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Endogenous Reverse Transcription Assays Reveal High-Level Resistance to the Triphosphate of (−)2′-Dideoxy-3′-Thiacytidine by Mutated M184V Human Immunodeficiency Virus Type 1

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Kinetic analysis showed that the K_i values and the K_i/K_m ratios for mutated, recombinant M184V human immunodeficiency virus type 1 reverse transcriptase (RT) for (−)2′-dideoxy-3′-thiacytidine triphosphate (3TCTP) were 35-fold higher than the equivalent values for wild-type RT but only about twice as high as the equivalent values for each of the triphosphates of ddC (ddCTP) and ddA (ddATP). Fully endogenous RT assays showed that viruses containing the M184V substitution were highly resistant to 3TCTP, with an increase in the 50% inhibitory concentration of 250-fold in comparison with wild-type recombinant virus.

Although mutations in the human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) are responsible for resistance to nucleoside analogs (10, 14, 18, 22), the molecular mechanisms involved are not well understood. For example, RT isolated from 3′-azido-3′-deoxythymidine (AZT)-resistant variants of HIV-1 displayed the same level of sensitivity to AZT triphosphate (AZTTP) in cell-free kinetics and endogenous enzyme assays as did wild-type (wt) material (17). In contrast, both recombinant L74V RT and K65R RT are less sensitive to each of ddITP, ddCTP, ddATP, and AZTTP than is wt RT in such assays (13, 20).

We have pursued this subject further by using fully endogenous RT reactions and enzyme kinetics to compare wt viruses and enzymes with those containing the M184V mutation. The fully endogenous RT reaction may be more physiologically relevant than enzyme kinetics assays, since virion RNA acts as a template and since viral proteins other than RT may influence the synthesis of viral DNA products (1, 7). M184V is located in the "palm" subdomain of RT, within a conserved YMDD motif thought to make up part of the polymerase active site (4, 16). Viruses that contain this mutation display high-level resistance (i.e., 250- to 1,000-fold) to (−)2′-dideoxy-3′-thiacytidine (3TC) (10, 14, 21, 22) and low-level resistance (i.e. 3- to 10-fold) to each of ddC and ddI in tissue culture (10, 14). We now report that the endogenous RT assay is a more sensitive means of determining levels of HIV resistance to dideoxynucleoside triphosphates (ddNTPs) than are enzyme kinetics measurements, at least in regard to 3TCTP.

M184V and wt RTs have similar RDDP and DDDP activity. Four homopolymeric templates, i.e., poly(rA-$dT_{12–18}$), poly (rC-$dG_{12–18}$), poly (rI-$dC_{12–18}$), and poly (dC-$dG_{12–18}$), and one heteropolymeric RNA template were used to study RNA- and DNA-dependent DNA polymerase activities. The AZTTP used for this work was a gift of Wellcome Laboratories, Research Triangle Park, N.C. 3TC and 3TCTP were gifts of Glaxo Group Research, Greenford, United Kingdom. Other ultrapure dNTPs, ddNTPs, and homopolymer template/primers (t/p), i.e., poly (rC)/oligo $(dG)_{12–18}$, poly(dC)/oligo$(dG)_{12–18}$, and poly(rA)/oligo$(dT)_{12–18}$ were obtained from Pharmacia Biotech, Inc., Montreal, Canada. Poly(rI)/oligo$(dC)_{12–18}$ was prepared from poly(rI) and oligo$(dC)_{12–18}$ (Pharmacia Biotech). [^{3}H]dNTPs (22 Ci/mmol) and [α-^{32}P]dNTPs (3,000 Ci/mmol) were purchased from ICN Biomedical Inc., Montreal, Canada, and Du Pont Inc., Mississauga, Canada. RNA template was prepared, using a MEGAscript transcription kit (Ambion, Austin, Tex.), from linearized plasmid pHIV-PBS, which consists of a 497-bp HIV-1 sequence spanning the R region of HIV-1 long terminal repeat and a portion of *gag* (2). Heteropolymeric RNA t/p was prepared as described previously (13) by annealing RNA template and a chemically synthesized 18-nucleotide DNA primer, complementary to the HIV-1 primer-binding sequence. Recombinant wt and mutated RTs and relevant plasmids were expressed and purified as described previously (24). RNA-dependent DNA polymerase (RDDP) and DNA-dependent DNA polymerase (DDDP) activities were measured as described previously (12, 13). The specific activities of the recombinant purified wt and M184V RT molecules used in this study were similar.

