Biochemical Pharmacology, Vol. 46, No. 12, pp. 2307–2313, 1993.
Printed in Great Britain.

0006-2952/93 $6.00 + 0.00
Pergamon Press Ltd

IDENTIFICATION OF THE AMINO ACID IN THE HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 REVERSE TRANSCRIPTASE INVOLVED IN THE PYROPHOSPHATE BINDING OF ANTIVIRAL NUCLEOSIDE TRIPHOSPHATE ANALOGS AND PHOSPHONOFORMATE

IMPLICATIONS FOR MULTIPLE DRUG RESISTANCE

GUANG-JIN IM, ENZO TRAMONTANO, CHARLES J. GONZALEZ and YUNG-CHI CHENG*
Department of Pharmacology, Yale University School of Medicine, New Haven, CT 06510, U.S.A.

(*Received* 30 *March* 1993; *accepted* 8 *July* 1993)

Abstract—A recombinant clone of human immunodeficiency virus type 1 reverse transcriptase (HIV-1 RT) with reduced sensitivity to 3′-azido-3′-deoxythymidine 5′-triphosphate (AZTTP) and phosphonoformate (PFA), a pyrophosphate analog, has been obtained from the RNA of HTLV-IIIB infected cells using the polymerase chain reaction. The mutant HIV-1 RT retained polymerase activity and was cross-resistant to triphosphate forms of other nucleoside analogs including 2′,3′-dideoxycytidine 5′-triphosphate, 2′,3′-dideoxyadenosine 5′-triphosphate, and 3′-deoxy-2′,3′-didehydrothymidine 5′-triphospate (D4TTP), but remained sensitive to the non-nucleoside HIV-1 RT inhibitors, such as nevirapine and TIBO R82150. Sequence analysis of the mutant HIV-1 RT revealed a single amino acid substitution (Val → Ala) at amino acid 90. The substitution of amino acid 90 by the closely related amino acids, such as Thr and Gly, also showed decreased sensitivity to AZTTP, D4TTP, and PFA. All these mutations at amino acid 90 also caused an alteration of K_m for thymidine triphosphate. These results suggest that Val at this site plays a role in determining the interaction of the HIV-1 RT enzyme with the pyrophosphate group of deoxynucleoside triphosphate (dNTP) and that the hydrophobicity of the amino acid at this position was the most important determinant in the binding of HIV-1 RT to dNTP.

Human immunodeficiency virus type 1 (HIV-1)† is the etiological agent for the acquired immunodeficiency syndrome (AIDS). Nucleoside HIV-1 reverse transcriptase (HIV-1 RT) inhibitors, such as 3′-azido-3′-deoxythymidine (AZT), dideoxycytidine (ddC), and dideoxyinosine (ddI), are now used clinically to treat AIDS patients. HIV-1 RT participates in converting the virus RNA genome to a double-stranded DNA intermediate which integrates into host cell DNA [1]. Triphosphate forms of nucleoside HIV-1 RT inhibitors formed through the action of host kinases are incorporated into the DNA intermediate by HIV-1 RT and then block the DNA polymerase activity of HIV-1 RT by chain termination of the template-primer [2–5]. AZT, however, cannot inhibit completely viral replication in chronically infected cells [6], and AZT- and ddI-resistant HIV-1 have both emerged on long-term therapy [7, 8]. Recently, non-nucleoside inhibitors of HIV-1 RT, including nevirapine [9] and tetrahydroimidazole [4,5,1-*jk*][1,4]-benzodiazepin-2(1*H*)-one and thione (TIBO) derivatives [10], have undergone clinical evaluation. These drugs selectively inhibit HIV-1 RT by a mechanism different from that of nucleoside analogs [11, 12] and, therefore, could be effective against AZT- or ddI-resistant mutant viruses. Unfortunately, strains of HIV-1 resistant to non-nucleoside HIV-1 RT inhibitors can be developed rapidly *in vitro* [13–15] and *in vivo*, the latter within as early as 4 weeks of therapy.

HIV-1 RT is a heterodimer composed of two subunits, p66 and p51, that have identical amino termini [16, 17]. It is a multifunctional viral enzyme that has RNA-dependent DNA polymerase, DNA-dependent DNA polymerase, and ribonuclease (RNase) H activities [18]. Recently, a 3.5 Å resolution electron density map of HIV-1 RT complexed with the non-nucleoside analog, nevirapine, was reported [19]. This three-dimensional crystalline structure, with its tentative locations of subdomains in the linear sequence of the enzyme, should guide the design of anti-HIV drugs targeted on the HIV-1 RT. Mutagenesis studies, however, will continue to complement and provide insight to the putative

* Corresponding author: Dr. Yung-Chi Cheng, Department of Pharmacology, Yale University School of Medicine, 333 Cedar St., New Haven, CT 06510. Tel. (203) 785-7119; FAX (203) 785-7129.

