses (1). A set of six specific substitutions on RT (M41L, D67N, K70R, T215Y or F, L210W, and K219E or Q) gives rise to high level AZT resistance (2), the appearance of T215F or Y being the most important substitution. A long awaited mechanism of AZT resistance because of these mutations has been proposed, based on biochemical studies using purified reverse transcriptase: AZT-resistant RT is able to catalyze a primer-unblocking reaction related to pyrophosphorolysis (3, 4) to remove the chain-terminating AZTMP. This “repair” reaction allows the RT to resume elongation of the primer DNA.

To circumvent limitations because of resistance and to increase the efficacy of antiretroviral regimens, several other nucleoside analogues such as 2',3'-dideoxyxycloises (ddNs) have been developed and used in the clinic. Unfortunately, combination therapies using a mixture of AZT and ddNs often give rise to multiple dideoxyxylcise-resistant viruses that are no longer sensitive to either AZT or dideoxyxylcises (5). Multidrug-resistant RTs isolated from these viruses carry another set of five mutations (A62V, V75I, F77L, F116Y, and Q151M) that were shown to significantly reduce sensitivity to AZT, ddI, ddC, and d4T in vivo (6, 7). Q151M is a key mutation in this type of multiple dideoxyxylcise resistance (8, 9). Q151M appears first during combination therapy with a concomitant increase in the virus load of infected patients. Multidrug resistance and viral fitness are increased further with the sequential appearance of up to four additional amino acid substitutions mentioned above. Q151M-based reverse transcriptase variants have been studied at the biochemical level (10, 11). Steady-state kinetics of single nucleotide incorporation showed that resistance levels of A62V/N75I/F77L/F116Y/Q151M RT (here referred to as Q151M<sub>complex</sub> RT) had risen up to 10-fold for ddATP and 14-fold for AZTTP. These results suggested that multidrug resistance is because of an altered recognition of the incoming nucleotide analogue, as opposed to

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1 The abbreviations used are: HIV, human immunodeficiency virus; RT, reverse transcriptase; AZT, 3'-azido-3'-deoxycytidine; ddT, 2',3'-dideoxy-2',3'-dideoxythymidine; 3TC, (-)-β-l,2',3'-dideoxy-3',β-Thiacytidine; AZTTP, 3'-azido-3'-deoxycytidine 5'-monophosphate; AZTTP, 3'-azido-3'-deoxycytidine 5'-triphosphate; ddATP, 2',3'-dideoxyadenine 5'-triphosphate; ddCTP, 2',3'-dideoxythymidine 5'-triphosphate; d4T, 2',3'-dideoxynucleoside 5'-triphosphate; ddNTP, 2',3'- dideoxynucleoside 5'-triphosphate; PP<sub>i</sub>, inorganic pyrophosphate; BH<sub>3</sub>, borano.
the resistance induced by the repair reaction described previously in the case of AZT.

Discrimination of the 5'-triphosphate analogue at the RT active site has been described as a mechanism of nucleoside drug resistance. The incorporation efficiency of an analogue into DNA, and hence its DNA termination potency, depends on two factors. First, the nucleotide analogue has to compete for binding to the RT active site with its natural nucleotide counterpart. This binding competition is reflected in the comparison of the analogue dissociation constant \(K_d\) with that of its natural nucleotide competitor. Second, once the nucleotide analogue is bound to the RT active site, it has to be incorporated into DNA with a favorable catalytic rate constant \(k_{cat}\). The overall efficiency of incorporation of a given analogue is thus the ratio \(k_{cat}/K_d\). This ratio offers a precise comparison between the chain termination properties of the analogues. Knowledge of the intracellular nucleotide analogue concentrations in the target cell allows one to predict the average number of chain termination events during viral DNA replication (12). The resistance of Q151M and Q151M\(_{\text{omplex}}\) RT toward various analogues has been studied using purified RT, but steady-state kinetics do not give access to the dissociation and catalytic rate constants \(K_d\) and \(k_{cat}\), respectively. Therefore, the precise mechanism by which Q151M and related substitutions confer nucleotide resistance to RT remains to be detailed.