Table 1 shows that the two enzymes possessed similar K_m and V values for each of the four dNTPs with all of the t/p used. This indicates that they had similar affinities for dNTPs. We considered that the location of codon 184 in the YMDD motif, thought to be part of the polymerase active site (16), might have affected the affinity of the enzyme for template. This possibility was examined by using both homopolymeric and heteropolymeric templates, yet no significant differences in K_m values were observed between the two enzymes (data not shown).

M184V RT displays resistance to 3TCTP. The K_i values for different ddNTPs, including AZTTP and 3TCTP, are given in Table 2. The K_i value of 3TCTP for M184V RT, with RNA heteropolymeric t/p, was 41.4 μM, an increase of about 35-fold over that for wt RT (1.15 μM) (Table 2). The K_i value of

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TABLE 1. K_m and V values of dNTPs for wild-type and M184V RTs

t/p[a]	Substrate	K_m (μM)[b]		V[b,c]	
		wt	M184V	wt	M184V
Poly(rA-dT_{12-18})	dTTP	2.47 ± 0.09	2.54 ± 0.14	5.47 ± 0.19	5.28 ± 0.61
Poly(rC-dG_{12-18})	dGTP	1.99 ± 0.24	3.71 ± 0.09	4.02 ± 0.35	4.47 ± 0.32
Poly(dC-dG_{12-18})	dGTP	1.37 ± 0.19	1.49 ± 0.14	2.75 ± 0.22	3.01 ± 0.43
Poly (rI-dC_{12-18})	dCTP	28.65 ± 1.44	26.35 ± 0.31	7.30 ± 0.47	7.48 ± 0.52
Heteropolymeric RNA	dTTP	1.22 ± 0.28	1.42 ± 0.19	2.15 ± 0.68	2.01 ± 0.34
	dCTP	3.58 ± 0.21	3.54 ± 0.32	1.85 ± 0.45	1.78 ± 0.30
	dGTP	0.23 ± 0.01	0.27 ± 0.06	0.90 ± 0.05	0.97 ± 0.04
	dATP	3.36 ± 0.33	4.39 ± 0.30	1.54 ± 0.16	1.67 ± 0.24

[a] In experiments with homopolymeric t/p, the latter were used at a concentration of 0.1 U/ml. For heteropolymeric RNA, a fixed concentration of 18 nM t/p was used and dNTP, for which the K_m value was to be determined, was added at a variety of concentrations. For heteropolymeric t/p, three dNTPs, other than the one to be measured, were added at a constant concentration of 7 μm.
[b] Values are means ± standard deviations for at least three separate experiments.
[c] V is expressed as nanomoles of dNMP incorporated per 30 min per microgram of RT.

ddATP, the active intracellular form of ddI, for M184V RT with heteropolymeric templates was threefold higher than that for wt RT. The K_i for ddCTP was increased only twofold. For AZTTP, ddGTP, and ddTTP, a twofold increase in K_i was also observed for M184V RT. No differences in the K_i or K_i/K_m values of ddITP were detected for wt and M184V RTs.