† Abbreviations: AIDS, acquired immunodeficiency syndrome; AZT, 3′-azido-3′-deoxythymidine; AZTTP, 3′-azido-3′-deoxythymidine 5′-triphosphate; ddC, dideoxycytidine; ddCTP, dideoxycytidine 5′-triphosphate; ddI, dideoxyinosine; ddATP, dideoxyadenosine 5′-triphosphate; D4TTP, 3′-deoxy-2′,3′-didehydrothymidine 5′-triphosphate; dNTP, deoxynucleoside triphosphate; HIV-1, human immunodeficiency virus type 1; HIV-1 RT, human immunodeficiency virus type 1 reverse transcriptase; k_{cat}, kinetic enzymatic turnover number; K_m, Michaelis constant; PCR, polymerase chain reaction; PFA, phosphonoformic acid; RT, reverse transcriptase; and TIBO, tetrahydroimidazole [4,5,1-*jk*] [1,4]-benzodiazepin-2(1*H*)-one and thione.

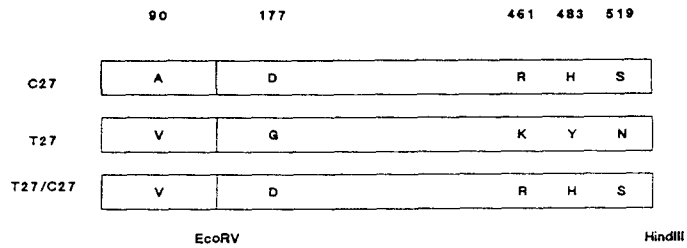

Fig. 1. Schematic representation of the control T27/C27 plasmid construction. The region encoding RT, flanked by *Eco*RV and *Hind*III sites, was exchanged between the drug-sensitive clone T27 and the drug-resistant clone C27 to construct the control RT clone T27/C27.

functional regions of HIV-1 RT [20–22]. The isolation and characterization of diverse mutants are, therefore, an important means to further define the functional sites on the HIV-1 RT enzyme.

In our laboratory, one recombinant HIV-1 RT cloned from chronically HIV-1 infected cells showed cross-resistance to 3′-azido-3′-deoxythymidine 5′-triphoshate (AZTTP) and phosphonoformic acid (PFA), a pyrophosphate analog. This mutant enzyme was analyzed in detail in an effort to better understand the putative deoxynucleoside triphosphate (dNTP) binding site.

MATERIALS AND METHODS

Compounds. AZTTP was obtained from R. F. Schinazi, Emory University (Atlanta, GA). Dideoxycytidine 5′-triphosphate (ddCTP), dideoxyadenosine 5′-triphosphate (ddATP), and PFA were purchased from the Sigma Chemical Co. (St. Louis, MO). 3′-Deoxy-2′,3′-didehydrothymidine 5′-triphosphate (D4TTP) was obtained from W. H. Prusoff, Yale University (New Haven, CT). Nevirapine was obtained from Boehringer Ingelheim Pharmaceuticals (Ridgefield, CT). The TIBO derivative R82150 was synthesized by K. Parker, Brown University (Providence, RI).

Cloning of HIV-1 RT. HIV-1 RT clones were prepared as described previously [15]. Briefly, total RNA was extracted from chronically HIV-1 (HTLV-IIIB strain; R. C. Gallo) infected H9 cells and was used as a template for polymerase chain reaction (PCR) amplification. The 1.7-kb RT product was digested with *Nco*I and *Hind*III, gel purified, and ligated into the plasmid expression vector pKK233-2 (Pharmacia, Piscataway, NJ); the resulting plasmid was used to transform *Escherichia coli* JM109. These clones express an active 66-kDa RT polypeptide after induction with isopropyl-β-D-thiogalactopyranoside.

To elucidate the role of amino acid 90 of RT in terms of RT sensitivity to inhibitors, a chimeric RT plasmid was prepared from the drug-sensitive clone T27 and the drug-resistant clone C27 (see Fig. 1). The plasmid C27 was restricted with *Eco*RV and *Hind*III, gel purified, and then the small HIV-1 RT fragment was ligated into the linearized plasmid T27 previously digested with *Eco*RV and *Hind*III.