We have previously described nucleotide analogues that are modified on their α-phosphate by a boran (BH\(_2\)) group (13). The \(R_c\) diastereoisomer of these \(BH_2\)-dNTPs analogues acts as a substrate for the RT, and as a chain terminator when it is devoid of a 3'OH group. Recently, we have determined the precise basis of dideoxynucleotide analogue resistance promoted by the K65R substitution in RT (14). K65R RT is responsible for a decrease in the rate of incorporation of the nucleotide analogues specifically. We have also shown that this resistance can be suppressed by the presence of the BH\(_2\) group at the α-position of ddNTPs. Indeed, the presence of the BH\(_2\) group does not influence the binding of the analogue to the RT active site, but greatly enhances the catalytic rate constant, \(k_{cat}\), of incorporation of the deoxy analogue specifically. This suppression of drug resistance provides mechanistic proof for the involvement of the catalytic step, but is also an elegant way to overcome resistance in vitro. These results allowed us to propose that these analogues could be similarly useful with other RTs showing nucleotide analogue resistance by a related mechanism. In addition to an ill defined resistance mechanism, the incorporation properties of BH\(_2\)-dNTP analogues are not known for these clinically important Q151M variants.

In this study, we show that a decrease in the rate constant of the phosphodiester bond formation is critical in the mechanism of multidrug resistance by Q151M and Q151M\(_{\text{omplex}}\) RT. Based on this result, we make use of BH\(_2\)-nucleotide analogues to overcome resistance and recover sensitivity to these nucleotide analogue inhibitors.

### EXPERIMENTAL PROCEDURES

**Plasmid Constructions, Enzyme Preparations, and Reagent**—The wild-type RT gene construct p66RTB served as a template for directed mutagenesis using the QuikChange kit from Stratagene, to obtain both wild-type, Q151M, or Q151M\(_{\text{omplex}}\) RT (100 nM) and nucleotides (5 αM AZTTP, and 25 μM each of dATP, dCTP, and dGTP) for 15 min at 37°C in RT buffer supplemented with 6 mM MgCl\(_2\). The unblocking reaction was started by adding dTTP to reach a final concentration of 25 μM in the presence of either pyrophosphate (PP\(_i\)) or ATP. In this manner, unblocking 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 were analyzed using denaturing gel electrophoresis.

### RESULTS

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

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 enzyme turnover rate, of incorporation of either pyrophosphate (PP\(_i\)) or ATP. In this manner, unblocking 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 were analyzed using denaturing gel electrophoresis. The % of unblocked primer is the ratio of extension products larger than 25 nucleotides over those larger than 24 nucleotides, multiplied by 100.

**Molecular Modeling and Structural Analysis**—Modeling was based on the crystal structure of the complex of HIV-1 reverse transcriptase with a DNA template/primer and a deoxynucleosine triphosphate (17) (Protein Data Bank code 1RTD). Structures were displayed, modified, and analyzed using the graphic program TURBO (18). Mutations were manually built and the whole structure was subsequently submitted to several rounds of minimization of the energy function, in which the crystallographic contribution has been removed, using the program crystallography NMR software (19). Preliminary validation of the method was performed on the deposited coordinates of the complex using an identical protocol for minimization.
nucleotide selectivity has been implicated as the mechanism of Q151M-based resistance (23), as in the case of the single amino acid substitutions M184V (resistance to 3TC (24)) and K65R (resistance to ddI and ddC (14)). However, it is not known how discrimination is achieved at the Q151M complex RT active site. In other words, it is not known whether nucleotide analogues are poorly bound to the resistant RT nucleotide binding site relative to their natural counterparts, or are bound to resistant RT with the same affinity as their natural counterparts but poorly incorporated into DNA at the catalytic step, as it is the case for K65R RT and ddNTPs (14). On the other hand, it has been shown that AZT-resistant RT (bearing also multiple substitutions such as D67N/K70R/T215F or Y or K219E or Q) is acting through a primer rescue (or unblocking) mechanism preferentially using NTP as pyrophosphate donors (4). It was thus of interest to determine whether the substitutions A62V/V75I/F77L/F116Y/Q151M would increase the natural ability of wild-type RT to unblock the analogue-terminated primer.

