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Antiviral Research 66 (2005) 153–158
Antiviral Research
www.elsevier.com/locate/antiviral

Effects of HIV Q151M-associated multi-drug resistance mutations on the activities of (−)-β-D-1′,3′-dioxolan guanine

Joy Y. Feng [a,*], Florence Myrick [a], Boulbaba Selmi [b], Jérôme Deval [b], Bruno Canard [b], Katyna Borroto-Esoda [a]

[a] *Gilead Science, 4 University Place, 4611 University Drive, Durham, NC 27707, USA*
[b] *Centre National de la Recherche Scientifique and Universites d'Aix-Marseille I et II, UMR 6098, Architecture et Fonction des Macromolecules Biologiques, ESIL-Case 925, 163 Avenue de Luminy, 13288 Marseille cedex 9, France*

Received 30 September 2004; accepted 15 March 2005

Abstract

The multi-drug resistance HIV-1 genotype A62V/V75I/F77L/F116Y/Q151M is associated with resistance to many nucleoside reverse transcriptase inhibitors including AZT, ddI, ddC, d4T, abacavir, and 3TC. In this study, we evaluated the antiviral activity of (−)-β-D-1′,3′-dioxolan guanine (DXG) towards mutant HIV-1 containing V75I/F77L/F116Y/Q151M ($V75I_{complex}$) and A62V/V75I/F77L/F116Y/Q151M ($A62V_{complex}$) in MT-2 cells. We further investigated the mechanism of resistance by studying the incorporation of DXG 5′-triphosphate (DXG-TP) during DNA synthesis by mutant enzymes containing single mutations at Q151M or A62V, and the $V75I_{complex}$ and $A62V_{complex}$ using pre-steady state kinetic analysis. Our studies showed that mutant virus containing $V75I_{complex}$ and $A62V_{complex}$ were both more than 23-fold resistant to DXG, and this correlated with the 68- and 20-fold resistance changes observed in the enzymatic assay. Compared to the wild-type enzyme, DXG-TP was incorporated 39- and 21-fold less efficiently by the mutant enzyme containing $V75I_{complex}$ and $A62V_{complex}$, mainly due to decreases in the rate of incorporation. The A62V mutation significantly increased the rate of incorporation (k_{pol}) for both dGTP (3-fold) and DXG-TP (7.9-fold), while the binding affinity of A62V HIV-1 RT for DXG-TP was decreased 14-fold. At the enzyme level, the addition of the A62V mutation to V75I/F77L/F116Y/Q151M moderately (3.4-fold) reversed the resistance to DXG-TP.

Keywords: DAPD; Amdoxovir; DXG; Pre-steady state; HIV-1 reverse transcriptase; Multi-drug resistance; Q151M

1. Introduction

Ever since the outbreak the HIV epidemic over two decades ago, nucleoside reverse transcriptase inhibitors (NRTIs) have been key components of antiviral therapy. Once inside the cells, NRTIs are phosphorylated by cellular enzymes to their 5′-triphosphates in order to serve as alternative substrate inhibitors for HIV-1 reverse transcriptase (RT). These approved NRTIs are significantly diverse in their chemical structures, activation pathways and primary resistance profiles (Beach, 1998; Pillay et al., 2000; Stein and Moore, 2001). However, a constellation of A62V, V75I, F77L, F116Y, and Q151M has been shown to confer cross-resistance to most of the NRTIs. This set of mutation, called the Q151M complex or multi-drug resistance (MDR) mutations, was found in HIV-infected individuals receiving AZT plus ddI or ddC and other ddI-containing regiments (Kavlick et al., 1998; Schmit et al., 1996, 1998; Shafer et al., 1995; Shirasaka et al., 1993). MDR mutations resulted in high-level resistance to AZT, ddI, ddC, d4T, and abacavir (Deval et al., 2004; Kavlick et al.,