RT assays. HIV-1 RT enzyme was expressed from recombinant plasmids in bacteria as described previously with minor modification [23]. The bacteria pellets were resuspended in lysis buffer (0.5 M NaCl, 10% glycerol, 0.1% Triton X-100, 2 mM EDTA, 5 mM dithiothreitol, 50 mM Tris, pH 7.8, 1 mM phenylmethylsulfonyl fluoride, 1 mg/mL lysozyme) and were incubated on ice for 30 min. After centrifugation, the supernatants were purified by using DEAE-52 and P-11 columns. For the RNA-dependent DNA polymerase assay, the partially purified enzyme was incubated in a 50 μL reaction mixture [50 mM Tris, pH 7.8, 50 mM KCl, 6 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mg/mL bovine serum albumin, 0.0025 units of template-primer [poly(rA)-oligo$(dT)_{10}$ or poly(rC)-oligo$(dG)_{12-18}$], 0.01 mM [^{3}H]dTTP or dGTP (sp. act. 1 Ci/mmol) for 30 min at 37°. The labeled product was precipitated by ice-cold 10% trichloroacetic acid, collected on Whatman glass GF/A filters, and counted. When DNase I-activated calf thymus DNA [24] was used as the template-primer, assay conditions were the same as described above except that 10 μM each of dATP, dTTP, and dGTP, and 1 μM [^{3}H]-dCTP (sp. act. 10 Ci/mmol) were used for ddCTP. For ddATP and AZTTP, assay conditions were the same as those for ddCTP except that different combinations of dNTPs were used. The kinetic analysis was made according to Lineweaver–Burk plots.

Construction of HIV-1 RT variants with mutation at amino acid 90 of RT. Different HIV-1 RT plasmids with mutation at amino acid 90 were made by oligonucleotide-directed mutagenesis [25] of the coding sequence for HIV-1 RT using protocols available commercially (Bio-Rad, Richmond, CA). The 1.7-kb HIV-1 RT gene coding region was cloned into the *Nco*I/*Hind*III sites of phagemid vector pSL1190 (Pharmacia), and the mutant clones were selected by nucleotide sequence analysis. The construct was then subcloned into the expression vector pKK233-2. The mutation was again confirmed by dideoxynucleotide sequencing.

RESULTS

When 13 recombinant HIV-1 RTs cloned from HIV-1 were screened for AZTTP and PFA resistance, heterogeneity of sensitivity of these cloned HIV-1 RTs was observed and one clone

Table 1. Sensitivity of HIV-1 RT clones to AZTTP and PFA

Clone	% RT activity	
	AZTTP (20 nM)	PFA (6 μM)
C4	69	21
C7	55	15
C10	25	9
C11	20	7
C13	29	6
C17	24	8
C19	46	4
C20	34	5
C22	66	10
C25	26	7
C27	90	59
C28	25	10
T27	33	7

(C27) showed resistance to both drugs (Table 1). To characterize this C27 HIV-1 RT, a chimeric HIV-1 RT plasmid (T27/C27) was constructed to be used for comparison by exchanging the region encoding RT, flanked by *Eco*RV and *Hind*III sites, between the drug-sensitive T27 and drug-resistant C27 clones (Fig. 1). The HIV-1 RT enzyme was then expressed in bacteria and purified with DEAE-52 and P-11 columns. Analysis of the partially purified enzyme by sodium dodecyl sulfate–polyacrylamide gel electrophoresis showed that more than 80% of protein had a molecular weight of 66 kDa.

The sensitivity of C27 and T27/C27 enzymes to antiviral nucleoside triphosphate analogs and PFA was tested by an *in vitro* assay using the appropriate templates. In a relative sense, the C27 HIV-1 RT showed cross-resistance to antiviral nucleoside triphosphate analogs, such as AZTTP, D4TTP, ddATP, and ddCTP, with 50% inhibitory concentration (IC_{50}) values 6- to 22-fold higher than the T27/C27 RT (Table 2). The C27 HIV-1 RT was also resistant to PFA with an IC_{50} value 20-fold higher than the control enzyme. This C27 HIV-1 RT, however, remained sensitive to non-nucleoside RT inhibitors, nevirapine and TIBO derivative R82150. To assess whether there is any difference between different templates used in this study in terms of the relative sensitivity of these two enzymes to inhibitors, the HIV-1 RT enzyme was tested against AZTTP using either poly(rA)-oligo$(dT)_{10}$ or an activated DNA as the template-primer. The results showed no significant difference between the two template-primers in terms of the ratio of the alteration of the IC_{50} value, although the absolute IC_{50} values were higher with activated DNA. The 66-kDa/51-kDa heterodimers prepared from pKRT2, which contains the HTLV-III BH10 RT gene [23], were also examined side-by-side with the 66-kDa/66-kDa homodimers from the wild-type control C27/T27. Similar results were obtained with these two different forms of reverse transcriptases (data not shown).