Q151M-based Resistance to AZTTP Does Not Involve a Primer Unblocking Reaction—We tested the efficiency of pyrophosphorytic and ATP-mediated repair of an AZTMP-terminated DNA primer by RT. A 5'-32P-labeled 21-mer primer annealed to a specific 35-mer template is extended using RT and a mixture of nucleotides in which dTTP is replaced by AZTTP (Fig. 1A, step 1). For the rescue reaction, the AZTMP-terminated 25-mer is incubated at various times in the presence of dTTP and ATP or PPi. ATP or PPi unblocks the primer, allowing RT to use dTTP now present in the reaction mixture and resume elongation up to a 35-mer (Fig. 1A, step 2). In the case of ATP as the pyrophosphate donor, the unblocking activity exhibited by wild-type and Q151M RT are comparable (Fig. 1, B and C). The Q151M complex RT shows a slightly impaired ATP-mediated ability to unblock the primer, relative to wild-type. Similar results are obtained for pyrophosphorylation using PPi, as the unblocking agent (Fig. 1, B and C). None of the substituted RT seems to have any improved primer unblocking ability that could account for resistance to AZTTP under various experimental conditions (not shown). We conclude that primer rescue is not involved in multinucleoside drug resistance exhibited by RT bearing Q151M-type substitutions. This finding is consistent with the results detailed below showing that discrimination of nucleotide analogues at the active site can account for drug resistance.

Pre-Steady State Kinetics of RT Carrying the Q151M Substitution—Using pre-steady state kinetics with a single nucleotide incorporation, we tested the possibility that either $k_d$ or $k_{pol}$ are different between wild-type RT and Q151M-based mutants. Fig. 2 shows a typical data set of rapid incorporation of a saturating concentration of dTTP or AZTTP by Q151M RT, with constant values reported in Table I. $K_a$, the nucleotide affinity, is calculated as the nucleotide concentration that gives half of the maximum incorporation rate $k_{pol}$ (Fig. 2). The incorporation efficiency ($k_{pol}/K_a$) is used to calculate the selectivity factor: $(k_{pol}/K_a)^{ATTP}/(k_{pol}/K_a)^{analogue}$. A selectivity factor greater than 1 means that the enzyme discriminates the analogue over the natural nucleotide. Finally, the resistance of the RT to the inhibitor is the ratio between the selectivity of the mutant and the selectivity of the wild-type enzyme. The enzyme affinity for dTTP ($K_a = 14 \mu M$) is very similar to that for AZTTP ($K_a = 18 \mu M$). However, the $k_{pol}$ (AZTTP) value drops from 17 s$^{-1}$ for dTTP to 4.3 s$^{-1}$. This means that the rate of a step before or at the creation of the phosphodiester bond decreases 4-fold when AZTTP is incorporated in comparison with its natural counterpart dTTP.

Q151M-based Resistance to AZTTP Is Because of a Discrimination of the Nucleotide Analogue—Wild-type RT does not discriminate AZTTP relative to dTTP (Table I, part A). Catalytic rate constants for both substrates are the same (13 s$^{-1}$). However, the nucleotide affinity is better for AZTTP than dTTP. Wild-type RT has a slightly higher overall incorporation efficiency ($k_{pol}/K_a$) for AZTTP (1.8 s$^{-1}$ $\mu M^{-1}$) than for dTTP (0.7 s$^{-1}$ $\mu M^{-1}$). Q151M RT, as shown above (Fig. 2), does not alter nucleotide affinity significantly but induces a drop in $k_{pol}$ from 17 s$^{-1}$ for dTTP to 4.3 s$^{-1}$ for AZTTP, decreasing the incorporation efficiency 5-fold. Comparison of incorporation efficiencies of wild-type and Q151M RT leads to a 12-fold resistance mainly provided by a $k_{pol}$ effect. When the four other mutations (A62V, V75I, F77L, and F116Y) are added to the Q151M background, the $k_{pol}$ constant further decreases 7-fold from 7.7 s$^{-1}$ for dTTP to 1.1 s$^{-1}$ for AZTTP. On the other hand, the affinity for the Q151M complex RT is unchanged ($K_a = 10 \mu M$). Taken together, these constants bring the AZTTP incorporation efficiency down to 0.11 s$^{-1}$ $\mu M^{-1}$, which is responsible for a 7.2-fold discrimination, and a 17-fold resistance. We conclude that resistance to AZTTP is observed with the Q151M mutation on reverse transcriptase at the catalytic step, and this resistance is further increased in the case of the Q151M complex RT.
Mechanism and Suppression of Q151M RT Drug Resistance

