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Biochemical Studies on the Mechanism of Human Immunodeficiency Virus Type 1 Reverse Transcriptase Resistance to 1-(β-D-Dioxolane)Thymine Triphosphate▽

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A large panel of drug-resistant mutants of human immunodeficiency virus type 1 reverse transcriptase (RT) was used to study the mechanisms of resistance to 1-(β-D-dioxolane)thymine triphosphate (DOT-TP) and other nucleotide analogs. RT containing thymidine analog-associated mutations (TAM) or RT with a T69S-SG insertion in combination with TAM removed 3′-azido-3′-deoxythymidine-5′-monophosphate or tenofovir more efficiently than DOT-monophosphate from chain-terminated DNA primer/template through ATP-mediated pyrophosphorolysis. For non-ATP-dependent discrimination toward DOT-TP, high levels of resistance were found for RT bearing the Q151M mutation with family mutations, while RT bearing only the M184V or the Y115F mutation conferred no resistance to DOT-TP. A lower degree of resistance to DOT-TP than to tenofovir diphosphate or carbovir-TP was found for RT containing the K65R mutation. In the present studies, 1-(β-D-dioxolane)guanine triphosphate, another nucleotide with a dioxolane sugar moiety, showed a resistance profile similar to that of DOT-TP. The results suggest that DOT, compared with other approved nucleoside analogs, is overall more resilient to mutations such as TAM, M184V, and K65R, which are commonly found in viruses derived from subjects failing multinucleoside therapy.

Nucleoside analog reverse transcriptase inhibitors (NRTI) remain the central class of drugs included in highly active antiretroviral therapy (HAART) cocktails used in the treatment of human immunodeficiency virus type 1 (HIV-1) infections. NRTI in their metabolically activated form, NRTI-5′-triphosphate (NRTI-TP), inhibit HIV-1 reverse transcriptase (RT) viral synthesis by competing with the natural 2′-deoxy nucleosides-5′-triphosphates (dNTP) and/or by chain termination, since they lack the 3′-hydroxyl group (11). Drug resistance is the major limitation of NRTI treatment, as has been reported for all classes of HIV-1 inhibitors (16, 17, 31). Our present understanding is that NRTI resistance occurs by mechanisms requiring that mutant RTs discriminate against the NRTI better than wild-type RTs, either before or after the incorporation of NRTI into the primer strand. The latter mechanism, referred to as pyrophosphorolysis, excision, or primer unblocking, involves an enhanced removal of the incorporated NRTI-monophosphate (MP), e.g., 3′-azido-3′-deoxythymidine-5′-MP (AZT-MP), thus, freeing the primer strand for further elongation (1, 24, 25). ATP at a physiological concentration of 3.2 mM (36) is considered the major pyrophosphate donor in cells for this mechanism (20, 24). The mutations in HIV-1 RT generally associated with this mechanism are M41L, D67N, T70R, L210W, T215Y/F, and/or K219Q, where T215Y is the key mutation. These mutations were first found in individuals treated with AZT or 2′,3′-dideoxy-2′,3′-didehydrothymidine (d4T) and are termed thymidine analog-associated mutations (TAM). The hypothesis is that the side chain switch of residue 215 threonine to tyrosine or phenylalanine improves the position of the ATP molecule for the catalytic attack of the incorporated NRTI-MP. Thus, the NRTI-MP is more easily removed as a dinucleoside tetraphosphate product (5, 7, 23, 29). Enhanced primer unblocking of NRTI-MP can also be achieved with mutations at residue 69 in the HIV polymerase as a two-amino-acid insertion (19, 20, 23). In contrast to the primer unblocking mechanism, mutations such as M184V, K65R, and Q151M are involved in direct substrate discrimination before the incorporation of NRTI (18, 31, 33). In fact, some of these mutations have been reported to suppress primer unblocking (31).

In the decade since HAART was introduced, a wealth of data has accumulated by which trends in the prevalence of drug resistance mutations can be established. Virus samples from treated individuals experiencing virologic failure have demonstrated the following prevalence of NRTI mutations: M184V, 48%; T215Y/F (representing TAM), 45%; T69D, 6.4%; L74V, 6%; Q151M, 2.7%; K65R, 1.9%; Y115F, 1.1%; and T69S-XX insertions, 0.8% (Stanford HIV Drug Resistance Database, http://hivdb.stanford.edu). However, since protocols have been updated from previous regimens consisting of AZT/d4T with β-L-2′,3′-dideoxy-3′-thiacytidine or 2′,3′-dideoxyinosine to the more up-to-date regimens with tenofovir (TFV)-emtricitabine (Emtriva [FTC]) or β-L-2′,3′-dideoxy-3′-thiacytidine and abacavir, there has been a slight decrease in the frequency of mutations TAM, T69D, Q151M, and residue 69 insertions, while there is a minor trend toward higher frequencies of mutations M184V, K65R, and Y115F (R. W. Shafer, personal communications).

Hence, there is still a need for additional NRTI that, besides

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being potent and less toxic, have a greater genetic barrier to the current common resistance mutations. Moreover, such an NRTI should be suitable for the inclusion of future combination therapies. Acknowledging the need to replace the thymidine kinase (TK)-dependent AZT and d4T therapies, which can be toxic on prolonged use and not active against the frequent TAM, this report focuses on (−)-(2*R*,4*R*)-1-(2-hydroxymethyl-1,3-dioxolan-4-yl)thymine (also known as DOT), a potent and thymidine kinase-1 dependent NRTI (8, 30). The structure of DOT differs from that of other NRTI since it contains a dioxolane instead of a sugar moiety (8). Another dioxolane nucleoside, β-D-dioxolane-guanine (DXG), the deaminated form of the prodrug β-D-2,6-diaminopurine dioxolane (DAPD), has selective anti-HIV activity and is nontoxic to mammalian and human cells (12, 35). DXG was found in cell culture studies to be active against HIV-1 strains containing TAM and/or M184V mutations in the Pol gene product (15). These DXG resistance results support data obtained with DOT, using structure modeling (9).

The focus of this work was to probe the biochemical mechanisms of resistance to DOT-triphosphate (TP) using a novel nonradioactive polymerase assay. A panel of purified HIV-1 RT containing mutations (TAM and M184V) that have been commonly observed in clinical samples obtained from HIV-infected individuals treated with nucleoside analogs was used. In addition, RT with mutations that are involved in multidrug resistance (Q151M and T69S-SG insertion) or that are increasing in prevalence, although still at a low frequency (K65R and Y115F), were also studied. The overall objective was to elucidate the level of resistance to DOT-TP compared to those of other approved NRTI-TP and DXG-TP for a broad range of RT mutants and to study the mechanism of resistance to these analogs.