Sequence analysis showed a unique nucleotide change of GTT (Val^{90}) to GCT (Ala) in the C27 RT gene, which was postulated to be responsible for the change of sensitivity to antiviral nucleoside triphosphate analogs and PFA. To further analyze the role of amino acid 90 in HIV-1 RT in terms of interaction with antiviral nucleoside triphosphate analogs and PFA, the amino acid Val was replaced with amino acids that were closely related, aliphatic, and uncharged at physiologic pH, Leu, Thr, and Gly, using oligonucleotide-directed mutagenesis. The partially purified HIV-1 RT enzymes were prepared from each of the mutant forms of HIV-1 RT, and the sensitivity to anti-HIV nucleoside triphosphate analogs, such as AZTTP and D4TTP, and PFA was determined. These were 66-kDa/66-kDa homodimer preparations. Substitution of Val by the uncharged and relatively more hydrophilic amino acid Thr decreased the level of drug sensitivity similar to that seen with the C27 HIV-1 RT (Table 3). Substitution of Val by the much smaller, uncharged, and less hydrophobic amino acid Gly further decreased the drug sensitivity. By contrast, introduction of the larger, uncharged, hydrophobic amino acid Leu produced no significant change in the sensitivity to AZTTP and D4TTP, but a 2-fold

Table 2. Sensitivity of recombinant HIV-1 RTs to RT inhibitors and PFA

Compound	IC_{50}		Fold resistance
	T27/C27	C27	
AZTTP*	5.6 ± 1.3 nM	36 ± 11 nM	6
AZTTP†	58 ± 10 nM	490 ± 14 nM	8
D4TTP*	12 ± 4 nM	80 ± 18 nM	7
ddATP†	0.11 ± 0.07 μM	2.4 ± 1.1 μM	22
ddCTP†	0.21 ± 0.04 μM	3.3 ± 0.1 μM	16
PFA*	0.30 ± 0.09 μM	5.9 ± 1.7 μM	20
Nevirapine‡	109 ± 30 nM	111 ± 47 nM	1
TIBO R82150‡	186 ± 6 nM	154 ± 46 nM	1

*–‡ Template-primer: * poly(rA)-oligo$(dT)_{10}$; † activated DNA; and ‡ poly(rC)-oligo$(dG)_{12-18}$. Calculations of IC_{50} and standard deviations were done from at least three determinations and the average values ± range are presented.

Table 3. Comparative studies of sensitivity of HIV-1 RT variants with a different amino acid at 90

Amino acid at 90	R*	IC_{50} AZTTP (nM)	IC_{50} D4TTP (nM)	IC_{50} PFA (μM)
Val (T27/C27)	$-CH-CH_3$ CH_3	5.6 ± 1.3	12 ± 4	0.30 ± 0.09
Leu	$-CH_2-CH-CH_3$ CH_3	7.0 ± 0.8	9.8 ± 1.9	0.63 ± 0.05
Thr	$-CH-CH_3$ OH	27 ± 5	48 ± 9	4.0 ± 0.1
Ala (C27)	CH_3	36 ± 11	80 ± 18	5.9 ± 1.7
Gly	-H	142 ± 11	235 ± 8	29 ± 1

All the reaction rates were normalized to the reaction obtained using the template-primer, poly(rA)-oligo(dT)$_{10}$. The calculations of IC_{50} and standard deviations were done from at least three determinations with the exception of Thr in which only two determinations were performed and the average values ± range are presented.

* R–CH–COOH
  NH_2

increase in the IC_{50} value with PFA. These results suggest that a decrease in amino acid hydrophobicity and/or side-chain volume at this site confers decreased sensitivity of the recombinant HIV-1 RT to anti-HIV nucleoside triphosphate analogs and PFA.

The kinetic studies of mutant HIV-1 RT enzymes that have Thr, Ala, or Gly substitutions at amino acid 90 showed an increase in the Michaelis constant (K_m) values for thymidine triphosphate (dTTP), indicating a decrease in binding of dTTP compared with T27/C27 HIV-1 RT (Table 4). The inhibition constant (K_i) values for inhibition by AZTTP and PP_i also increased. The turnover rates of mutant HIV-1 RTs, however, were not changed significantly compared with control. Inhibition by AZTTP was competitive and inhibition by PP_i was noncompetitive with respect to dTTP (data not shown).

The HIV-1 RT prefers Mg^{2+} as divalent metal cation for optimal enzyme activity. In contrast, Mn^{2+} is preferred by the RTs of the murine leukemia viruses. To test whether there is an alteration of dependence in RT activity on the divalent cation with mutation at amino acid 90, purified HIV-1 RTs were assayed under identical conditions with various concentrations of Mg^{2+} and Mn^{2+}. The control T27/C27 HIV-1 RT was more active with Mg^{2+}. By contrast, the Gly mutant HIV-1 RT seemed to prefer Mn^{2+} as divalent cation, whereas the other three mutant HIV-1 RTs showed no significant preference for Mg^{2+} or Mn^{2+} (Fig. 2). The effects of different KCl concentrations on the mutant HIV-1 RT activity were also tested, and the results showed that the activity of the mutant HIV-1 RTs was less responsive to different KCl concentrations than the control T27/C27 HIV-1 RT.