**Multiple Dideoxynucleotide Resistance Is kpol Dependent**—Fig. 3 summarizes the evolution of RT-mediated resistance by discrimination of AZTTP and ddNTPs. Resistance is mostly acquired by the Q151M substitution in the case of AZTTP (12-fold), and ddCTP (2.3-fold). On the other hand, the addition of the other four mutations is required to reach high ddATP resistance levels (9-fold), whereas Q151M alone is responsible for only a 2.5-fold increase. The affinity constant $K_a$ does not play any significant role in the discrimination process. Table I shows that there is no drop in affinity for ddATP, ddCTP, nor AZTTP upon the appearance of resistance substitutions. Instead, there is a direct dependence of Q151M-based resistance to AZTTP and ddNTPs upon the catalytic constant $k_{pol}$ (Fig. 3, A and B). 90% of the initial $k_{pol}$(ddATP) value drops as a result of the sole methionine substitution in position 151 (Fig. 3B). In the case of AZTTP, 66 and 91% of the catalytic rate constant values are lost with the appearance of Q151M and A62V/N75I/F77L/P116Y/Q151M substitutions, respectively. We conclude that resistance phenotypes of Q151M-based mutants observed at the biochemical level are directly linked to the variation of the catalytic rate constant $k_{pol}$ of incorporation of nucleotide analogues into DNA.

**BH$_3$-Nucleotide Analogues Suppress the Resistance to AZTTP and ddATP**—We had previously studied BH$_3$-nucleotide analogues for their ability to circumvent RT-mediated resistance against nucleotide analogues in biochemical assays. We have recently determined the molecular mechanism of resistance of K65R RT to ddNTPs (14). Relative to dATP, this resistant enzyme discriminates ddATP at the catalytic step of the incorporation. However, K65R RT is not able to discriminate BH$_3$-ddATP from dATP (14). To further characterize the unique properties of this particular class of compounds, we assayed BH$_3$ analogues with Q151M RT. When BH$_3$-AZTTP is incorporated by Q151M RT, the $k_{pol}$ value increases up to 13-fold, from 1.1 s$^{-1}$ for $k_{pol}$(AZTTP) to 14 s$^{-1}$ for $k_{pol}$(BH$_3$-AZTTP), with an overall catalytic efficiency of 1.1 s$^{-1}$ M$^{-1}$ (Fig. 4A, Table II). Thus, there is no discrimination against BH$_3$-AZTTP by the Q151M complex, RT, and no in vitro resistance either. As a consequence, the resistance of Q151M RT is suppressed when a BH$_3$ group is present on the $\alpha$-phosphate of AZTTP. The suppression of resistance is even more dramatic when BH$_3$-ddATP is used (Fig. 4B). The Q151M complex RT has a $k_{pol}$(BH$_3$-ddATP) of 28.5 s$^{-1}$, 74-fold higher than $k_{pol}$(ddATP), and 4-fold higher than $k_{pol}$(ddATP) of wild-type RT (Table II). This means that BH$_3$-ddATP is a better substrate for Q151M complex RT than dTTP, dCTP, and AZTTP for wild-type RT. The effects of BH$_3$ groups on $k_{pol}$ values are summarized graphically in Fig. 4C. A structural and mechanistic interpretation of this rate enhancement has been presented previously (14) and holds in the case of Q151M RT (see “Discussion”).