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MATERIALS AND METHODS

Site-directed mutagenesis and expression and purification of HIV-1 RT. Site-directed mutagenesis of the RT from different HIV-1 wild-type backgrounds (Table 1) were generated using a QuikChange method (Stratagene), but different sources of expression vectors and purification methods were used. The first eight RT described in Table 1 were prepared using the isolate HXB2(a) background based on the pKK233-2 expression vector (kindly provided by D. K. Stammers) and were expressed and purified to heterodimer p66/p51 as previously described, using anion and cation exchange chromatography (20, 34). A C-terminal histidine-tagged expression vector similar to p6HRT-PROT based on the expression vector pT5m (kindly provided by S. H. Hughes) was used to express the HXB2(b) and 41L/67N/70R/215Y/219Q mutant RTs. The plasmid simultaneously expressed HIV-1 RT p66 and HIV-1 protease and thereby expresses both the RT p66 and p51 forms, as p66 is processed by the protease. A HiTrap chelating Ni^{2+} column (Amersham/GE Health Care) purification method was used (3). The same expression vector and purification method were also used to clone and purify the pNL4-3 wild-type RT and the Y115F mutant. The K65R RT and its equivalent wild-type isolate LAI RT were supplied as purified homodimer p66 form RT (a generous gift from P. Meyer, W. A. Scott, and J. W. Mellors). The purification of these N-terminal histidine-tagged products has previously been described (24). All of the RT products had a purity of >90% according to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. All the preparations, except for the K65R mutant and the LAI wild-type RTs, were in p66/51 heterodimeric form, with the p66 form at levels of 55 to 75% and the p51 at levels of 25 to 45%.

Heteropolymeric DNA polymerase assay. Two different nonradioactive RT assays were used, the DNA polymerase assay and the poly(rA)/oligo(dT) (prA/odT) assay, to study the NRTI-TP inhibition of the purified RT mutants. Separate kit components such as covalently linked DNA microtiter plates or prA plates (from a high-sensitivity type RT assay) and tracer solution (alkaline phosphate [AP]-conjugated anti-bromodeoxyuridine [BrdU] antibody) were obtained from Cavidi Tech, Uppsala, Sweden.

The DNA polymerase assay is based on a DNA primer with a specific sequence that is covalently bound to the well of the 96-well microtiter plate; an 18-base 5′-GTC-CCT-GTT-CCG-GCG-CCA-3′ sequence linked at the 5′ end to the primary amine by a C6 spacer arm. The amount of primer bound was approximately 10 ng/well (110 nM). The RT assay reaction mixture (total volume 150 μl/well) contained HEPES 50 mM (pH 7.3); $MgCl_2$, 10 mM; Triton X-100, 0.5%; bovine serum albumin, 0.1 mg/ml; dATP, dGTP, dCTP, and 5-bromo-2′-deoxyuridine-5′-triphosphate (BrdUTP), 1.0 μM each (where BrdUTP replaces TTP) (all from Sigma); and a 50-base DNA template (5′-AAA-AAA-AAA-AAA-GTC-AGT-CAG-TCA-GTC-AGT-CAT-GGC-GCC-CGA-ACA-GGG-AC-3′), 50 ng/well (190 nM) (i.e., a 5′-A_{12}-3′ tail with a 5′-$(GTCA)_5$-3′ repeat as template) (Integrated DNA Technologies). The reason for positioning the pdA_{12} region at the template 5′ end was to have a variable sequence before the major signal for product detection with tracer antibody. To obtain a significant ATP primer unblocking reaction in the assay, the ATP (Amersham/GE Health Care) concentration was set to 5 mM. We found the ATP effect for the 41L/67N/70R/210W/215Y mutant resistance to AZT-TP and TFV-diphosphate (DP) to vary in a linear fashion between 0 and 6 mM and to plateau with maximum ATP-dependent resistance at approximately 8 mM ATP (data not shown).

The reaction was started with the addition of RT in the 30-to-100-pM range. The recombinant HIV-1 polymerases had an activity within 1,500 to 3,000 U/mg as defined by the standard Lenti RT assay (Cavidi Tech) (10). To achieve reproducible kinetics between wild-type and mutant RTs, approximately 1.5 to 4.5 mU/well of each enzyme was used. It should be noted that a 10-fold broader range had very limited influence on the 50% inhibitory concentration (IC_{50}) values for the NRTI-TP (data not shown). In order to further improve the comparison between wild-type and mutant RTs, the same sources of vectors, purification methods, and p66/p51 dimerization were used as described in Table 1. The RT reaction mixture was incubated at 33°C for 180 min and terminated by a water wash of the plates. Since the anti-BrdU antibodies (tracer AP-conjugated) bound more favorably to incorporated BrdUMP as a single-strand rather than a double-strand DNA product, incubation with 2 M NaOH (3 times 200 μl/well for 5 min) was performed to dehybridize the DNA template. After a thorough wash with water, tracer was added and incubated for 90 min at 33°C. The detection step for color absorbance at 405 nm was performed as previously described (20). The level of resistance to NRTI-TP by the various RT mutants compared to that of corresponding wild-type RT was determined as IC_{50} values of RT activity (Table 1 and Table 3). Resistance values (*n*-fold) were determined by dividing the IC_{50} for the mutant by the IC_{50} for the respective wild type. Steady-state kinetics could be assumed since the RT reaction was linear for more than 8 h (data not shown).