DISCUSSION

Given the poor fidelity of HIV-1 RT [26, 27], it is not surprising that the HIV-1 strains from different laboratories are heterogeneous with respect to their nucleotide sequences. Thirteen recombinant HIV-1 RTs, obtained from RNA of HTLV-IIIB infected H9 cells via the PCR technique, showed heterogeneity in their sensitivity to AZTTP and PFA. Sequence analysis of some of the HIV-1 RT clones also showed variation in the nucleotide sequences (data not shown). This supports the notion that HIV-1 RTs in the HIV-1 subpopulation are heterogeneous, and also implies that a multi-drug resistant viral phenotype may pre-exist in the HIV-1 subpopulation given that the C27 clone with a pleiotropic HIV-1 RT phenotype was obtained without prior drug selection. Although we cannot rule out that the HIV-1 RT clone, C27, is an artifact resulting from

Table 4. Kinetic and inhibitor constants of HIV-1 RT variants

Amino acid at 90	K_m dTTP (μM)	k_{cat} (min^{-1})	K_i AZTTP (nM)	K_i PP_i (mM)
Val (T27/C27)	5	72	3	0.1
Ala (C27)	27	102	25	0.4
Thr	20	67	18	ND*
Gly	65	86	113	2.2

* Not done.

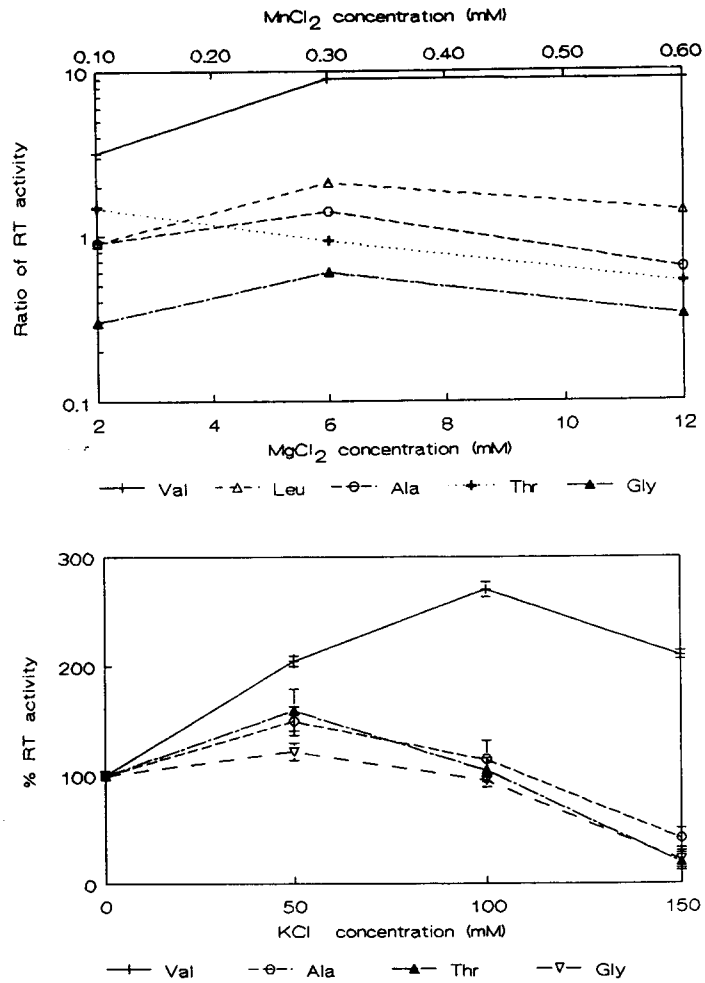

Fig. 2. Top panel: Dependence of HIV-1 RT activity on divalent cations. The control and mutant HIV-1 RTs were assayed as described in Materials and Methods with various concentrations of Mg^{2+} or Mn^{2+}. The RT activity was presented as a ratio of RT activities from three different sets of $MgCl_2$/$MnCl_2$ concentrations (2 mM/0.1 mM, 6 mM/0.3 mM, 12 mM/0.6 mM). Bottom panel: Effect of KCl concentration on HIV-1 RT activity, using the template-primer poly(rC)-oligo$(dG)_{12-18}$. The control and mutant HIV-1 RTs were assayed as described in Materials and Methods with different concentrations of KCl. The calculations of standard deviations were determined after the assays were performed twice in triplicate and averaged. The values for 50, 100 and 150 mM KCl concentrations are, respectively: Val 205 ± 5, 270 ± 7, 210 ± 4; Ala 150 ± 13, 115 ± 17, 42 ± 9; Thr 160 ± 19, 105 ± 11, 20 ± 8; and Gly 122 ± 8, 95 ± 6, 22 ± 8 (Leu was not assayed). The RT activity is presented as percent activity compared with that in the absence of KCl.

the PCR technique, it should be noted that there was no significant variation in the nucleotide sequences when the identical technique was employed to study the structure of DNA topoisomerase I (unpublished data).