**A Model for Q151M-based Multidrug Resistance**—We used the crystal structure of Huang et al. (17) to understand our results at a molecular level. In this structure, glutamine 151 is located into the dNTP binding pocket of the RT active site. Its side chain carbonyl interacts with the 3'-OH of the incoming dTTP. This 3'-OH also interacts with one nonbridging oxygen of the $\beta$-phosphate. This particular internal bond is thought to be critical in the catalytic step of nucleotide incorporation, by facilitating the in-line attack of the 3'-OH of the primer onto the $\alpha$-phosphate of the incoming nucleotide (14, 25). Because dideoxynucleotides lack this 3'-OH, there is no internal bond with the oxygen in the $\beta$-phosphate. This lack of interaction may explain why ddNTPs are less efficient substrates for the incorporation than dNTPs, as observed with ddATP (Table I, part B, and Ref. 16).
Mechanism and Suppression of Q151M RT Drug Resistance

Pre-steady state kinetic constants of WT RT, Q151M RT, and Q151M_complex RT mutants for DNA templates

The resistance is determined by the ratio of selectivity WT RT/selectivitymutant.

| Table I | Pre-steady state kinetic constants of WT RT, Q151M RT, and Q151M_complex RT mutants for DNA templates |
|---------|---------------------------------------------------------------------------------------------------|
| A       |                                                                                                   |
| Nucleotide | WT RT | Q151M RT | Q151M_complex RT |
|----------|--------|----------|------------------|
|          | dTTP   | AZTTP    | dTTP             | AZTTP |
|          |        |          |                  |       |
| $k_{p}$ (μM)$^a$ | 17$^b$ | 7.1      | 14               | 18    |
| $k_{p}/K_{d}$ ($s^{-1}$)$^a$ | 13$^b$ | 13       | 17               | 4.3   |
| $k_{p}/K_{d}$ ($s^{-1}$ $μM^{-1}$) | 0.75   | 1.8      | 1.2              | 0.24  |
| Selectivity$^c$ | 0.4X   | 5X       | 12X              | 17X   |
| Resistance$^c$ |        |          |                  |       |
| B       |                                                                                                   |
| Nucleotide | WT RT | Q151M RT | Q151M_complex RT |
|----------|--------|----------|------------------|
|          | dATP   | ddATP    | dATP             | ddATP |
|          |        |          |                  |       |
| $k_{p}$ (μM)$^a$ | 7.5$^b$ | 8.0      | 15               | 3.9   |
| $k_{p}/K_{d}$ ($s^{-1}$)$^a$ | 50$^b$ | 7.2      | 50               | 0.69  |
| $k_{p}/K_{d}$ ($s^{-1}$ $μM^{-1}$) | 6.7    | 0.91     | 3.2              | 0.17  |
| Selectivity$^c$ | 7.4 X  | 18 X     | 2.4X             | 66 X  |
| Resistance$^c$ |        |          |                  |       |
| C       |                                                                                                   |
| Nucleotide | WT RT | Q151M RT | Q151M_complex RT |
|----------|--------|----------|------------------|
|          | dCTP   | ddCTP    | dCTP             | ddCTP |
|          |        |          |                  |       |
| $k_{p}$ (μM)$^a$ | 7.9$^b$ | 5.4      | 12               | 4.6   |
| $k_{p}/K_{d}$ ($s^{-1}$)$^a$ | 7.3$^b$ | 1.9      | 8.6              | 0.51  |
| $k_{p}/K_{d}$ ($s^{-1}$ $μM^{-1}$) | 0.93   | 0.34     | 0.69             | 0.11  |
| Selectivity$^c$ | 2.7X   | 6.3X     | 2.3X             | 6.4X  |
| Resistance$^c$ |        |          |                  |       |

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

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

$^c$ Selectivity and resistance were determined as described under “Results.” The selectivity is the ratio of $k_{p}/K_{d}$ (nucleotide analogue)/$k_{p}/K_{d}$ (nucleotide).

