The K65R Mutant Reverse Transcriptase of HIV-1 Cross-resistant to 2',3'-Dideoxyctydine, 2',3'-Dideoxy-3'-thiacytidine, and 2',3'-Dideoxyinosine Shows Reduced Sensitivity to Specific Dideoxynucleoside Triphosphate Inhibitors in Vitro*

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The K65R mutation in human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) confers cross-resistance to 2',3'-dideoxyctydine (ddC), 2',3'-dideoxy-3'-thiacytidine (3TC), and 2',3'-dideoxyinosine (ddI). We characterized the in vitro sensitivities of recombinant wild type (wt) and K65R mutant RT to dideoxynucleoside triphosphate (ddNTP) inhibitors, using a variety of primer-templates. With poly(rA)-oligo(dT), the K65R mutant showed slight increases in $K_\text{m}$ for ddTTP and 3'-azido,3'-deoxythymidine triphosphate (AZTTP) compared to wt RT, but neither wt nor K65R RT was inhibited by ddCTP or ddATP. With poly(rA)-oligo(dC), the K65R mutant showed a 2-fold increase in $K_\text{m}$ for ddGTP and a 20-fold increase in $K_\text{m}$ for ddCTP compared to wt, whereas ddATP, ddTTP, and AZTTP failed to inhibit either enzyme. With a heteropolymeric primer-template, the K65R mutant showed 10-fold reduced sensitivities to ddCTP, 3TCTP, and ddATP, and 4-fold reduced sensitivity to AZTTP, compared to wt. In contrast, both enzymes were equally inhibited by ddTTP and ddGTP. HIV-1 cross-resistance to ddC/3TC/ddI resulting from the K65R mutation may therefore involve selective alterations in substrate/inhibitor recognition. Additionally, competitive inhibition by ddNTPs noncomplementary to the template base appears to be unimportant in the mechanism of inhibition of HIV-1 RT by dideoxynucleoside analogs.

The human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) is essential for the conversion of viral genomic RNA into double-stranded DNA that can then be integrated into the infected host cell genome. This enzyme provides a primary target for the development of antiviral compounds such as the nucleoside analogs 3'-azido,3'-deoxythymidine (AZT), 2',3'-dideoxyinosine (ddI), and 2',3'-dideoxyctydine (ddC), and 2',3'-dideoxy-3'-thiacytidine (3TC). These nucleoside analogs, once intracellularly metabolized to their triphosphate forms, are believed to inhibit HIV-1 RT by acting both as chain terminators of the nascent DNA chain and as competitive inhibitors with respect to the natural dideoxynucleoside triphosphate (dNTP) substrates (Goody et al., 1991). Although such drugs reduce viral load in HIV-1-infected individuals, their use has unfortunately resulted in the appearance of drug-resistant strains of HIV-1 (reviewed by Richman (1993)). In all cases to date, this resistance correlates with mutations in HIV-1 RT (Lander and Kemp, 1989; St. Clair et al., 1991; Fitzgibbon et al., 1992; Gu et al., 1992, 1994). The molecular mechanisms of the antiviral drug resistance of HIV-1 are not yet clear. The RT in lysates of HIV-1 strains that show more than 100-fold resistance to AZT in cell culture has identical sensitivity to AZTTP in vitro compared to RT in lysates of wild type virus (Wainberg et al., 1990; Lacey et al., 1992). Martin and co-workers (1993) have used purified recombinant RT to demonstrate that the L74V mutation, responsible for single-drug resistance to ddI, may result in altered substrate/inhibitor recognition by the enzyme. Our recent studies have implied that a subdomain of HIV-1 RT, in which five mutations correlated with nucleoside analog drug resistance occur, may be important for the correct binding of the dNTP complementary to the cognate template base residue (Wu et al., 1993).

We and others have shown that cross-resistance to ddC, 3TC, and ddI results from a K65R mutation in the viral RT (Gu et al., 1994; Zhang et al., 1994). To elucidate the mechanism of resistance caused by this mutation, we used site-directed mutagenesis to introduce the K65R substitution into vectors allowing expression of both p66 and p51 subunits of HIV-1 RT. We report here that purified recombinant p51-p66 K65R mutant RT shows significantly decreased in vitro sensitivity to ddCTP, 3TCTP, and ddATP (the intracellular active form of ddI) but little or no change in sensitivity to ddTTP, AZTTP, or ddGTP, compared to wt enzyme.