The NRTI-TP used were DOT-TP, DXG-TP, AZT-TP, carbovir (CBV)-TP (the active metabolite of abacavir), and TFV-DP. AZT-TP and TFV-DP were kindly provided by Cavidi Tech. The other nucleotides were synthesized in our laboratory using a rapid method from the corresponding nucleoside analog (21). Purity (>92%) was confirmed by high-performance liquid chromatography and mass spectrometry.

prA/odT primer unblocking assay. The prA/odT assay, previously described (19, 20, 32), was used to further investigate the ATP-dependent primer unblocking for the RT mutants containing TAM and residue 69 insertion. This assay is limited to thymidine analogs, as prA_{300} was used as the RNA template, and thereby only DOT-TP and AZT-TP were studied. In the reaction mixture, $odT_{12–18}$ (Sigma, St. Louis, MO) was added as primer together with BrdUTP (used as the only dNTP). Besides these changes, the same reaction buffer was used as for the DNA polymerase assay, together with the same procedure and assay time, but the NaOH step was omitted. The concentration of $odT_{12–18}$ was 0.22 ng/well (0.2 nM), since this odT amount was on the plateau of the V_{max} curve (95% relative V_{max}) when was studied the K_m of odT (data not shown). A lower ATP concentration (3.2 mM) than in the DNA polymerase assay was used, since previous studies (19, 20) had indicated that this amount was enough to display sufficient primer unblocking. The K_m (BrdUTP) and K_i (DOT-TP or AZT-TP) values were obtained by fitting the data to the Michaelis-Menten competitive inhibition equation using Grafit 5.0 (Erithacus Software, Horley Surrey, United Kingdom). The level of resistance by the RT mutant was expressed as an increase of the K_i/K_m value compared to the corresponding wild-type ratio. The concentration ranges of BrdUTP and DOT-TP or AZT-TP are described in Table 2. The RT amount used was 4 to 12 pM/well. The RT activity

TABLE 1. Resistance to NRTI-TP of RT mutants by the DNA polymerase assay[a]

Mutations in respective wild-type background	NRTI-TP used[b]									
	DOT-TP		AZT-TP		DXG-TP		CBV-TP		TFV-DP	
	IC_{50} ± SE	Fold increase	IC_{50} ± SE	Fold increase	IC_{50} ± SE	Fold increase	IC_{50} ± SE	Fold increase	IC_{50} ± SE	Fold increase
Assay with ATP										
Wild-type HXB2(a)	0.38 ± 0.04	1.0	0.46 ± 0.05	1.0	0.15 ± 0.03	1.0	1.5 ± 0.6	1.0	3.3 ± 0.2	1.0
184V	0.5 ± 0.05	1.3	0.52 ± 0.1	1.1	0.2 ± 0.04	1.3	8.4 ± 2[c]	5.6	2.3 ± 0.2[c]	0.7
41L/67N/70R/210W/215Y	0.5 ± 0.2	1.3	2.3 ± 0.7[c]	5.0	0.12 ± 0.03	0.8	1.5 ± 0.1	1.0	13 ± 2[c]	3.7
41L/67N/70R/184V/210W/215Y	0.7 ± 0.03[c]	1.8	0.9 ± 0.3	2.0	0.19 ± 0.05	1.3	4.5 ± 1[c]	3.0	5.9 ± 0.7[c]	1.8
41L/69S-SG/210W/215Y	1.6 ± 0.2[c]	4.2	4.4 ± 1.2[c]	9.6	0.34 ± 0.04[c]	2.4	5.1 ± 0.05[c]	3.4	24 ± 5[c]	7.3
41L/69S-SG/184V/210W/215Y	1.4 ± 0.6[c]	3.7	1.2 ± 0.8	2.6	0.26 ± 0.02[c]	1.6	6.4 ± 0.9[c]	4.3	ND	ND
75I/77L/116Y/151M	6.1 ± 2[c]	16	9 ± 1[c]	20	1.2 ± 0.5[c]	10	11 ± 2[c]	7.3	11 ± 2[c]	3.3
75I/77L/116Y/151M/184V	15 ± 6[c]	39	22 ± 3[c]	48	2.4 ± 0.05[c]	18	18 ± 7[c]	12	4.6 ± 0.9	1.4
Wild-type HXB2(b)	0.38 ± 0.02	1.0	0.48 ± 0.1	1.0	ND	ND	ND	ND	3.2 ± 0.3	1.0
41L/67N/70R/215Y/219Q[d]	0.84 ± 0.1[c]	2.2	2.5 ± 0.2[c]	5.2	ND	ND	ND	ND	10.5 ± 2[c]	3.3
Wild-type LAI	0.19 ± 0.01	1.0	0.3 ± 0.06	1.0	0.2 ± 0.01	1.0	0.9 ± 0.5	1.0	1.3 ± 0.2	1.0
65R[d]	0.75 ± 0.2[c]	3.9	0.61 ± 0.1[c]	2.0	1.3 ± 0[c]	6.5	5.5 ± 2[c]	6.1	21 ± 4[c]	16
Wild-type pNL4-3	0.3 ± 0.03	1.0	0.42 ± 0.1	1.0	ND	ND	1.8 ± 0.1	1.0	4 ± 0.5	1.0
115F[d]	0.2 ± 0.05[c]	0.6	0.55 ± 0.3	1.3	ND	ND	3.6 ± 0[c]	2.0	3.7 ± 0.7	0.9
Assay without ATP										
Wild-type HXB2(a)	0.11 ± 0.05	1.0	0.18 ± 0.02	1.0	0.12 ± 0.02	1.0	1.1 ± 0.4	1.0	1.6 ± 0.3	1.0
184V	0.18 ± 0.06	1.6	0.25 ± 0.1	1.4	0.2 ± 0.05	1.7	5.5 ± 0.6[c]	5.0	2.2 ± 0.2[c]	1.4
41L/67N/70R/210W/215Y	0.12 ± 0.04	1.0	0.14 ± 0.03	0.8	0.11 ± 0.02	0.9	0.9 ± 0.02	0.8	1.4 ± 0.3	0.9
41L/67N/70R/184V/210W/215Y	0.16 ± 0.03	1.5	0.34 ± 0.1[c]	1.9	0.14 ± 0.01	1.2	2.7 ± 0.1[c]	2.5	1.4 ± 0.1	0.9
41L/69S-SG/210W/215Y	0.11 ± 0.01	1.0	0.16 ± 0.02	0.9	0.15 ± 0.01	1.3	0.9 ± 0.3	0.8	3.7 ± 0.4[c]	2.3
41L/69S-SG/184V/210W/215Y	0.22 ± 0.03[c]	2.0	0.21 ± 0.04	1.2	0.14 ± 0.04	1.2	3.4 ± 0.04[c]	3.1	ND	ND
75I/77L/116Y/151M	2.1 ± 1[c]	19	5.1 ± 0.6[c]	28	2.5 ± 1[c]	21	6.6 ± 3[c]	6.0	8 ± 1[c]	5.0
75I/77L/116Y/151M/184V	7.1 ± 2[c]	65	10.6 ± 2[c]	59	7.2 ± 2[c]	60	54 ± 5[c]	49	2.9 ± 0.3[c]	1.8
Wild-type HXB2(b)	0.12 ± 0.1	1.0	0.20 ± 0.04	1.0	ND	ND	ND	ND	ND	ND
41L/67N/70R/215Y/219Q[d]	0.13 ± 0.02	1.1	0.19 ± 0.03	1.0	ND	ND	ND	ND	ND	ND
Wild-type LAI	0.11 ± 0.02	1.0	0.08 ± 0.01	1.0	0.18 ± 0.01	1.0	0.68 ± 0.2	1.0	0.55 ± 0.1	1.0
65R[d]	0.46 ± 0.2[c]	4.2	0.35 ± 0.1[c]	4.3	0.62 ± 0[c]	3.4	5 ± 0.6[c]	7.4	12 ± 0.4[c]	22
Wild-type pNL4-3	0.10 ± 0.01	1.0	0.13 ± 0.01	1.0	ND	ND	1.2 ± 0.2	1.0	1.2 ± 0.05	1.0
115F[d]	0.08 ± 0[c]	0.8	0.13 ± 0.02	1.0	ND	ND	2.6 ± 0.4[c]	2.2	1.1 ± 0.1	0.9

