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Resistance to Nucleoside Analogs of Selective Mutants of Human Immunodeficiency Virus Type 2 Reverse Transcriptase

MICHAL PERACH, TAMI RUBINEK, AND AMNON HIZI*

Department of Cell Biology and Histology, Sackler School of Medicine, Tel Aviv University, Tel Aviv 69978, Israel

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We have studied selected mutants of human immunodeficiency virus type 2 (HIV-2) reverse transcriptase (RT) in a cell-free system in order to assess whether the mutant proteins exhibit a reduction in the sensitivity to nucleoside analog inhibitors similar to that of HIV-1 RT. We have modified, by site-directed mutagenesis, several of those amino acid residues so that their equivalent substitutions in HIV-1 RT have led to the formation of HIV-1 RT variants with the highest degree of resistance to dideoxynucleoside triphosphates (i.e., Glu-89→Gly, Leu-74→Val, and Ser-215→Tyr [which is comparable to the Thr-215→Tyr mutation of HIV-1 RT] and the double mutations Glu-89→Gly/Ser-215→Tyr and Leu-74→Val/Ser-215→Tyr). The similarity found between resistance of the newly generated HIV-2 RT mutants to nucleoside analogs and that of the comparable mutants of HIV-1 RT can support the notion that the overall protein folding around the DNA polymerase active site in HIV-2 RT is quite similar to that of HIV-1 RT.

Human immunodeficiency viruses type 1 and type 2 (HIV-1 and HIV-2), the etiologic agents of AIDS (2, 6, 11), encode, like other retroviruses, the enzyme reverse transcriptase (RT). RT copies the single-stranded viral RNA genome into linear double-stranded DNA, that is subsequently integrated in the host cell chromosomal DNA (13, 34, 35). This essential step in the life cycle of retroviruses is carried out by both the RNA-dependent and DNA-dependent DNA polymerase (RDDP and DDDP, respectively) activities and by the ribonuclease H activity, all of which are exhibited by the viral RT (13, 18, 24). Most anti-HIV drugs tested so far, including several that have already been approved for clinical use, exhibit anti-HIV RT activities (23). These inhibitors can be divided into two groups. The first one, the nucleoside analogs, such as the 2′,3′-dideoxynucleoside analogs (dideoxynucleoside triphosphates [ddNTPs]) (e.g., 3′-azido-2′,3′-dideoxythymidine [AZT], 2′,3′-dideoxythymidine [ddT], 2′,3′-dideoxyguanosine [ddG], and 2′,3′-dideoxycytidine) serve as competitive inhibitors. The inhibitors, which need to be converted by cellular kinases to their 5′-triphosphate forms, are potent chain terminators of DNA synthesis catalyzed by RT (10, 23). Both HIV-1 RT and HIV-2 RT are highly sensitive to these nucleoside analogs (18, 33). The second group of HIV RT inhibitors are nonnucleoside inhibitors. They do not require any intracellular conversion and are not competing with the substrate binding site of the RT, and most of them are potent inhibitors of HIV-1 RT but not of HIV-2 RT (8, 23).

One of the major difficulties in using anti-HIV RT drugs to treat AIDS is the genetic flexibility of the virus that leads to the rapid emergence of drug-resistant RT mutants. Since most of the research conducted so far on HIV RTs has been done with HIV-1 RT, many mutations that confer drug resistance to HIV-1 RT have already been characterized (3, 5, 8, 9, 12, 22, 23, 29). We have previously expressed in bacteria the RT from the Rod 1.12 strain of HIV-2 (17). Biochemical studies of this HIV-2 RT compared with the RT of the BH-10 clone of HIV-1 (14) reveal a similarity in many enzymatic properties such as sensitivity to ddNTPs and sulfhydryl reagents and the relatively low fidelity of DNA synthesis. On the other hand, the two enzymes show substantial differences in other properties, including the specific activity of ribonuclease H and heat stability (1, 18). Very few studies have been carried out on the effects of mutations in HIV-2 RT on the resistance to nucleoside analogs. In one study it was shown that, as in the case of HIV-1 RT, a Glu-89→Gly (E89G) mutation in HIV-2 RT renders the enzyme resistant to both ddGTP and phosphonoformic acid (33).