Abbreviations: AZT, 3′-azido-3′-deoxythymidine; AZT^R, AZT-resistant; ddC, 2′,3′-dideoxycytidine; ddI, 2′,3′-dideoxyinosine; d4T, 2′,3′-didehydro-3′-deoxythymidine; DAPD, (−)-β-D-2,6-diaminopurine dioxolane; DXG, (−)-β-D-1′,3′-dioxolane guanosine; D30/D45, DNA/DNA primer/template 30/45-mer; MP, 5′-monophosphate; MDR, multi-drug resistance; dNTP, 2′-deoxynucleoside 5′-triphosphate, a general term for the natural nucleoside 5′-triphosphates; ddNTP, 2′,3′-dideoxynucleoside 5′-triphosphate, a general term for the analog nucleoside 5′-triphosphates; RT, reverse transcriptase; 3TC, (−)β-L-2′,3′-dideoxy-3′-thiacytidine; TP, 5′-triphosphate; wt, wild-type

* Corresponding author. Tel.: +1 919 294 7647; fax: +1 919 294 7661.
E-mail address: joy.feng@gilead.com (J.Y. Feng).

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doi:10.1016/j.antiviral.2005.03.001

1998; Pillay et al., 2000; Schmit et al., 1998; Shirasaka et al., 1995), low cross-resistance to 3TC (Deval et al., 2004; Garcia-Lerma et al., 1999; Schmit et al., 1998), and no resistance to tenofovir (Deval et al., 2004; McColl and Miller, 2003). The prevalence of the MDR mutations in treatment-experienced patients appears low (<2%), however, it is expected to rise in the future and could pose a serious treat to the efficacy of anti-HIV therapy (Pillay et al., 2000).

The emergence and the role for each individual mutation in the Q151M complex in viral resistance have been the focus of many studies (Iversen et al., 1996; Shafer et al., 1994; Shirasaka et al., 1995; Ueno et al., 1995). The Q151M complex develops in a sequential and cumulative pattern over the course of a 2–4-year observation period. Q151M is the first mutation to develop and it confers partial resistance to some of the nucleoside analogs tested (AZT, ddI, ddC and d4T). With time, mutations at position F116Y and F77L develop, followed by emergence of two other mutations A62V and V75I. The V75I, F77L and F116Y mutations have little or no effect on drug susceptibility by themselves, but their co-occurrence with Q151M results in high-level resistance to many NRTIs. The biochemical properties of RT carrying all or a subset of the Q151M complex have also been studied in detail. Steady state kinetic analyses by Ueno et al. demonstrated that the Q151M complex mutations (mainly Q151M) altered substrate recognition by the HIV RT and accounted for the resistance observed in vitro (Ueno and Mitsuya, 1997; Ueno et al., 1995). Pre-steady state kinetic studies by Deval et al. (2002) revealed that Q151M and Q151M complex confer resistance to 2′,3′-dideoxynucleotide analogs mainly through decreased rates of incorporation during DNA synthesis, while having no effect on pyrophosphorolytic and ATP-mediated excision of the incorporated ddNMP.

Amdoxovir ((−)-β-D-2,6-diaminopurine dioxolane, DAPD) (Fig. 1) is a selective inhibitor of HIV-1 replication in vitro. DAPD is deaminated by adenosine deaminase to the guanosine analog dioxolane guanine (DXG), which is subsequently phosphorylated to the corresponding 5′-triphosphate (DXG-TP) (Furman et al., 2001). DXG-TP is a potent alternative substrate inhibitor of HIV-1 reverse transcriptase (Furman et al., 2001). The two primary mutations associated with DXG-resistance are K65R and L74V, which have been studied in detail by recombinant HIV cell culture assays and enzyme kinetic studies (Bazmi et al., 2000; Gu et al., 1999; Jeffrey et al., 2003). A pre-steady state kinetic analysis showed that HIV-1 RT containing the Q151M mutation (alone or with the K65R or K103N) is 7–23-fold resistant to DXG-TP (Jeffrey et al., 2003). In the current study, we evaluated the antiviral activity of DXG against recombinant HIV containing the V75I/77L/116Y/151M ($V75I_{complex}$) and A62V/75I/77L/116Y/151M ($A62V_{complex}$) mutations. We also studied the incorporation of dGTP and DXG-TP by mutant HIV-1 RTs containing $V75I_{complex}$ and $A62V_{complex}$ using pre-steady state kinetic analysis.