[a] Resistance of RT mutants to NRTI-TP was measured with the DNA polymerase assay of RT, with and without ATP (5 mM).
[b] The IC_{50} values are expressed as μM of NRTI-TP. The IC_{50} are averages from at least two separate experiments conducted in duplicate. The IC_{50} values were determined using seven different concentrations of NRTI-TP adjusted optimally for each mutant's expected IC_{50} value. Standard errors (± SE) are indicated. ND, not determined. Fold increase was calculated by dividing the mutant RT IC_{50} values by the respective wild-type IC_{50} values.
[c] Differs statistically from matched wild-type IC_{50} ($P < 0.05$).
[d] Mutations 41L/67N/70R/215Y/219Q, 65R, and 115F in HXB2(b), LAI, and pNL4-3 background, respectively.

TABLE 2. Resistance of RT mutants to DOT-TP and AZT-TP measured with the prA/odT assay[a]

Mutations in respective wild-type background	NRTI-TP[b]							
	DOT-TP				AZT-TP			
	K_i ± SE	K_m ± SE	K_i/K_m	Fold increase	K_i ± SE	K_m ± SE	K_i/K_m	Fold increase
Wild-type HXB2(a)	0.0028 ± 0.001	0.32 ± 0.04	0.0088	1.0	0.0054 ± 0.0008	0.31 ± 0.06	0.017	1.0
41L/67N/70R/210W/215Y	0.013 ± 0.001	0.51 ± 0.1	0.025	2.8	0.13 ± 0.04	0.43 ± 0.04	0.3	17.6
41L/69S-SG/210W/215Y	0.059 ± 0.007	0.5 ± 0.03	0.12	13.6	0.33 ± 0.008	0.53 ± 0.04	0.62	36.5
Wild-type HXB2(b)	0.0018 ± 0.0005	0.24 ± 0.03	0.0075	1.0	0.004 ± 0.0004	0.29 ± 0.004	0.014	1.0
41L/67N/70R/215Y/219Q[c]	0.011 ± 0.002	0.42 ± 0.04	0.026	3.5	0.095 ± 0.02	0.49 ± 0.03	0.19	13.6

[a] Resistance of RT mutants to DOT-TP and AZT-TP relative to that of the wild type, measured with the prA/odT assay, with 3.2 mM ATP.
[b] The K_i values are expressed in μM of DOT-TP or AZT-TP and K_m values as μM of BrdUTP. All values are the averages calculated from at least two separate experiments conducted in duplicate. Standard errors (SE) are indicated. Fold increase values were calculated as K_i/K_m values relative to those of the wild-type corresponding ratio. The K_m values were determined by using six different concentrations of BrdUTP, from 0.074 μM to 16 μM, in approximately threefold increments. The K_i values were determined for DOT-TP/AZT-TP by using four different concentrations adjusted optimally for each mutant's expected K_i value.
[c] In the HXB2(b) background, from a different vector and purification method.

was linear during the assay time within the substrate range used, and thus, steady-state kinetics were assumed.

Statistical analyses. Average IC_{50} values in Table 1 and Table 3 were calculated using data that contained between two and four separate experiments, conducted in duplicate. The IC_{50} values of mutants were compared with the matched wild-type IC_{50} values, using at least four observations (pooled data). Outcomes were considered to differ statistically from matched wild-type IC_{50} values if P values of <0.05 were calculated based on the two-tailed Student's t test with equal variances.

RESULTS

A panel of 10 site-directed RT mutants was studied with the DNA polymerase assay. The novel inhibitors DOT-TP and DXG-TP (as T and G analogs) were compared to the antiviral NRTI-TP of currently approved drugs such as AZT-TP, CBV-TP, and TFV-DP (as T, G, and A analogs). The results are described below and are summarized in Table 1. The substrate discrimination effect was measured in assays without ATP and the primer unblocking effect by adding ATP in the RT assay. Hence, it is the combined resistance effect of substrate discrimination and primer unblocking, i.e., measured in the presence of ATP, that balance out to the final data related to the resistance level observed.

Mutations involved in ATP-dependent primer unblocking. The levels of resistance of the 41/67/70/210/215 mutant in the HXB2(a) background and the 41/67/70/215/219 mutant in the HXB2(b) background were determined. As expected for these TAM mutants, increased resistance to NRTI was found only in the presence of ATP (Table 1). Thus, in the absence of ATP, these mutants behaved like the wild type, i.e., they were involved only in primer unblocking and not in substrate discrimination (Table 1). When ATP (5 mM) was added to the DNA polymerase assay, the order of fold increase for the two TAM mutants was AZT-TP (5.0- and 5.2-fold, respectively) > TFV-DP (3.7- and 3.3-fold, respectively) > DOT-TP (1.3- and 2.2-fold, respectively) > CBV-TP (1.0-fold) > DXG-TP (0.8-fold) (Table 1). In addition, the 41/67/70/210/215 mutant with d4T-TP resulted in a 4.1-fold level of resistance (data not shown). The same order of increase of the NRTI-TP was found with the 41/69S-SG/210/215 mutant enzyme. Addition of the residue 69 insertion gave a more pronounced primer unblocking for all analogs, which is in agreement with previous studies (4, 19, 20, 23).