In the current study we have investigated selected mutants of HIV-2 RT in vitro in order to assess whether the purified protein shows a reduction in sensitivity to nucleoside inhibitors similar to that of the comparable mutants of HIV-1 RT, since there are no reports on the emergence and characterization of drug-resistant HIV-2 RT. We have modified by site-directed mutagenesis those amino acid residues in HIV-2 RT that are homologous to residues in HIV-1 RT (31), and their substitutions resulted in the formation of HIV-1 RT variants with the highest degree of resistance to ddNTPs (7, 8, 23). To this aim, we generated the following mutants: E89G, Leu-74→Val (L74V), Ser-215→Tyr (S215Y) (which is comparable to the Thr-215→Tyr [T215Y] of HIV-1 RT), and the double mutants L74V/S215Y and E89G/S215Y.

Recombinant HIV-2 RT and its mutants were expressed in the DH5α strain of *E. coli*, by using the expression vector pUC12N as described previously (16, 17). The genes encoding the single mutants, E89G, L74V, and S215Y, were generated by the PCR-based megaprimer technique, as described in detail previously (15, 16). The genes encoding the double mutants E89G/S215Y and L74V/S215Y were constructed by replacing the 606-bp *Nco*I-*Pst*I or 557-bp *Nco*I-*Eco*RV DNA fragments of the S215Y-encoding genome with the compatible DNA fragments of the L74V- and E89G-encoding genomes, respectively. The coding regions of all five mutants and wild-type HIV-2 RT were then modified to include six consecutive histidine codons at the 5′ end of the genome, between the initiation methionine codon and the proline codon at the amino terminus of the protein (17). The bacteria were grown and harvested as described previously (15, 17, 32). The proteins underwent a rapid purification procedure by the metal chelate chromatography method (19, 25), and they were

* Corresponding author. Phone: 972-3-6409974. Fax: 972-3-6407432.

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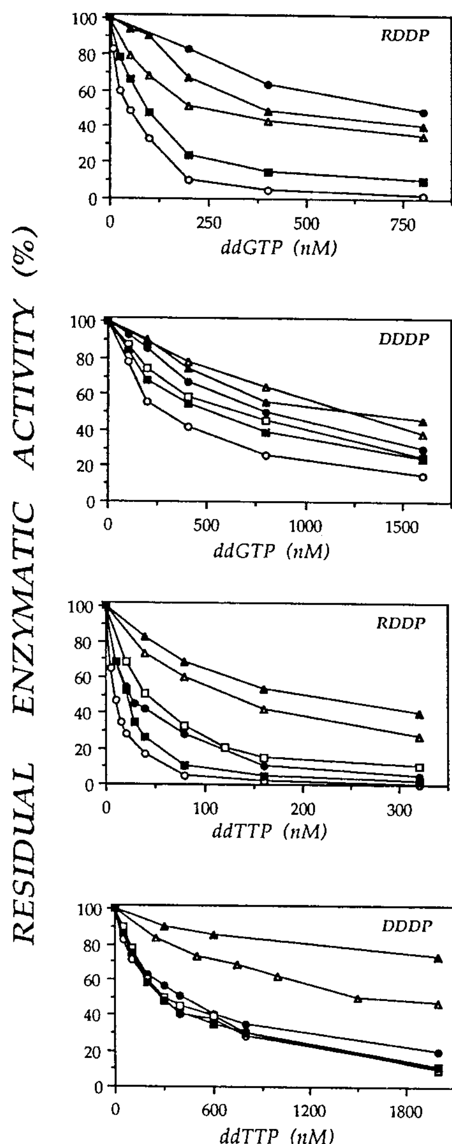

FIG. 1. Effects of ddGTP and ddTTP on DNA polymerase activities of wild-type and mutant HIV-2 RTs. The enzymes were expressed in bacteria and purified as described in the text. The RDDP activity was assayed with either poly(rC)$_n$-oligo(dG)$_{12-18}$ and ddGTP or with poly(rA)$_n$-oligo(dT)$_{12-18}$ and ddTTP. The DDDP activity was assayed with herring testis-activated DNA. Increasing concentrations of either ddNTP were added to the reaction mixtures, and the initial enzymatic activities were calculated as a percentage of similar reactions without inhibitors. ○, wild-type HIV-2 RT; ●, L74V mutant; □, E89G; ■, S215Y; △, L74V/S215Y; ▲, E89G/S215Y.