M184V-containing viruses are resistant to 3TCTP in endogenous RT reactions. MT-2 cells were used to generate viruses containing either the M184V or K65R substitution in RT as described previously (14). For endogenous RT reactions, viruses were harvested from the culture fluids of 2×10^8 infected MT-2 cells as follows: low-speed centrifugation of cells at $1,000 \times g$ for 30 min, and ultracentrifugation of supernatant fluids at $88,000 \times g$ for 1 h at 4°C. Pelleted viruses were resuspended in 0.2 ml of TN buffer (10 mM Tris [pH 7.8], 100 mM NaCl), further purified as described previously (23) with 2-ml columns of Sephacel S-1000 (Pharmacia, Montreal, Canada) that had been prebalanced with TN buffer, and eluted with TN buffer. Purified viruses were quantified and kept at 4°C until use.

Endogenous RT assays were modified from previously described procedures (3, 8) and were performed with a total volume of 30 μl containing 50 mM Tris (pH 7.8), 5 mM $MgCl_2$, 60 mM KCl, 10 mM dithiothreitol, 10 mM NaCl, 1 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 0.1% Nonidet P-40, 0.4 mM each dATP, dGTP and dTTP, 10 μM dCTP, 10 μCi of [α-^{32}P]dCTP, 20 μl of purified HIV normalized on the basis of p24 content (i.e., 280 ng of p24, representing approximately 2×10^7 cpm of RT activity per ml per reaction), and variable concentrations of inhibitors. After 6 h at 39°C, reactions were terminated by addition of an equal volume of stop buffer (1% sodium dodecyl sulfate, 50 mM EDTA, 0.2 M NaCl). The reaction mixture was digested with 20 μg of protease at 56°C for 30 min, extracted with phenol-chloroform, and precipitated with ethanol. Products were boiled and separated on a 1% denaturing agarose gel (20 mM NaOH, 1 mM EDTA), visualized by autoradiography (Fig. 1), and analyzed by molecular imaging (Bio-Rad, Mississauga, Canada) (Fig. 2).

We found that the 50% inhibitory concentration (IC_{50}) of 3TCTP was 1,970 μM for M184V versus 7.8 μM for wt virus, an increase of about 250-fold (Fig. 2). The recombinant K65R virus displayed less resistance than the M184V virus to 3TCTP in endogenous RT reactions (Fig. 1 and 2), i.e., a 13-fold difference in comparison with wt virus (Fig. 2). Similar results were obtained on at least three separate occasions.

Mutated RTs that contain a YVDD instead of a YMDD motif almost fully retained DNA polymerase activity, consistent with a recent report that also documented a slight decrease in the processivity of mutated M184V RT (5). Viruses

TABLE 2. K_i values for wt and M184V RTs

t/p[a]	dNTP[a]	Inhibitor[a]	K_i (μM)[b]		K_i/K_m[b]	
			wt	M184V	wt	M184V
Poly(rA-dT_{12-18})	dTTP	AZTTP	0.007	0.014	0.003	0.005
Poly(rA-dT_{12-18})	dTTP	ddTTP	0.007	0.017	0.003	0.007
Poly(rC-dG_{12-18})	dGTP	ddGTP	0.029	0.083	0.015	0.022
Poly(rI-dC_{12-18})	dCTP	ddCTP	0.24	0.32	0.008	0.012
Poly(dC-dG_{12-18})	dGTP	ddGTP	0.008	0.017	0.006	0.011
Heteropolymeric RNA	dNTP	AZTTP	0.014	0.042	0.011	0.029
		ddTTP	0.016	0.037	0.013	0.026
		ddCTP	0.19	0.42	0.053	0.118
		3TCTP	1.15	41.4	0.32	11.69
		ddGTP	0.006	0.013	0.019	0.048
		ddATP	0.57	1.63	0.17	0.37
		ddITP	19.6	21.4	5.83	4.87

[a] The concentration of dNTPs was 7 μM, and that of t/p was 0.1 U/ml. Inhibitor was added at variable concentrations.
[b] Values are means determined on the basis of at least three separate experiments.