It is known that incorporation of a dNTP to the next nucleotide position on the template can prevent primer unblocking

TABLE 3. The DNA polymerase assay with 5 mM ATP and with modification of dNTP concentration or template sequence

Mutations in wild type background	Assay modifications of dNTP concn or template sequence	NRTI-TP[a]					
		DOT-TP		TFV-DP		AZT-TP	
		IC_{50} ± SE	Fold increase	IC_{50} ± SE	Fold increase	IC_{50} ± SE	Fold increase
Wild-type HXB2(a)	1 μM dNTP	0.38 ± 0.04	1.0	3.3 ± 0.2	1.0	0.46 ± 0.05	1.0
Wild-type HXB2(a)	20 μM dNTP	2.0 ± 0.1	1.0	19 ± 0.5	1.0	3.3 ± 0.7	1.0
41L/67N/70R/210W/215Y	1 μM dNTP	0.5 ± 0.2	1.3	13 ± 2[b]	3.7	2.3 ± 0.7[b]	5.0
41L/67N/70R/210W/215Y	20 μM dNTP	2.4 ± 0.3	1.2	49 ± 7[b]	2.6	16 ± 3[b]	4.8
Wild-type HXB2(a)	5′-A_{12}-$(GTCA)_5$-3′	0.38 ± 0.04	1.0	3.3 ± 0.2	1.0	0.46 ± 0.05	1.0
Wild-type HXB2(a)	5′-A_{12}-$(GACT)_5$-3′	0.31 ± 0.01	1.0	2.8 ± 0.3	1.0	ND	ND
41L/67N/70R/210W/215Y	5′-A_{12}-$(GTCA)_5$-3′	0.5 ± 0.2	1.3	13 ± 2[b]	3.7	2.3 ± 0.7[b]	5.0
41L/67N/70R/210W/215Y	5′-A_{12}-$(GACT)_5$-3′	0.4 ± 0.03[b]	1.3	8.6 ± 1[b]	3.1	ND	ND
41L/69S-SG/210W/215Y	5′-A_{12}-$(GTCA)_5$-3′	1.6 ± 0.2[b]	4.2	24 ± 5[b]	7.3	4.4 ± 1.2[b]	9.6
41L/69S-SG/210W/215Y	5′-A_{12}-$(GACT)_5$-3′	0.78 ± 0.1[b]	2.5	17 ± 1[b]	6.0	ND	ND

[a] Mean IC_{50} values ± standard errors (± SE) are expressed as μM from at least two separate experiments conducted in duplicate. ND, not determined. Fold increase was calculated by dividing the mutant RT IC_{50} values by respective wild-type IC_{50} values and assay modification.
[b] Differs statistically from matched wild-type IC_{50} ($P < 0.05$) values.

by a so-called dead-end-complex (DEC) (26). Therefore, we studied the 41/67/70/210/215 mutant by using higher dNTP concentrations (20 μM) and found a similar increase toward that of the wild-type level for DOT-TP and AZT-TP but a lower resistance (2.6-fold) for TFV-DP (Table 3).

It has been reported that the upstream template sequence can have an effect on ATP-dependent primer unblocking (22). Therefore, the effects on primer unblocking of DOT-MP and TFV were determined with the RT mutants 41/67/70/210/215 and 41/69S-SG/210/215 in an assay with a different template sequence, i.e., a repeat sequence of 5′-$(GACT)_5$-3′ instead of the 5′-$(GTCA)_5$-3′ sequence described in Materials and Methods. A similar or a slightly lower range of *n*-fold increased values was found for each of the TAM mutants DOT-TP (1.3- and 2.5-fold, respectively) and TFV-DP (3.1 and 6.0-fold, respectively) (Table 3).

A further investigation of DOT-TP and AZT-TP resistance was performed with the prA/odT assay. As shown in Table 2, the levels of resistance, measured as the mutant K_i/K_m ratio over that of the wild type, were overall higher for both nucleotides. However, DOT-TP demonstrated a smaller *n*-fold increase for the two TAM mutant enzymes (2.8- and 3.5-fold, respectively) in comparison with that of AZT-TP (17.6- and 13.6-fold, respectively), and the same trend applied for the residue 69 insertion mutant (13.6- compared to 36.5-fold, respectively).

Mutations involved in non-ATP-dependent discrimination. The DNA polymerase assay with and without ATP was used to study the resistance mechanism involved in the reduced ability to incorporate NRTI relative to natural substrate (Table 1). A large increase of non-ATP-dependent DOT-TP resistance was found with the Q151M mutants, from 16- to 65-fold, depending on the inclusion of the M184V mutation and on added ATP (Table 1). RTs carrying the K65R mutation appeared to be associated with some decreased discrimination to DOT-TP with and without ATP (3.9- and 4.2-fold, respectively). However, this decrease was less than that for CBV-TP (6.1- and 7.4-fold, respectively) and not of the same magnitude as that for TFV-DP (16- and 22-fold, respectively). The RT carrying the M184V mutation alone did not exhibit any apparent resistance for the NRTI-TP studied, except for CBV-TP (5-fold). The abacavir-associated mutation Y115F showed no resistance against DOT-TP, and only a twofold binding discrimination against CBV-TP was noted (Table 1).

Mutations involved in the suppression of resistance. Addition of the M184V mutation to the 41/67/70/210/215 mutant suppressed resistance to AZT-TP from 5.0- to 2.0-fold and resistance to TFV-DP from 3.7- to 1.8-fold (Table 1). Furthermore, a comparison of results from assays performed in the presence and absence of ATP indicated slightly lower resistance levels with RTs bearing only the M184V mutation for all NRTI (except for CBV-TP) (Table 1). This reduced effect of M184V in mutants without TAM seemed more evident with the 75/77/116/151M/184V mutant in the presence of ATP. The most prominent reductions were observed for DXG-TP and CBV-TP. However, the 75/77/116/151M mutant by itself without M184V was also found to be involved in decreased resistance to almost the same degree. Therefore, a comparison of assay results in the presence and absence of ATP suggests that Q151M and its family of mutations may contribute toward a suppressed resistance profile in a manner similar to that of M184V. Furthermore, the same ATP-dependent suppression effect was obtained with the K65R mutation for most NRTI-TP.