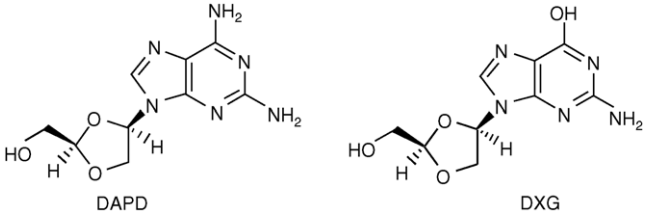

Fig. 1. Structure of DAPD and DXG.

2. Materials and methods

2.1. Construction, purification, and kinetic analysis

The wild-type RT gene construct p66RTB served as a template for directed mutagenesis using the QuikChange Kit (Stratagene, La Jolla, CA) to obtain the Q151M, $V75I_{complex}$, and $A62V_{complex}$ RTs. The recombinant RTs were co-expressed with HIV protease in *Escherichia coli* to get p66/p51 heterodimers and then purified by affinity chromatography as described (Boretto et al., 2001). All enzymes were quantified by active-site titration before biochemical studies.

2.2. Recombinant HIV-1 production and antiviral assay

The plasmid constructs described above were used as templates for the PCR amplification of the 1460-bp p66RTB fragment. The amplified RT genes were then cloned into a HIV-1_{LAI} backbone (Shi and Mellors, 1997). The resulting recombinant proviral clones were electroporated into MT-2 cells for preparation of viral stocks as previously described (Mewshaw et al., 2002). Genotypic analysis of viral isolates was performed using di-deoxy sequencing on the ABI-377 system. Recombinant viruses were analyzed for phenotypic sensitivity to DXG, abacavir, ddI, AZT, D4T, and 3TC on MT2 cells using the XTT colorimetric assay (Weislow et al., 1989). MT2 cells were infected with either the mutant virus or wild-type HIV-1_{LAI} at a multiplicity of infection of 0.03 in RPMI 1640 medium containing 10% fetal bovine serum, 20 μg of gentamicin/mL (Life Technologies), and 2 μg of polybrene/mL (Sigma) for 3 h at 37 °C. Following infection, cells were seeded into 96-well plates containing test compounds at 3×10^4 cells/well. Within each 96-well plate, test compounds were analyzed in triplicate at five-fold serial dilutions. The infected cells were cultured for 5 days in the presence of test compounds. On day 5, XTT {2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5[(phenylammino)carbonyl]-2*H*-tetrazolium hydroxide} was added, and the plates were incubated for 3 h at 37 °C and then analyzed for absorption (A_{450}). A dose-response curve for each individual compound was generated by using the absorption values of the uninfected cell controls as 100% protection and virus-infected cells not treated with drugs as 0% protection. From the dose-response curve, a 50% effective concentration (EC_{50}) was calculated and defined

as the concentration of drug that inhibited the virus-induced cytopathic effect by 50%.

2.3. Reagents

PAGE-purified DNA primer (30-mer, 5′-GCC TCG CAG CCG TCC AAC CAA CTC AAC CTC-3′) and DNA template (45-mer, 3′-CGG AGC GTC GGC AGG TTG GTT GAG TTG GAG CTA GGT TAC GGC AGG-5′) were obtained from Integrated DNA Technologies Inc. (Coralville, IA). ^{32}P-labeling of DNA 30-mer and annealing of DNA/DNA 30/45-mer primer/template (D30/D45) were conducted as previously reported (Feng and Anderson, 1999). The stock solution of dGTP was from Sigma. [γ–^{32}P]ATP was purchased from Amersham Biosciences. DXG-TP was synthesized by TriLink BioTechnologies (San Diego, CA) in the form of lithium salt. All other buffers and reagents were of analytical grade.