Modeling was performed using dTTP and ddTTP as nucleotides present in the RT active site for three reasons. First, dTTP is the nucleotide described in the ternary complex structure of Huang et al. (17). Second, ddTTP is also a thymine nucleotide. Third, as shown above, $k_{p}$ (dTTP) is the kinetic constant most relevant to resistance and with a value of 13 s$^{-1}$, it is between that of dCTP ($k_{p}$ (dCTP) = 7.3 s$^{-1}$) and ddATP ($k_{p}$ (ddATP) = 50 s$^{-1}$) for wild-type RT (Table I). An energy minimization (removing the crystallographic contribution) was run on the coordinates of the structure deposited by Huang et al. (17). The aim of this minimization was to validate our modeling procedure. We obtained a new model in which the side chains of two residues located in the vicinity of the dTTP, namely Arg-72 and Gln-151, adopt a slightly different conformation than in the original model. Two hypotheses can account for this. First, the small differences observed may be because of some constraints originating from the crystallographic term. Second, the crystal structure has been solved at a medium resolution (3.2 Å); this means that some imprecision and/or alternative conformation can appear for certain side chains, and that some constraints have been introduced during the refinement procedure. Because structure factors have not been deposited in the Protein Data Bank, it is not possible to calculate the electron density map and therefore check that a particular amino acid of the model can actually have one conformation only. The consequences of the medium resolution data is reflected, for instance, by the fact that the distance between the side chain oxygen atom of Gln-151 and the 3′-OH of the dTTP ribose differs by 0.7 Å between the 2 complexes of the asymmetric unit. In conclusion, despite the small differences between the model obtained after minimization and the coordinates deposited in the Protein Data Bank, the previously mentioned interactions between the incoming nucleotide and residues Arg-72 and Gln-151 are still present, which, with the previous considerations, validates our minimization and gives us a basis for molecular modeling (Fig. 5A).

We then introduced the Q151M substitution and performed a new minimization. The ribose moiety is subjected to a tiny movement toward Tyr-115, whereas both Arg-72 and Lys-65 are kept in the same conformation, showing that the Q151M substitution is compatible with the actual structure of the active site and that high amplitude movements are not necessary to accommodate this mutation (Fig. 5B). However, it is clear that the electrostatic interaction that previously existed (between the carbonyl of the Gln-151 side chain and the 3′-OH of the ribose) is absent. The positioning of the base moiety and the ribose therefore relies on (i) the hydrogen bond between the 3′-OH of the ribose moiety of the dTTP and the main chain nitrogen of Tyr-115 and (ii) hydrophobic interactions between the base moiety of the nucleotide with the template base, and the ribose of the nucleotide with the aromatic ring of Tyr-115. We conclude that the Q151M substitution removes one of the two hydrogen bonds in charge of positioning both the ribose and base of the dTTP.

We then replaced dTTP by ddTTP in the wild-type structure and performed a new minimization (Fig. 5C). The active site structure is similar to what was described in Fig. 5B. The absence of the interaction between the 3′-deoxy of the incoming nucleotide and the main chain nitrogen of Tyr-115 does not seem to affect the position of the nucleotide. Likewise, the latter does not change when the dTTP and the Gln-151 residue are simultaneously replaced by the ddTTP and methionine residue, respectively (Fig. 5D). However, there are no more electrostatic interactions involved in the positioning of the base
and ribose moieties of the nucleotide. The positioning of nucleo-
base and ribose moieties relies on hydrophobic interactions
only. We conclude that both the dideoxyribose moiety and the
leaving pyrophosphate group lack a stable positioning in the
Q151M RT active site (Fig. 5D), explaining why the catalytic
constant $k_{pol}$ is both selectively and severely altered, and why
a BH$_3$ group at the $\alpha$-phosphate suppresses resistance (see
"Discussion").