DISCUSSION

DOT was previously found to have a more potent activity than AZT and TFV against viruses with TAM in studies using primary human lymphocytes (9, 30). In the present study, we confirmed that DOT-TP had greater activity against RTs with TAM than against AZT-TP and TFV-DP, using the DNA polymerase assay (Table 1). To further explore this difference, extra dNTPs (20 μM) were added in the assay. It is known that high AZT resistance in cell-based systems expressing TAM in HIV-1 RT can be attributed to AZT's bulky azido group, preventing DEC formation (26). The formation of the DEC (in the presence of high dNTP concentration) inhibits ATP removal of incorporated NRTI-MP. This has previously been demonstrated for d4T-MP and TFV (but not for AZT-MP) with RTs bearing TAM (26, 27). In the DNA polymerase assay, the lower sensitivity to dNTP inhibition for the 41/67/70/210/215 mutant on primers chain terminated with AZT-MP was assessed. A 30% higher sensitivity for dNTP inhibition with chain-terminated TFV was noted (Table 3), while the TAM mutant was generally less resistant to DOT-TP, irrespective of the dNTP concentration used. Since the intracellular dNTP concentration is estimated to be 0.3 to 5 μM in resting peripheral blood mononuclear cells and 3 to 26 μM in activated cells (13, 36), the 1 μM of the dNTP used in the DNA polymerase assay (Table 1) more closely represented the human situation, whereas the 20 μM used (Table 3) more closely represented the situation in cell culture assays. It should be noted that RTs bearing the residue 69 insertion mutations have a decreased sensitivity to dNTP inhibition of the primer unblocking activity, which could explain why these mutants give higher and broader NRTI resistance in both the cell culture and the RT assay (23).

It is known that the template sequence has an effect on the ATP-dependent primer unblocking (22). In particular, large variations have been reported for primers terminated with a ddA-MP, ddC-MP, or ddG-MP analog, while T analogs such as AZT-MP and ddT-MP were found to be markedly less influenced by the template sequence (22). Therefore, our study was limited to only two repeats (GACT and GTCA). Comparisons of DOT-TP and TFV-DP using RTs bearing TAM or TAM with residue 69 insertion suggest that primer-incorporated DOT-MP maintains a favorable reduced ability for primer unblocking (Table 3).

Kinetic parameters from our previous studies of RT with TAM and residue 69 insertions, using a prA/odT assay, had revealed an approximately twofold higher AZT-TP resistance than the data for DNA polymerase as presented above (20). In the present study, using the prA/odT assay, a higher ATP-dependent resistance for AZT-TP and DOT-TP was found (Table 1 and Table 2). A larger primer unblocking capacity between the mutant and the wild-type RTs in the prA/odT assay than in the DNA polymerase assay may be explained by the long homopolymeric prA_{300} template strands, which allow more multiple incorporations than the shorter heteropolymeric DNA_{32} template. It is noteworthy that the present prA/

odT assay study generated an almost twofold higher AZT-TP resistance than the previous AZT-TP data described, using a similar prA/odT assay with related mutants (20). This may have resulted from using a new type of prA plate (High Sensitivity) and/or a different assay buffer condition. Irrespective of the DNA or prA/odT assay used, the primer unblocking rate of incorporated DOT-MP was, overall, fourfold lower than the removal of AZT-MP in RTs bearing TAM.

High levels of resistance to DOT-TP and DXG-TP by RT bearing the Q151M mutation were found through a non-ATP-dependent discrimination mechanism (Table 1). This is in agreement with the high levels of DOT resistance observed for viral drug susceptibility cell-based assays using virus containing the Q151M and similar family mutations (30). This suggested that the methionine residue 151 could make direct contact with the incoming DOT's dioxolane sugar ring. In contrast, the multidrug-resistant Q151M family mutations demonstrated very low resistance against TFV-DP compared to that of other NRTI (Table 1), in agreement with virus drug susceptibility assay data (31).

A fourfold range of DOT-TP resistance was found with the K65R mutant, both with and without ATP, which was lower than that for CBV-TP and TFV-DP (Table 1). However, the AZT-TP data also showed a somewhat elevated resistance (2-fold with ATP and 4.3-fold without ATP). Since the K65R mutant has previously been reported to be susceptible to AZT (onefold resistance or less) (28, 37), the data are somewhat unclear on how important K65R is for discriminating DOT-TP.

It is well recognized that mutations M184V and K65R diminished the ability of HIV-1 RT to perform the primer unblocking of incorporated NRTI-MP such as AZT-MP (6, 14, 37). It is thereby established that M184V and K65R mutants have less primer unblocking ability than wild-type RT (6, 14, 37). A comparison of the results in the presence and absence of ATP demonstrated that primer unblocking was decreased in the presence of K65R or M184V, which resulted in an overall lower NRTI-TP resistance in the presence of ATP (Table 1). Furthermore, we demonstrated the novel finding that Q151M and its family of mutations (without M184V) also had this ability (Table 1). It is notable that mutations involved in the suppression of primer unblocking clearly demonstrate that the final resistance level is dependent upon both resistance mechanisms (substrate discrimination and primer unblocking) working in concert. Furthermore, the ability of the 41/67/70/210/215 mutant to enable primer unblocking of incorporated AZT-MP and TFV was reduced with the addition of the M184V mutation (Table 1 [with ATP]). Surprisingly, the presence of mutation M184V in combination with Q151M and family mutations significantly increased the binding discrimination for DOT-TP, AZT-TP, and DXG-TP (also CBV-TP, but not TFV-DP), in comparison to that of the Q151M mutant without M184V (Table 1). However, an increase (from 13- to 21-fold) in AZT resistance for the same clones was noted when studied in a viral drug susceptibility assay (unpublished data).

It is hypothesized that the abacavir-associated mutation Y115F (tyrosine 115 phenylalanine) could be involved in DOT resistance and give rise to a different resistance profile. It has previously been predicted, using modeling simulations of the three-dimensional structure of HIV-1 RT, that the amino back bone of the tyrosine residue 115 could stabilize the 3′-oxygen in the D-like dioxolane ring (9). This could well be the case, but the mutation switch occurs in the side chain and not in the backbone. It is known that the side chain of tyrosine 115 interacts with the 2′ group in the deoxynucleotide sugar moiety and that a switch to the smaller phenylalanine side chain can induce misincorporation of ribonucleotides (2). The phenylalanine 115 residue apparently discriminates CBV-TP slightly but not DOT-TP. We are currently selecting for resistance mutations toward DOT in HIV-1-infected primary human lymphocytes. To date, after 40 passages in the presence of DOT, no mutation has been selected.