2.4. Pre-steady state kinetic for single nucleotide incorporation into DNA

Transient kinetic experiments were performed by the rapid quench method as described previously using a KinTek Instrument Model RQF-3 rapid-quench-flow apparatus (Kati et al., 1992). Unless noted otherwise, all concentrations referred to the final concentration after mixing. Under pre-steady state burst conditions, the reactions were carried out by mixing a solution containing the pre-incubated complex of HIV-1 RT (100 nM) and 5′-^{32}P-labeled D30/D45 primer/template duplex (300 nM) with a solution containing 10 mM $MgCl_2$ and various concentrations of the dNTP. When single-turnover conditions were used, the reactions were carried out by mixing a solution containing the pre-incubated complex of HIV-1 RT (200–250 nM) and 5′-^{32}P-labeled D30/D45 duplex (50 nM) with a solution of 10 mM $MgCl_2$ and various concentration of the dNTP. The reactions were quenched with 0.3 M EDTA at time intervals ranging from 3 ms to 2 min.

The products from each quench reaction were resolved by electrophoresis (14% acrylamide, 8 M urea) followed by phosphor imaging (Bio-Rad Personal Molecular Imager FX). Data were fitted by non-linear regression (KaleidaGraph 3.51). Under burst conditions, the product formation occurred in a fast exponential phase, followed by a slower linear phase. Data from burst experiments were fit to a burst equation:

$$[\text{Product}] = A[1 - \exp(-k_{\text{obsd}}t) + k_{\text{ss}}t]$$

A represents the amplitude of the burst that correlates with the concentration of enzyme in the active form, k_{obsd} is the observed first-order rate constant for dNMP incorporation, k_{ss} is the observed steady state rate constant, and t is the reaction time. Data from single-turnover experiments were fit to a single exponential equation:

$$[\text{Product}] = A[1 - \exp(-k_{\text{obsd}}t)]$$

The dissociation constant, K_{d}, for dNTP binding to the enzyme–DNA complex was calculated by fitting the data to the hyperbolic equation:

$$k_{\text{obsd}} = \frac{k_{\text{pol}}[\text{dNTP}]}{K_{\text{d}} + [\text{dNTP}]}$$

k_{pol} is the maximum rate of dNTP incorporation and [dNTP] is the corresponding concentration of dNTP.

3. Results

3.1. Virologic studies

In this study, we examined the antiviral activity of DXG against HIV-1 containing the multi-drug resistant mutation Q151M, along with the Q151M-containing V75I$_{\text{complex}}$ and A62V$_{\text{complex}}$. Fold changes in EC_{50} values for each of the mutant virus as compared to wild type were evaluated. Due to the variability inherent in these assays, changes in EC_{50} less than three-fold were considered to be insignificant. As shown in Table 1, the single mutation Q151M conferred low to moderate resistance to DXG as well as AZT and d4T. Addition of the V75I/77L/116Y mutations (V75I$_{\text{complex}}$) resulted in greater resistance to DXG and AZT while decreasing resistance to d4T. The virus containing the V75I$_{\text{complex}}$ was also resistant to abacavir, ddI, and 3TC. Addition of the A62V mutation to the V75I$_{\text{complex}}$ decreased the resistance to abacavir but increased resistance to d4T. No additional effects on the antiviral of DXG, AZT, ddI or 3TC were observed with addition of A62V to the V75I$_{\text{complex}}$, partially due to the experimental design.