mainly composed of p68/p55 heterodimers (data not shown). The purified RTs were assayed for the effects of two ddNTP chain terminators, ddGTP and ddTTP, on the extent of DNA synthesis. As found previously (18, 26), ddTTP and AZT-3P gave always similar results (data not shown); therefore, ddTTP was used to represent both. We have assessed the DNA polymerase activities by two different assay systems. RDDP activity was assayed by using two different synthetic template primers (i.e., poly(rC)$_n$-oligo(dG)$_{12-18}$ with dGTP and the inhibitor ddGTP and poly(rA)$_n$-oligo(dT)$_{12-18}$ with dTTP and the inhibitor ddTTP), and DDDP activity was assayed with gapped DNA (31) and all four dNTPs with either ddTTP or ddGTP.

The effects of ddTTP and ddGTP on the DNA polymerase functions of mutants and wild-type HIV-2 RT are presented in Fig. 1. In most cases the mutants show reduced sensitivity to both inhibitors relative to that of the wild-type RT-associated DNA polymerase activities. The inhibitor concentrations leading to a 50% inhibition of the initial enzymatic activity (IC_{50} values), calculated from Fig. 1, are presented in Table 1. In addition, we calculated the K_i, K_m, and K_i/K_m values for all mutant and wild-type RTs with the two competitive inhibitors (Table 1). K_m values for dGTP and dTTP were determined from the double reciprocal plots (Lineweaver-Burk) of the initial enzymatic velocities versus dNTP concentrations. The K_i values were calculated from secondary plots of the K_m (apparent) values (calculated from the double reciprocal plots with the inhibitors) versus inhibitor concentrations. In the case of the E89G mutant, the RDDP activity with poly(rC)$_n$-oligo (dG)$_{12-18}$ was too low to give any accurate values, and for the double mutant E89G/S215Y we could accurately calculate only the IC_{50} values for both the poly(rC)$_n$-oligo(dG)$_{12-18}$-directed synthesis and the DDDP activity with ddTTP.

The behavior of the mutant L74V RT relative to that of wild-type HIV-2 RT in the two assay systems indicates that there is, nearly always, a significant reduction in the sensitivity to both ddTTP and ddGTP (Fig. 1 and Table 1). The highest decrease in the inhibition, as expressed by changes in the increase of both the IC_{50} and K_i/K_m values, is observed for ddGTP in the RDDP reaction (almost 17-fold). This mutation has a milder effect on the sensitivity of the RT to ddTTP. These results are compatible with the resistance observed for the L74V mutant of HIV-1 RT (27). The newly generated S215Y mutant of HIV-2 RT often shows decreased susceptibilities to both ddGTP and ddTTP, with the most significant difference in the K_i/K_m value of the RDDP reaction in the presence of ddGTP (11.3-fold). As in the former case of L74V HIV-2 RT, a more pronounced decrease relative to that of wild-type RT is obtained with ddGTP rather than with ddTTP. These results are in accordance with those obtained with HIV-1 RT (22, 27). The E89G mutant of HIV-2 RT exhibits the highest degree of resistance (as expressed by the K_i/K_m values) to either ddGTP or ddTTP in the DDDP activity. A previous study has shown that such a mutant of HIV-2 RT (fused at its amino terminus to the *trpE* gene product protein and to a 43-residue peptide of the integrase at its carboxyl terminus) shows a resistance to both ddGTP and phosphonoformic acid (33). As expected, the double mutants of HIV-2 RT, L74V/S215Y and E89G/S215Y, show a reduction in the susceptibility to both analogs relative to that of wild-type RT, which is often higher than that observed with the comparable singly substituted proteins. This is apparent mainly from the increase of the IC_{50} values (Fig. 1 and Table 1). The most-prominent resistance to ddTTP is obtained with the E89G/S215Y mutant in the RDDP activity (the IC_{50} values are 17.4- or 6.3-fold higher than those obtained with S215Y or E89G mutant RTs, respectively). A similar phenomenon was observed for the K_i values of the single mutant L74V and the L74V/T215Y double mutant of HIV-1 RT (27).