Whether the original C27 clone is an artifact or not, the amino acid 90 in HIV-1 RT is implicated by this study to play a critical role in determining the sensitivity of HIV-1 RT to triphosphate forms of antiviral nucleoside analogs and PFA. In the C27 clone, a relatively large change of the sensitivity to antiviral nucleoside triphosphate analogs and PFA resulted from the replacement of valine's larger and hydrophobic residue by a smaller and less hydrophobic residue such as alanine. The correlation between decrease in side-chain volume and/or hydrophobicity at this site and reduced sensitivity to both anti-HIV nucleoside triphosphate analogs and PFA was further substantiated when Val was replaced with Thr and Gly. The change of drug sensitivity due to the less hydrophobic Thr mutation at this site paralleled the result of the Ala mutation in which both side-chain volume and hydrophobicity were decreased, suggesting that diminished hydrophobicity and not side-chain volume is the predominant determinant with the Thr mutation. The Gly mutation, which further decreases both side-chain volume and hydrophobicity in comparison

with the Ala mutation, showed an even higher degree of drug resistance. In contrast, when the Val was substituted by the larger and more hydrophobic Leu, there was no significant change in the drug sensitivity.

When the hydrophobicity was decreased by substitution at amino acid 90, the K_m values for dTTP increased without a significant effect on the kinetic enzymatic turnover number (k_{cat}), suggesting that this amino acid may be involved primarily in binding the dNTP substrate. These results are consistent with the suggestion that the association of the dNTP ligands with HIV-1 RT is predominantly hydrophobically driven [28], because the decrease in hydrophobicity induced by mutation at amino acid 90 accompanied a decrease in binding of dTTP. The substitution of amino acid 90 could also alter the binding of the PP_i analog PFA and PP_i, a component of dNTP, suggesting that the amino acid 90 plays an important role especially in the binding of the pyrophosphate group of dNTP. This idea may explain the reason why the substitution of amino acid 90 confers multi-drug resistance to both all antiviral nucleoside triphosphate analogs tested in this study and PFA. Our previous observation that the inhibition of HIV-1 RT by AZTTP and PFA is kinetically mutually exclusive also supports this idea [4]. A recent study [29] has reported that a variant HIV-1 RT enzyme that is resistnat to 2′,3′-dideoxyguanosine triphosphate (ddGTP) has Gly instead of Glu at amino acid 89 and demonstrates broad cross-resistance to antiviral nucleoside analogs and PFA. This report substantiates the importance of positions 89 and 90 with respect to the dNTP binding domain. To relate our findings to the newly crystallized HIV-1 RT, amino acid 90 lies at the purported juncture of the "fingers" and "palm" subdomains [19]. Its location here, in a hinged groove, would appear to align the pyrophosphate group, while the elongation site of the template primer is positioned in the full cleft of the polymerase active site formed by the designated "fingers," "palm," and "thumb" domains.

It is also of interest that there was an alteration in the dependence of activity of mutant RTs on its divalent cation. In *E. coli* DNA polymerase I, the divalent cation is coordinated with the γ-phosphoryl group of dNTPs to form the initial complex, which is then bound to the enzyme to form a metal-pyrophosphate chelate ring [30]. However, it is not clear whether a similar complex is formed in HIV-1 RT. The role of divalent cations in HIV-1 RT action requires further studies.

The emergence of HIV-1 resistant to the currently available HIV-1 RT inhibitors is a major problem in the treatment of AIDS patients [7, 8]. It is thus noteworthy that the mutation of a single amino acid on the recombinant HIV-1 RT could confer a significant level of cross-resistance to the triphosphate forms of all anti-HIV nucleoside analogs studied. It would be alarming if such a simple mutation could confer broad cross-resistance to nucleoside HIV-1 RT inhibitors and PFA *in vivo*.

The discrepancy between the mutant HIV-1 RT and proviral HIV-1 with the same mutation in terms of drug sensitivity suggests that the drug sensitivity of HIV-1 RT *in vitro* may not be applicable to the drug sensitivity of HIV in cell culture. Specific mutations in the HIV-1 RT gene have been found in AZT-resistant isolates of HIV-1 from AZT-treated AIDS patients. The mutations are sufficient to confer the drug resistance on the virus in culture when introduced into a cloned wild-type genome [31]. By contrast, the mutant HIV-1 RT isolated from the resistant virus or expressed in bacteria does not show any significant changes in its sensitivity to AZTTP. The mutant HIV-1 RT with a mutation at amino acid 89 did not show increased resistance to 2′,3′-dideoxyguanosine, although the mutant HIV-1 RT was resistant to ddGTP [29]. Therefore, we are currently pursuing proviral HIV-1 constructs with mutation at amino acid 90 in the HIV-1 RT to assess the infectivity and sensitivity of these HIV-1 variants to nucleoside HIV-1 RT inhibitors and PFA. Preliminary results, in collaboration with John W. Mellors at the University of Pittsburg, show that the recombinant proviral HIV-1 with the Ala mutation at amino acid 90 was able to propagate in both MT-2 and H9 cells. The efficiency of ths propagation as measured by p24 levels and tissue cytotoxic inhibitory $dosage_{50}$ appears, as expected from our kinetic analysis, less that the control construct with Val at position 90. The sensitivity of the virus from the transfected cells to the nucleoside analogs and PFA is currently being explored.