Table 1
Drug susceptibility for wild-type and Q151M-containing mutant HIV-1

Mutation	EC_{50} (μM)[a] (fold changes from wild-type virus)					
	DXG	Abacavir	AZT	D4T	ddI	3TC
WT (xxLAI)	2.2 ± 0.9	2.5 ± 1.3	0.5 ± 0.4	5.6 ± 2.8	12 ± 8	2.6 ± 1.5
Q151M	21 ± 4 (9.6)	5.5 ± 3.7 (2.2)	2.4 ± 0.3 (4.8)	26 ± 6 (4.6)	32 ± 3 (2.7)	3.7 ± 2.6 (1.2)
V75I$_{\text{complex}}$	>50 (>23)	>20 (>8)	>10 (>20)	18 ± 2 (3.2)	>100 (>8.3)	25 ± 3 (8.3)
A62V$_{\text{complex}}$	>50 (>23)	7.0 ± 0.6 (2.8)	>10 (>20)	>50 (>8.9)	>100 (>8.3)	29 ± 12 (9.7)

[a] Values are mean ± standard deviation from at least three experiments.

Table 2
Kinetic differences between wild-type and mutant HIV-1 RT in DNA-dependent incorporation of dGTP or DXG-TP

HIV-1 RT	dGTP			DXG-TP			Selectivity factor[a]	Resistance fold changes[b]
	k_{pol} (s^{-1})	K_d (μM)	k_{pol}/K_d ($\mu M^{-1}\ s^{-1}$)	k_{pol} (s^{-1})	K_d (μM)	k_{pol}/K_d ($\mu M^{-1}\ s^{-1}$)		
WT	15.9 ± 0.5[c]	6.1 ± 0.9	2.6 ± 0.4[d]	1.46 ± 0.03	2.5 ± 0.2	0.58 ± 0.05	4.5 ± 0.8[d]	1
Q151M[e]	26 ± 2	6.8 ± 1.8	3.8 ± 1.0	0.34 ± 0.02	2.9 ± 0.4	0.12 ± 0.02	33 ± 10	7
A62V	48 ± 2	8.1 ± 1.1	5.9 ± 0.8	11.6 ± 0.5	36 ± 4	0.32 ± 0.04	18 ± 3	4
$V75I_{complex}$	42 ± 2	9.2 ± 1.2	4.6 ± 0.6	0.066 ± 0.004	4.4 ± 0.7	0.015 ± 0.003	300 ± 70	68
$A62V_{complex}$	60 ± 6	25 ± 5	2.4 ± 0.5	0.090 ± 0.009	3.3 ± 0.8	0.027 ± 0.007	90 ± 30	20

[a] Defined as $(k_{pol}/K_d)_{dGTP}/(k_{pol}/K_d)_{DXG\text{-}TP}$.
[b] Defined as $(\text{selectivity factor})_{\text{mutant RT}}/(\text{selectivity factor})_{\text{wild-type RT}}$.
[c] Values are average ± standard error.
[d] Errors were calculated using standard methods (Harris, 1995).
[e] From Jeffrey et al. (2003).

3.2. Enzymatic studies

Detailed mechanistic studies of the DNA-dependent incorporation of DXG-MP by wt and mutant HIV-1 RTs were conducted using pre-steady state kinetic analysis. The results are summarized in Table 2. The k_{pol} value represents the maximum rate of incorporation at the saturating concentration of a dNTP analog. The K_d value represents the nucleotide concentration that is required to reach half of k_{pol}. Therefore, a lower value of K_d indicates a tighter binding affinity of the enzyme to the dNTP analog. The ratio of k_{pol}/K_d is defined as incorporation efficiency and a higher value of k_{pol}/K_d for a dNTP analog indicates that it is a better substrate for HIV-1 RT.