Analysis of the three-dimensional structure of HIV-1 RT indicates that most mutations that confer resistance to ddNTPs do not lie in the vicinity of the DNA polymerase active site but rather are located within the "fingers" and "palm" subdomains (20). Thus, Leu-74 resides in the β sheet, designated β4 in the fingers. Glu-89 is located in β5a, and Thr-215 is located in β11a, both at the base of the fingers in the palm subdomain. It was suggested that the fingers subdomain is involved in the appropriate positioning of the template strand (20, 21, 28). Consequently, modification of these residues can influence the

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TABLE 1. Inhibition of wild-type and mutant HIV-2 RT by dNTP analogs

Assay and HIV-2 RT source	Result with inhibitor[a]:							
	ddGTP				ddTTP			
	IC_{50}	K_i	K_m	K_i/K_m	IC_{50}	K_i	K_m	K_i/K_m
RDDP								
Wild type	45	21	5.7	0.0037	9	36	10.2	0.0035
L74V	760 (16.9)	319	5.2	0.0613 (16.5)	24 (2.7)	57	13.8	0.0041 (1.2)
E89G	ND	ND	ND	ND	40 (4.4)	87	23	0.0038 (1.1)
S215Y	96 (2.1)	376	9	0.0418 (11.3)	14.5 (1.6)	35	17.5	0.002 (0.6)
L74V/S215Y	201 (4.5)	155	11	0.0141 (3.8)	120 (13.3)	239	18.6	0.0128 (3.7)
E89G/S215Y	352 (7.8)	ND	ND	ND	252 (28)	165	25	0.0066 (1.9)
DDDP								
Wild type	252	215	4.5	0.0478	280	376	2.6	0.1446
L74V	802 (3.2)	546	2.7	0.2022 (4.2)	410 (1.5)	1,320	1.9	0.6947 (4.8)
E89G	751 (3.0)	563	2	0.2815 (5.9)	301 (1.1)	660	0.8	0.825 (5.7)
S215Y	525 (2.1)	525	3.6	0.1458 (3.1)	275 (1)	1,110	2.6	0.4269 (2.9)
L74V/S215Y	1,510 (6.0)	922	8.5	0.1085 (2.3)	875 (3.1)	1,021	0.98	1.042 (7.2)
E89G/S215Y	2,020 (8.0)	1,108	6.5	0.1705 (3.6)	>2,400 (>8.6)	ND	ND	ND

[a] The IC_{50} values were calculated from the data in Fig. 1. The IC_{50} and K_i values are expressed as the nanomolar concentrations of ddNTP, and K_m values are expressed as micromolar concentrations of dNTP. The values are averages calculated from at least two separate experiments. The figures in parentheses are the ratios of the values divided by the equivalent ones of wild-type HIV-2 RT. ND, not determined.

capability of the RT to accept or reject the incoming dNTP. A recent study of the sensitivity of HIV-1 RT to ddNTP depending on the length of the template extension (beyond the 3′ end of the primer strand) has suggested that resistance results from a change in conformation of the template primer. This leads to a reduction in the ability of the RT to incorporate ddNTP instead of the incoming dNTP into the nascent DNA (4). In view of the similarity observed in the drug sensitivity of the mutants of HIV-1 RT and HIV-2 RT studied, it is quite possible that the folding of HIV-2 RT in the DNA polymerase domain is very similar to that of HIV-1 RT.

The overall sequence identity between the p66 and p68 polypeptides of HIV-1 RT and HIV-2 RT, respectively, is about 61%. This figure is even higher (70%) after including conservative amino acid changes (32). An equivalent sequence similarity was determined for the DNA polymerase domains of the two RTs. It is important to study whether the sequence differences between the two HIV RTs lead to subtle dissimilarities in the folding of the proteins. Such putative differences can cause the disparities detected in several of the catalytic properties of these RTs (18). Recent epidemiological studies have shown that there is an increase in the spread of HIV-2 worldwide (30). Therefore, the treatment of HIV-2-infected AIDS patients with nucleoside analogs becomes a greater challenge. Since little is known about the emergence of drug-resistant variants of HIV-2 RT in AIDS patients, it is important to study the resistance of this enzyme to the inhibitors in use.

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