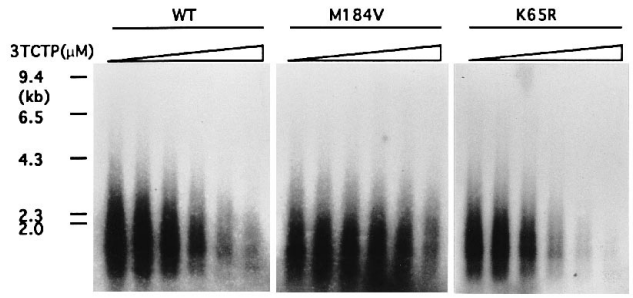

FIG. 1. Analysis of endogenous RT reactions. Concentrations of 3TCTP from the left for wt viruses were 0, 0.6, 4.0, 20, 60, and 120 μM. For M184V and K65R viruses, concentrations of 3TCTP were 0, 6, 40, 200, 600, and 2,400 μM.

that contain the M184V substitution replicate almost as well as wt viruses do (19, 24). However, this mutation confers high-level resistance to 3TC and 3TCTP in tissue culture (10, 14, 21, 22) and cell-free assays, respectively.

The products of our endogenous RT reaction must contain both minus- and plus-strand DNA, since substantial amounts of this material migrated between 9.4 and 23 kb on a neutral agarose gel (results not shown), a pattern similar to that previously reported for murine leukemia virus (11). In contrast (8, 17), most DNA products migrated faster than 4.3 kb on denaturing gels (Fig. 1), a result attributable to the fact that DNA products were partially double-stranded and/or because circular intermediate complexes formed during reverse transcription (11). Upon denaturation, relatively short single-stranded DNA molecules, including plus strands initiated at multiple sites (6), would be separated from minus strands and would move faster than intermediate complexes.

The similarity in V and V/K_m values between wt and M184V RTs indicates that the two enzymes possess comparable catalytic efficiencies. Furthermore, the M184V mutation may not affect the ability of the enzyme to bind to template, because the K_m of M184V RT for different t/p was similar to that of wt (results not shown). Recently, another "helix clamp" structure was proposed to be responsible for template binding (15).

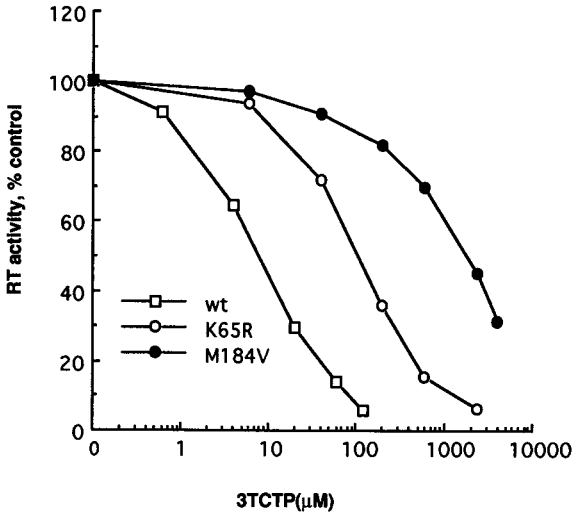

FIG. 2. Inhibition of endogenous RT reactions by 3TCTP. RT activities are expressed as percentages of those of controls performed in the absence of drug. Data are expressed as mean values of three separate experiments.

In contrast, significant differences in K_i values were shown to be associated with M184V with regard to 3TCTP (Table 2). M184V RT had a 35-fold-elevated K_i of 41.4 μM compared with 1.15 μM for wt. The K_i/K_m ratio, useful in determining the recognition of dNTP and ddNTP by an enzyme, was also increased by about 35-fold (11.69 for M184V versus 0.32 for wt RT), in agreement with results of earlier studies (9). As shown here, endogenous RT reactions showed even greater differences between M184V-containing and wt viruses, i.e., a 252-fold elevated IC_{50}. Thus, endogenous RT assays may, in some cases, represent a more sensitive means of determining the resistance to nucleoside analog triphosphates than kinetics assays performed with purified RTs. The former system may also be of greater physiological relevance, since viral RNA genome serves as the template and other viral proteins can interact with RT in the reaction.

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