In summary, the facile nonradioactive DNA polymerase assay proved useful for studies of the mechanism of HIV-1 resistance to DOT-TP and provided comparative data for the level of DOT-TP resistance to other NRTI-TP. The biochemical mechanism of HIV-1 resistance to NRTI-TP occurs in two different ways depending on the amino acid substitutions present in the RT. The data suggest that the accumulation of TAM involving primer unblocking can result in a low level of resistance to DOT-TP and that this level was found to be lower for AZT-TP and TFV-DP. The mechanism involving non-ATP-dependent discrimination demonstrated that RT bearing the Q151M cluster was highly resistant to DOT-TP, whereas discrimination was not found with RT bearing only the mutation M184V or Y115F. DOT-TP was more potent against HIV-1 RT containing K65R than TFV-DP and CBV-TP. Therefore, DOT-TP showed a resistance profile similarly favorable to that of DXG-TP. In view of these data, DOT merits further development as a potential TK1-dependent anti-HIV-1 agent.

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REFERENCES

1. **Arion, D., N. Kaushik, S. McCormick, G. Borkow, and M. A. Parniak.** 1998. Phenotypic mechanism of HIV-1 resistance to 3′-azido-3′-deoxythymidine (AZT): increased polymerization processivity and enhanced sensitivity to pyrophosphate of the mutant viral reverse transcriptase. Biochemistry **37:** 15908–15917.
2. **Boyer, P. L., and S. H. Hughes.** 2000. Effects of amino acid substitutions at position 115 on the fidelity of human immunodeficiency virus type 1 reverse transcriptase. J. Virol. **74:**6494–6500.
3. **Boyer, P. L., C. Tantillo, A. Jacobo-Molina, R. G. Nanni, J. Ding, E. Arnold, and S. H. Hughes.** 1994. Sensitivity of wild-type human immunodeficiency virus type 1 reverse transcriptase to dideoxynucleotides depends on template length: the sensitivity of drug-resistant mutants does not. Proc. Natl. Acad. Sci. USA **91:**4882–4886.
4. **Boyer, P. L., S. G. Sarafianos, E. Arnold, and S. H. Hughes.** 2002. Nucleoside