**DISCUSSION**

In the treatment of AIDS, the prolonged use of nucleoside
analogues selects drug-resistant and often multidrug-resistant
viruses. To optimize antiretroviral regimens, it is important to
identify mutations in the RT gene that promote drug resist-
ance. A view of the mechanisms by which drug-resistant RTs
escape DNA replication inhibition is emerging. Two types of
biochemical mechanisms of RT-mediated drug resistance have
been characterized so far. In the first type, the analogue is
incorporated efficiently in the nascent proviral DNA, but the
“mutant” or drug-resistant RT has an increased ability to un-
block the analogue-terminated DNA chain. The prototype
of this mechanism is that of AZT-resistance mutations (4). In
the second type, resistance occurs when drug-resistant RT exhibits
a biased selection against the analogue relative to its natural
counterpart. In this type of mechanism based on discrimina-
tion, either the nucleotide analogue could bind to RT with a
unfavorable affinity ($K_d$ effect) or binds with a favorable affinity
but is not incorporated into DNA with a favorable catalytic
rate ($k_{pol}$ effect, such as K65R and ddN (14)). Several of these
mechanisms can occur simultaneously. For example, the
M184V substitution may have both a $K_d$ (24) and a $k_{pol}$ (25)
effect toward 3TCTP. Likewise, the V75I substitution pro-
 motes both a discrimination of d4TTP and an increase in py-
rrophosphorolysis of the d4TMP-terminated DNA primer (26).        

In some instances, an additional mutation, termed suppress-
ive mutation, will re-sensitize a drug-resistant RT to another
drug. This phenomenon indicates that much is to be learned on
the interplay of drug resistance mechanisms that could be
useful in the rational combination of drugs. Coupling favorable
drug combinations observed in the clinic with mechanistic and
enzymatic data will ultimately lead to the rational synthesis of
second generation drugs eliciting no resistance or drugs active
against drug-resistant strains.

In this paper, we report the detailed mechanism of RT-
mediated multidrug resistance promoted by a set of substitu-
tions centered around Q151M. Although the most common AZT
resistance mechanism proceeds by primer unblocking mediated
by at least 4 mutations in the RT gene (the T215F/Y complex),
we show that the 5 substitutions present in the Q151M$_{\text{complex}}$
RT do not promote any increase in RT-mediated primer un-
blocking. Instead, our result shows that Q151M plays a central
role in promoting both AZTTP and ddNTP discrimination. In-
terestingly, Q151M alone promotes a much greater resistance
to AZTTP than to ddNTP, and the subsequent appearance of
the other mutations of the Q151M$_{\text{complex}}$ are at the benefit of
ddATP resistance almost exclusively.

Is our data at the molecular level in agreement with in vivo
studies? We have shown that Q151M RT exhibits a 12-fold
resistance to AZTTP. Using recombinant HIV-1$_{\text{NL4-3}}$ virus,
Maeda et al. (6) found a comparable 16-fold increase of the IC$_{50}$
because of the Q151M substitution. However, the 17-fold res-
istance of the Q151M$_{\text{complex}}$ RT in vitro is quite low compared
with the 100–200-fold increase of the IC$_{50}$ observed using re-
combinant viruses (6, 8). In the case of dideoxynucleotides, we
observed very similar results between our pre-steady state
experiments and the results described in Maeda et al. (6), i.e. a
2.4-fold resistance of purified Q151M RT to ddATP compared
with a 4-fold increase of the ddIC$_{50}$ for the Q151M$_{\text{complex}}$
 virus, and a 9-fold ddATP resistance of purified Q151M$_{\text{complex}}$
RT compared with a 9-fold increase in ddIC$_{50}$ for the
Q151M$_{\text{complex}}$ virus. A discrepancy is observed in the case of
ddCTP. The limited resistance (about 2.5-fold) of both resistant
enzymes differs from the 14–20-fold increase in IC$_{50}$ for
the Q151M$_{\text{complex}}$ Virus (6, 8). Thus, enzymatic data reflects better
low to medium viral resistance phenotypes than high level
resistance. Such an observation is also valid in the case of AZT
resistance where modest (3–20-fold) increases in primer un-
blocking translate into >100-fold resistance in vivo, indicating
that any mechanistic model still needs refinement in the case of
high level drug resistance.
Our results compare well with those of others obtained using steady-state kinetics (11). Using the latter type of experiments, the Q151M complex RT is 10-fold resistant to ddATP (9-fold in pre-steady state experiments as described here) and 14-fold resistant to AZTTP (14-fold in pre-steady state experiments as described here). Pre-steady state kinetic experiments allow us to pinpoint the precise step that is targeted by the drug-resistant RT. This step is the catalytic rate constant, and this knowledge is of importance in the elucidation of the mechanism as well as the design of bonified DNA synthesis inhibitors, as discussed below.