Mutant enzymes containing single mutations at Q151M or A62V, and the $V75I_{complex}$ and $A62V_{complex}$ incorporated the natural substrate dGTP at 1.6-, 3.0-, 2.6-, and 3.8-fold higher rates, respectively, than the wt RT. However, the binding affinity of the Q151M RT for dGTP was similar to wild-type enzyme, while the mutant enzymes containing A62V, $V75I_{complex}$, and $A62V_{complex}$ had 1.3-, 1.5-, and 4.1-fold weaker binding affinity, respectively, as compared to wt enzyme. These results were similar to the increased k_{cat} and K_m values reported for dGTP incorporation into a homopolymeric primer/template by mutant RT containing $V75I_{complex}$ and $A62V_{complex}$ (Ueno et al., 1995). Our data showed that dGTP was incorporated by the mutant enzymes at efficiencies that were either similar or slightly higher (up to 2.3-fold) than wt RT, which is consistent with the report that the incorporation efficiency of dTTP, dATP and dCTP were not affected by Q151M and $Q151M_{complex}$ (Deval et al., 2002, 2004). In contrast, DXG-TP was incorporated 4.8-, 2.5-, 39-, and 21-fold slower by the mutant enzymes Q151M, A62V, $V75I_{complex}$, and $A62V_{complex}$, respectively, than the wt enzyme. The binding affinities of mutant enzymes for DXG-TP were similar to that of wt enzyme, with the exception of A62V, which bound DXG-TP 14-fold less tightly than the wild-type (wt). Among the mutant enzymes, A62V was the only one that incorporated DXG-TP as efficiently as the wt enzyme. DXG-TP was incorporated 4.8-, 39-, and 21-fold less efficiently than the wt RT by mutant enzymes Q151M, $V75I_{complex}$, and $A62V_{complex}$, respectively.

The "selectivity factor" is defined by the ratio of the dGTP's k_{pol}/K_d value to the DXG-TP's k_{pol}/K_d value. The higher the selectivity factor, the greater the discrimination between the natural substrate and the nucleoside analog. In this study, the selectivity factor of the wt HIV-1 RT for DXG-TP was only 4.5, indicating that DXG-TP is a good substrate for HIV-RT relative to the natural substrate. In contrast, the selectivity factors were 33, 18, 300, and 90 for mutant enzymes Q151M, A62V, $V75I_{complex}$, and $A62V_{complex}$, indicating an increased preference for the natural substrate by the mutant HIV-1 RT. The overall "resistance fold change" for the RTs is defined as the ratio between the selectivity factors of the mutant enzymes and the selectivity factor of the wt enzyme. A resistance factor that is >1 indicates that the mutant enzyme becomes even more discriminating against the incorporation of DXG-TP versus the incorporation of the natural substrate dGTP. This enzymatic study demonstrated that the mutant enzymes with Q151M, A62V, $V75I_{complex}$, and $A62V_{complex}$ were 7-, 4-, 68-, and 20-fold resistant to DXG-TP.

4. Discussion

DXG showed favorable activities against virus harboring HIV-1 RT mutations associated with resistance to AZT (M41L/D67N/K70R/T215Y/K219Q), 3TC (M184V), abacavir (M41L/D67N, M184V/L210W/T215Y) and efavirenz (K103N) (Mewshaw et al., 2002). MDR has not been known to be associated with DXG-resistance in the clinical studies of DAPD. However, due the NRTI nature of the compound and the increasing prevalence of MDR, there is a need to study the antiviral activity of DXG for mutant viruses containing Q151M and Q151M-associated mutations (Pillay et al., 2000). Our in vitro studies showed that mutant HIV-1 containing either $V75I_{complex}$ or $A62V_{complex}$ was highly resistant to DXG. The results on other NRTIs were generally consistent with previously reported values (Deval et al., 2004; Garcia-Lerma et al., 1999; Iversen et al., 1996; Shirasaka et

al., 1995). Addition of the three or four mutations to the background of Q151M inevitably led to higher resistance to all of the NRTIs tested.