analog resistance caused by insertions in the fingers of human immunodeficiency virus type 1 reverse transcriptase involves ATP-mediated excision. J. Virol. **76:**9143–9151.
5. **Boyer, P. L., S. G. Sarafianos, E. Arnold, and S. H. Hughes.** 2001. Selective excision of AZTMP by drug-resistant human immunodeficiency virus reverse transcriptase. J. Virol. **75:**4832–4842.
6. **Boyer, P. L., S. G. Sarafianos, E. Arnold, and S. H. Hughes.** 2002. The M184V mutation reduces the selective excision of zidovudine 5′-monophosphate (AZTMP) by the reverse transcriptase of human immunodeficiency virus type 1. J. Virol. **76:**3248–3256.
7. **Chamberlain, P. P., J. Ren, C. E. Nichols, L. Douglas, J. Lennerstrand, B. A. Larder, D. I. Stuart, and D. K. Stammers.** 2002. Crystal structures of zidovudine- or lamivudine-resistant human immunodeficiency virus type 1 reverse transcriptases containing mutations at codons 41, 184, and 215. J. Virol. **76:**10015–10019.
8. **Chu, C. K., S. K. Ahn, H. O. Kim, J. W. Beach, A. J. Alves, L. S. Jeong, Q. Islam, P. V. Roey, and R. F. Schinazi.** 1991. Asymmetric synthesis of enantiomerically pure (−)-(1′R,4′R)-dioxolane-thymine and its anti-HIV activity. Tetrahedron Lett. **32:**3791–3794.
9. **Chu, C. K., V. Yadav, Y. H. Chong, and R. F. Schinazi.** 2005. Anti-HIV activity of (−)-(2*R*,4*R*)-1-(2-hydroxymethyl-1,3-dioxolan-4-yl)thymine against drug-resistant HIV-1 mutants and studies of its molecular mechanism. J. Med. Chem. **48:**3949–3952.
10. **Ekstrand, D. H., R. J. Awad, C. F. R. Kallander, and J. S. Gronowitz.** 1996. A sensitive assay for the quantification of reverse transcriptase activity based on the use of carrier-bound template and non-radioactive-product detection, with special reference to human-immunodeficiency-virus isolation. Biotechnol. Appl. Biochem. **23:**95–105.
11. **Furman, P. A., J. A. Fyfe, M. H. St. Clair, K. Weinhold, J. L. Rideout, G. A. Freeman, S. N. Lehrman, D. P. Bolognesi, S. Broder, H. Mitsuya, and D. W. Barry.** 1986. Phosphorylation of 3′-azido-3′-deoxythymidine and selective interaction of the 5′-triphosphate with human immunodeficiency virus reverse transcriptase. Proc. Natl. Acad. Sci. USA **83:**8333–8337.
12. **Furman, P. A., J. Jeffrey, L. L. Kiefer, J. Y. Feng, K. S. Anderson, K. Borroto-Esoda, E. Hill, W. C. Copeland, C. K. Chu, J. P. Sommadossi, I. Liberman, R. F. Schinazi, and G. R. Painter.** 2001. Mechanism of action of 1-β-D-2,6-diaminopurine dioxolane, a prodrug of the human immunodeficiency virus type 1 inhibitor 1-β-D-dioxolane guanosine. Antimicrob. Agents Chemother. **45:**158–165.
13. **Gao, W. Y., A. Cara, R. C. Gallo, and F. Lori.** 1993. Low levels of deoxynucleotides in peripheral blood lymphocytes: a strategy to inhibit human immunodeficiency virus type 1 replication. Proc. Natl. Acad. Sci. USA **90:**8925–8928.
14. **Götte, M., D. Arion, M. A. Parniak, and M. A. Wainberg.** 2000. The M184V mutation in the reverse transcriptase of human immunodeficiency virus type 1 impairs rescue of chain-terminated DNA synthesis. J. Virol. **74:**3579–3585.
15. **Gu, Z., M. A. Wainberg, N. Nguyen-Ba, L. L'Heureux, J. M. de Muys, T. L. Bowlin, and R. F. Rando.** 1999. Mechanism of action and in vitro activity of 1′,3′-dioxolanylpurine nucleoside analogues against sensitive and drug-resistant human immunodeficiency virus type 1 variants. Antimicrob. Agents Chemother. **43:**2376–2382.
16. **Johnson, V. A., F. Brun-Vezinet, B. Clotet, D. R. Kuritzkes, D. Pillay, J. M. Schapiro, and D. D. Richman.** 2006. Update of the drug resistance mutations in HIV-1: fall 2006. Top. HIV Med. **14:**125–130.
17. **Larder, B. A., and S. D. Kemp.** 1989. Multiple mutations in HIV-1 reverse transcriptase confer high-level resistance to zidovudine (AZT). Science **246:** 1155–1158.
18. **Larder, B. A., S. D. Kemp, and P. R. Harrigan.** 1995. Potential mechanism for sustained antiretroviral efficacy of AZT-3TC combination therapy. Science **269:**696–699.
19. **Lennerstrand, J., D. K. Stammers, and B. A. Larder.** 2001. Biochemical mechanism of HIV-1 reverse transcriptase resistance to stavudine. Antimicrob. Agents Chemother. **45:**2144–2146.
20. **Lennerstrand, J., K. Hertogs, D. K. Stammers, and B. A. Larder.** 2001. Correlation between viral resistance to zidovudine and resistance at the reverse transcriptase level found for a panel of human immunodeficiency virus type 1 mutants. J. Virol. **75:**7202–7205.
21. **Ludwig, J., and F. Eckstein.** 1989. Rapid and efficient synthesis of nucleoside 5′-O-(1-thiotriphosphates), 5′-triphosphates and 2′,3′-cyclophosphorothioates using 2-chloro-4H-1,3,2-benzodioxaphosphorin-4-one. J. Org. Chem. **54:**631–635.
22. **Meyer, P. R., A. J. Smith, S. E. Matsuura, and W. A. Scott.** 2004. Effects of primer-template sequence on ATP-dependent removal of chain-terminating nucleotide analogues by HIV-1 reverse transcriptase. J. Biol. Chem. **279:** 45389–45398.
23. **Meyer, P. R., J. Lennerstrand, S. Matsuura, B. A. Larder, and W. A. Scott.** 2003. Effects of dipeptide insertions between codons 69 and 70 of human immunodeficiency virus type 1 reverse transcriptase on primer unblocking, deoxynucleoside triphosphate inhibition, and DNA chain elongation. J. Virol. **77:**3871–3877.
24. **Meyer, P. R., S. E. Matsuura, A. G. So, and W. A. Scott.** 1998. Unblocking of chain-terminated primer by HIV-1 reverse transcriptase through a nucleotide-dependent mechanism. Proc. Natl. Acad. Sci. USA **95:**13471–13476.
25. **Meyer, P. R., S. E. Matsuura, A. M. Mian, A. G. So, and W. A. Scott.** 1999. A mechanism of AZT resistance: an increase in nucleotide-dependent primer unblocking by mutant HIV-1 reverse transcriptase. Mol. Cell **4:**35–43.
26. **Meyer, P. R., S. E. Matsuura, R. F. Schinazi, A. G. So, and W. A. Scott.** 2000. Differential removal of thymidine nucleotide analogues from blocked DNA chains by human immunodeficiency virus reverse transcriptase in the presence of physiological concentrations of 2′-deoxynucleoside triphosphates. Antimicrob. Agents Chemother. **44:**3465–3472.
27. **Naeger, L. K., N. A. Margot, and M. D. Miller.** 2002. ATP-dependent removal of nucleoside reverse transcriptase inhibitors by human immunodeficiency virus type 1 reverse transcriptase. Antimicrob. Agents Chemother. **46:**2179–2184.
28. **Parikh, U. M., D. I. Koontz, C. K. Chu, R. F. Schinazi, and J. W. Mellors.** 2005. In vitro activity of structurally diverse nucleoside analogs against human immunodeficiency virus type 1 with the K65R mutation in reverse transcriptase. Antimicrob. Agents Chemother. **49:**1139–1144.
29. **Ray, A. S., E. Murakami, A. Basavapathruni, J. A. Vaccaro, D. Ulrich, C. K. Chu, R. F. Schinazi, and K. S. Anderson.** 2003. Probing the molecular mechanisms of AZT drug resistance mediated by HIV-1 reverse transcriptase using a transient kinetic analysis. Biochemistry **42:**8831–8841.
30. **Schinazi, R. F., G. Asif, M. Detorio, B. Hernandez-Santiago, M. Ruckstuhl, V. Yadav, K. L. Rapp, M. Bennett, J. Grier, M.-Y. Xie, J. Lennerstrand, M. Kozlowski, S. Lutz, S. J. Hurwitz, and C. K. Chu.** 2005. 1-beta-D-Dioxolane)thymine (DOT) is a TK-dependent orally bioavailable nucleoside with specific activity against HIV-1 resistant variants. Antivir. Ther. **10**(Suppl.):S65.
31. **Shafer, R. W.** 2002. Genotypic testing for human immunodeficiency virus type 1 drug resistance. Clin. Microbiol. Rev. **15:**247–277.
32. **Shao, X. W., A. Malmsten, J. Lennerstrand, A. Sonnerborg, T. Unge, J. S. Gronowitz, and C. F. Källander.** 2003. Phenotypic drug susceptibility testing achieved directly on HIV-1 reverse transcriptase recovered from plasma. AIDS **17:**1463–1471.
33. **Shirasaka, T., M. F. Kavlick, T. Ueno, W. Y. Gao, E. Kojima, M. L. Alcaide, S. Chokekijchai, B. M. Roy, E. Arnold, R. Yarchoan, and H. Mitsuya.** 1995. Emergence of human immunodeficiency virus type 1 variants with resistance to multiple dideoxynucleosides in patients receiving therapy with dideoxynucleosides. Proc. Natl. Acad. Sci. USA **14:**2398–2402.
34. **Stammers, D. K., D. O. Somers, C. K. Ross, I. Kirby, P. H. Ray, J. E. Wilson, M. Norman, J. S. Ren, R. M. Esnouf, E. F. Garman, E. Y. Jones, and D. I. Staurt.** 1994. Crystals of HIV-1 reverse transcriptase diffracting to 2.2 A resolution. J. Mol. Biol. **242:**586–588.
35. **Thompson, M. A., H. A. Kessler, J. J. Eron, Jr., J. M. Jacobson, N. Adda, G. Shen, J. Zong, J. Harris, C. Moxham, and F. S. Rousseau.** 2005. DAPD-101 Study Group. Short-term safety and pharmacodynamics of amdoxovir in HIV-infected patients. AIDS **19:**1607–1615.
36. **Traut, T. W.** 1994. Physiological concentrations of purines and pyrimidines. Mol. Cell. Biochem. **140:**1–22.
37. **White, K. L., J. M. Chen, J. Y. Feng, N. A. Margot, J. K. Ly, A. S. Ray, H. L. Macarthur, M. J. McDermott, S. Swaminathan, and M. D. Miller.** 2006. The K65R reverse transcriptase mutation in HIV-1 reverses the excision phenotype of zidovudine resistance mutations. Antivir. Ther. **11:**155–163.