In summary, we demonstrated here that the mutation at amino acid 90 of HIV-1 RT can cause cross-resistance to antiviral nucleoside triphosphate analogs and PFA and that this amino acid is involved in binding of the pyrophosphate group of dNTP. The three-dimensional structure of the crystallized HIV-1 RT suggests that the amino acid 90 lies within the juncture of the "fingers" and "palm" subdomains of the polymerase cleft of HIV-1 RT near the putative catalytic site. It is clear, however, that this amino acid plays an important role with regard to the binding to the pyrophosphate group of dNTP. With the newly characterized three-dimensional structure of HIV-1 RT, the kinetic and mutational information obtained from this study will further the understanding of the molecular mechanism of action of HIV-1 RT.

Acknowledgement—This work was supported by National Cancer Institute Grant CA-44358.

REFERENCES

1. Varmus H, Retroviruses. *Science* **240**: 1427–1435, 1988.
2. Mitsuya H, Weinhold KJ, Furman PA, St. Clair MH, Nusinoff Lehrman S, Gallo RC, Bolognesi D, Barry DW and Broder S, 3′-Azido-3′-deoxythymidine (BW A509U): An antiviral agent that inhibits the infectivity and cytopathic effect of human T-lymphotropic virus type III/lymphadenopathy-associated virus *in vitro*. *Proc Natl Acad Sci USA* **82**: 7096–7100, 1985.
3. Cheng YC, Dutschman GE, Bastow KF, Sarngadharan MG and Ting RYC, Human immunodeficiency virus reverse transcriptase. *J Biol Chem* **262**: 2187–2189.
4. Starnes MC and Cheng YC, Inhibition of human immunodeficiency virus reverse transcriptase by 2′,3′-dideoxynucleoside triphosphates: Template dependence, and combination with phosphoformate. *Virus Genes* **2**: 241–251, 1988.