At the HIV-1 RT enzyme level, the Q151M, V75I$_{complex}$, and A62V$_{complex}$ mutations made the enzyme 7-, 68-, and 20-fold more resistant to DXG-TP, mainly due to a decrease in the rate of incorporation for DXG-TP. This was similar to the Q151M-associated resistance for AZT-TP, ddATP, and ddCTP reported previously (Deval et al., 2002). An earlier steady state study showed that the A62V mutant RT was up to two-fold more sensitive to ddCTP and AZT-TP than the wt enzyme (Ueno et al., 1995). Furthermore, the addition of the A62V to a background of V75I/77L/116Y/151M led to a nearly two-fold decrease in IC_{50} value for ddATP, indicating a re-sensitization effect by A62V. In this study, we found some very interesting properties associated with the A62V mutation. First, the A62V mutant RT incorporated DXG-TP eight-fold *faster* than the wt RT, which was unusual for 2′,3′-dideoxynucleotide analogs. Secondly, the incorporation efficiency of natural substrate dGTP by mutant A62V RT was two-fold higher than wt RT, solely due to an increase in k_{pol}. Thirdly, the addition of A62V to V75I/77L/116Y/151M enabled the RT to be less discriminating against DXG-TP, as seen by a 3.4-fold ($p < 0.01$) decrease in the resistance fold changes. This A62V-associated re-sensitization effect was only seen with abacavir in our in vitro studies, partially because some of the EC_{50} values of mutant viruses were roughly estimated based on the highest drug concentration tested. Nevertheless, our data were in agreement with the in vitro drug susceptibility studies for mutant HIV-1 to AZT, ddI and ddC published by Maeda et al. (1998). In their study, the drug resistance was HIV-$1_{75/77/116/151}$ > HIV-$1_{62/75/77/116/151}$ > HIV-1_{151}, with 1.5 (AZT), 3.3 (ddI), and 3.9-fold (ddC) decrease in EC_{50} values when A62V was added to a 75I/77L/116Y/151M background. It is likely that the extent of the re-sensitization effect of A62V varies between different NRTIs and is due to their different structure contacts with the enzyme with the Val substitution at A62. It is worthwhile pointing out that those mutations at A62V and V75I complex, which both caused resistance to DXG-TP individually, seemingly had an antagonistic effect on the resistance fold change. This was different from the re-sensitization effect of mutation K103N on DXG-TP incorporation by K65R, Q151M and K65R/Q151M mutant RTs, where K103N was slightly more sensitive to DXG-TP than the wt RT (Jeffrey et al., 2003).

In this study, natural dGTP was incorporated by the mutant enzymes as efficiently as the wt RT. Even though this is hardly indicative of the viral replication capacity in vitro, it is consistent with the report that mutant viruses containing Q151M, V75I$_{complex}$, and A62V$_{complex}$ replicate at rates comparable to wt virus in the absence of drugs (Maeda et al., 1998). The benefit for virus to acquire 62V has been shown by the increase of replication competence over V75I$_{complex}$ in the presence of AZT and ddI, at the cost of some loss in resistance (Kosalaraksa et al., 1999; Maeda et al., 1998). While our pre-steady state data on dGTP and DXG-TP incorporation do not directly support this observation, a study of the incorporation of dTTP/AZT-TP and dATP/ddATP (the active form of ddI) by the mutant RTs could be more relevant. In conclusion, our study demonstrated the mechanism for Q151M-associated DXG resistance at the molecular level and showed that the Q151M-associated resistance to DXG was due to significant decreases in the rate of incorporation of DXG-TP by RTs containing Q151M, V75I/77L/116Y/151M and 62V/75I/77L/116Y/151M mutations. This altered substrate discrimination at the enzyme level translated to a resistant phenotype for the corresponding mutant virus in the in vitro evaluations.

Acknowledgement

We thank Andrew Bae for DNA sequencing of the recombinant viruses.

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