The mechanism of resistance described here is the third example of drug resistance provided by a selective reduction in the catalytic rate constant \( k_{\text{pol}} \) of incorporation of the analogue. TABLE II

| BH3-AZTTP | BH3-ddATP |
|----------|----------|
| \( K_d (\mu M)^a \) | 13 | 7.6 |
| \( k_{\text{pol}} (s^{-1})^a \) | 14 | 28 |
| \( k_{\text{pol}}/K_d (s^{-1} \mu M^{-1}) \) | 1.1 | 3.7 |
| Selectivity\(^b\) | 0.71 \( X(\text{dTTP}) \) | 0.62 \( X(\text{dATP}) \) |

\(^a\) \( K_d \) and \( k_{\text{pol}} \) were determined as described under “Experimental Procedures.” Standard deviations were <20%.

\(^b\) Selectivity is the ratio of \( [k_{\text{pol}}/K_d (\text{nucleotide analogue})] /[k_{\text{pol}}/K_d (\text{nucleotide})] \).

Our results compare well with those of others obtained using steady-state kinetics (11). Using the latter type of experiments, the Q151M complex RT is 10-fold resistant to ddATP (9-fold in pre-steady state experiments as described here) and 14-fold resistant to AZTTP (14-fold in pre-steady state experiments as described here). Pre-steady state kinetic experiments allow us to pinpoint the precise step that is targeted by the drug-resistant RT. This step is the catalytic rate constant, and this knowledge is of importance in the elucidation of the mechanism as well as the design of bonified DNA synthesis inhibitors, as discussed below.

The mechanism of resistance described here is the third example of drug resistance provided by a selective reduction in the catalytic rate constant \( k_{\text{pol}} \) of incorporation of the analogue.
into DNA. The first example of this type of resistance is that provided by M184V toward 3TCTP (25). In this case, the decreased catalytic rate is provided by poor alignment of the reactive centers for 3TCTP selectively. The second example is provided by K65R toward ddNTPs (14, 27). In this second example, the K65R substitution serves to destabilize the leaving pyrophosphate moiety of a ddNTP selectively. It is very interesting to note that in this third example of Q151M-mediated resistance, a novel manner of decreasing the k_{pol} is used at the RT active site. It is the absence of the hydrogen bond network involving the 3'-OH, Tyr-115, Met-151, and the leaving pyrophosphate group that is at the origin of the selective decrease of k_{pol}. The absence of this hydrogen bond network is consistent with the fact that Q151M shows a decreased unblocking activity. Indeed, it is easy to understand from Fig. 5D that a pyrophosphate donor moiety (coming either from PP_{i} or a NTP) would lack precise positioning at the Q151M RT active site to perform pyrophosphorylase, the reverse of the incorporation reaction. Q151M represents the first and only example of AZT resistance promoted by discrimination of AZTTP at the RT active site.

In light of these mechanistic interpretations as well as of the K65R RT-mediated ddNTP resistance mechanism (14), we can add support to the mechanism of the BH3-mediated suppression of drug resistance. This mechanism has been described in detail in the case of K65R RT and resistance to ddNTPs, into which the BH3 group shows identical suppressive effects (14). The presence of the α-(R_{g})BH3 group on the incoming nucleotide, be it 3'-deoxy or not, removes binding and/or alignment constraints on the leaving pyrophosphate group. The rate of elimination of the pyrophosphate is then largely dependent on the chemical properties of the BH3 group, and not on the vicinal amino acids side chains (such as Arg-72 (28)) nor on the presence of the 3’-OH. These results are in agreement with the fact that R72A RT is able to efficiently incorporate either BH3-AZTTP or BH3-ddATTP into DNA but not any non-BH3-nucleotide 5'-triphosphate (13). Together with their use as chemical probes for the polymerization mechanism, the BH3 analogues confirm here their general interest in the suppression of RT-mediated drug resistance at the enzyme level.

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