5. Vazquez-Padua MA, Starnes MC and Cheng YC, Incorporation of 3'-azido-3'-deoxythymidine into cellular DNA and its removal in a human leukemia cell line. *Cancer Commun* **2**: 55–62, 1990.
6. Ho DD, Moudgil T and Alam M, Quantitation of human immunodeficiency virus type 1 in the blood of infected persons. *N Engl J Med* **321**: 1621–1625, 1989.
7. Larder BA, Darby G and Richman DD, HIV with reduced sensitivity to zidovudine (AZT) isolated during prolonged therapy. *Science* **243**: 1731–1734, 1989.
8. St. Clair MH, Martin JL, Tudor-Williams SG, Bach MC, Vavro CL, King DM, Kellam P, Kemp SD and Larder BA, Resistance to ddI and sensitivity to AZT induced by a mutation in HIV-1 reverse transcriptase. *Science* **253**: 1557–1559, 1991.
9. Merluzzi VJ, Hargrave KD, Labadia M, Grozinger K, Skoog M, Wu JC, Shih C-K, Eckner K, Hattox S, Adams J, Rosenthal AS, Faanes R, Eckner RJ, Koup RA and Sullivan JL, Inhibition of HIV-1 replication by a nonnucleoside reverse transcriptase inhibitor. *Science* **250**: 1411–1413, 1990.
10. Pauwels R, Andries K, Desmyter J, Schols D, Kukla MJ, Breslin HJ, Raeymaeckers A, Gelder JV, Woestenborghs R, Heykants J, Schellekens K, Janssen MAC, DeClerq E and Janssen PAJ, Potent and selective inhibition of HIV-1 replication *in vitro* by a novel series of TIBO derivatives. *Nature* **343**: 470–474, 1990.
11. Shih C-K, Rose JM, Hansen GL, Wu JC, Bacolla A and Griffin JA, Chimeric human immunodeficiency virus type 1/type 2 reverse transcriptases display reversed sensitivity to nonnucleoside analog inhibitors. *Proc Natl Acad Sci USA* **88**: 9878–9882, 1991.
12. Tramontano E and Cheng Y-C, HIV-1 reverse transcriptase inhibition by a dipyridodiazepinone derivative: BI-RG-587. *Biochem Pharmacol* **43**: 1371–1376, 1992.
13. Nunberg JH, Schleif WA, Boots EJ, O'Brien JA, Quintero JC, Hoffman JM, Emini EA and Goldman ME, Viral resistance to human immunodeficiency virus type-1 specific pyridinone reverse transcriptase inhibitors. *J Virol* **65**: 4887–4892, 1991.
14. Richman D, Shih C-K, Lowy I, Rose J, Prodanovich P, Goff S and Griffin J, Human immunodeficiency virus type 1 mutants resistant to nonnucleoside inhibitors of reverse transcriptase arise in tissue culture. *Proc Natl Acad Sci USA* **88**: 11241–11245, 1991.
15. Mellors JW, Dutschman GE, Im G-J, Tramontano E, Winkler SR and Cheng Y-C, *In vitro* selection and molecular characterization of human immunodeficiency virus-1 resistant to non-nucleoside inhibitors of reverse transcriptase. *Mol Pharmacol* **41**: 446–451, 1992.
16. Veronese FM, Copeland TD, DeVico AL, Rahman R, Oroszlan S, Gallo RC and Sarngadharan MG, Characterization of highly immunogenic p66/p51 as the reverse transcriptase of HTLV-III/LAV. *Science* **231**: 1289–1291, 1986.
17. Lightfoote MM, Coligan JE, Folks TM, Fauci AS, Martin MA and Venkatesan S, Structural characterization of reverse transcriptase and endonuclease polypeptides of the acquired immunodeficiency syndrome retrovirus. *J Virol* **60**: 771–775, 1986.
18. Gilboa E, Mitra W, Goff S and Baltimore D, A detailed model of reverse transcriptase and tests of crucial aspects. *Cell* **18**: 93–100, 1979.
19. Kohlstaedt LA, Wang J, Friedman JM, Rice PA and Steitz TA, Crystal structure at 3.5 Å resolution of HIV-1 reverse transcriptase complexed with an inhibitor. *Science* **256**: 1783–1790, 1992.
20. Larder BA, Kemp SD and Purifoy DJM, Infectious potential of human immunodeficiency virus type 1 reverse transcriptase mutants with altered inhibitor sensitivity. *Proc Natl Acad Sci USA* **86**: 4803–4807, 1989.
21. Boyer PL, Ferris AL and Hughes SH, Cassette mutagenesis of the reverse transcriptase of human immunodeficiency virus type 1. *J Virol* **66**: 1031–1039, 1992.
22. Lowe DM, Parmar V, Kemp SD and Larder BA, Mutational analysis of two conserved sequence motifs in HIV-1 reverse transcriptase. *FEBS Lett* **282**: 231–234, 1991.
23. D'Aquila RT and Summers WC, HIV-1 reverse transcriptase/ribonuclease H: High level expression in *Escherichia coli* from a plasmid constructed using the polymerase chain reaction. *J Acquir Immune Defic Syndr* **2**: 579–587, 1989.
24. Baril E, Mitchener J, Lee L and Baril B, Action of pancreatic DNase; Requirements for activation of DNA as a template-primer for DNA polymerase. *Nucleic Acids Res* **4**: 2641–2653, 1977.
25. Kunkel TA, Roberts JD and Zakour RA, Rapid and efficient site-specific mutagenesis without phenotypic selection. *Methods Enzymol* **154**: 367–382, 1987.
26. Preston BD, Poiesz BJ and Loeb LA, Fidelity of HIV-1 reverse transcriptase. *Science* **242**: 1168–1171, 1988.
27. Roberts JD, Bebenek K and Kunkel TA, The accuracy of reverse transcriptase from HIV-1. *Science* **242**: 1171–1173, 1988.
28. Painter GR, Wright LL, Hopkins S and Furman PA, Initial binding of 2'-deoxynucleoside 5'-triphosphates to human immunodeficiency virus type 1 reverse transcriptase. *J Biol Chem* **266**: 19362–19368, 1991.
29. Prasad VR, Lowy I, de los Santos T, Chiang L and Goff SP, Isolation and characterization of a dideoxyguanosine triphosphate-resistant mutant of human immunodeficiency virus reverse transcriptase. *Proc Natl Acad Sci USA* **88**: 11363–11367, 1991.
30. Ferrin LJ and Mildvan AS, NMR studies of the conformations and interactions of substrates and ribonucleotide templates bound to the large fragment of DNA polymerase I. In: *DNA Replication and Recombination* (Eds. McMacken R and Kelly TJ), pp. 75–87. Alan R. Liss, New York, 1987.
31. Larder BA and Kemp SD, Multiple mutations in HIV-1 reverse transcriptase confer high-level resistance to zidovudine (AZT). *Science* **246**: 1155–1158, 1989.