MATERIALS AND METHODS

AZTTP was purchased from Moravek Biochemicals. 3TCTP was a generous gift from Glaxo Group Research (Greenford, United Kingdom). Restriction enzymes were obtained from Boehringer Mannheim. The Megascript™ T7 polymerase transcription kit was a product of Ambion (Austin, TX). The expression vector pKK223-3, ultrapure dNTPs, and the homopolymeric primer-templates (P/T) poly(rA)-oligo(dT)16-17 and poly(rC)-oligo(dG)16-19 were purchased from Pharmacia Biotech Inc. The P/T poly(rA)-oligo(dC)16-19 was prepared from poly(rA) and oligo(dC)16-19 (Pharmacia) as described (Martin et al., 1993). DNA oligonucleotides were synthesized by GSD (Toronto, Canada). Heteropolymeric template-primer for RT RNA-dependent DNA polymerase activity was prepared from RNA transcripts of plasmid pHIV-PBS and a synthetic 18-nucleotide DNA primer, as described.
\textit{Escherichia coli} JM109 was transformed with either wt or

When RTeu was paired with reverse primer RT5ld (5'-CTAAGC(T-

al., 1989; Clark et al., 1990; Deibel et al., 1990) (see Table I).

\begin{table}[h]
\centering
\begin{tabular}{cccccc}
\hline
\textbf{Template/primers} & \textbf{Substrate} & \textbf{\(K_m\)} & \textbf{\(V\)} & \textbf{\(V/K_m\)}
\hline
Poly(r)-oligo(dT) & dTTP & 5.1 & 0.9 & 4.7 & 1.2 & 3.9 & 0.2 & 4.0 & 0.3 & 0.76 & 0.85
Poly(r)-oligo(dC) & dCTP & 12.2 & 1.6 & 26.4 & 4.8 & 20.5 & 1.3 & 15.1 & 1.2 & 1.7 & 0.6
Poly(C)-oligo(dG) & dGTP & 1.7 & 0.1 & 2.3 & 0.3 & 12.6 & 0.8 & 10.1 & 0.4 & 7.4 & 4.4
Heteropolymeric & dCTP & 3.3 & 0.8 & 10.2 & 0.9 & 1.1 & 0.1 & 1.3 & 0.1 & 0.33 & 0.13
 & dATP & 0.4 & 0.05 & 0.7 & 0.04 & 1.3 & 0.1 & 1.1 & 0.1 & 3.2 & 1.7
 & dGTP & 0.3 & 0.03 & 0.4 & 0.02 & 2.4 & 0.1 & 2.7 & 0.1 & 8.0 & 6.8
 & dTTP & 1.4 & 0.1 & 2.0 & 0.3 & 4.9 & 1.4 & 5.0 & 0.3 & 3.5 & 2.5
\hline
* Wild type
\footnote{Reported as nmol dNMP incorporated/30 min/µg p51/p66 RT heterodimer.}
\end{tabular}
\caption{Values are means ± standard errors of at least three separate determinations.}
\end{table}

(\textit{Arts et al.}, 1994). \(\text{PHI}^{+}\) and \(\alpha^{+}\)-DNSTPs were products of Amersham Corp. and DuPont.

\textbf{Cloning of Wild Type and Mutated pHTE6 and pHTE5.}--The HIV-1

provincial clone HXB2 was obtained from the AIDS Research and Reference

Reagent Program, Division of AIDS, NIAID, National Institutes of

Health, courtesy of Drs. B. Hahn and G. Shaw. We used the polymerase

chain reaction to amplify the complete RT coding sequence of HXB2

(nucleotides 2549-4228) using RTeu (5'-CTAAGC(T-GACTA)-CTAAGGATATTGAG-3') and RTld

(5'-CTAAGC(T-GACTA)-TAGTACTT-TCCTGATTCGAG-3'), respectively, of the RT coding region, giving rise to construct pRT66.

The initiation and stop codons (delineated by parentheses) were inserted immediately upstream and downstream, giving rise to construct pRT66. The amplified segment was digested with EcoRI and HindIII, restricted by homopolymeric and heteropolymeric PIT. Enzyme Assays--RT RNA-dependent DNA polymerase activity was measured as described (\textit{Wu et al.}, 1993), but with variable concentrations of ddNTP in the assay. With the heteropolymeric PIT, the concentration of dCTP, dATP, dTTP, or dGTP was varied while the concentrations of the other three ddNTPs were held constant at 5 µM each.

In assays of ddNTP inhibition, reactions included saturating concentrations of both wt and K65R and variable concentrations of ddNTPs.

\textbf{Kinetic parameters of the reconstituted RT enzymes were calculated as described previously (\textit{Wu et al.}, 1993). Inhibition constants were calculated by fitting the data to the equation} \(K_{\text{inhib}} = K_e + [S]/K_m\) (\textit{Segel}, 1975).

\textbf{RESULTS}

\textbf{Cloning and Expression of wt and K65R RT.}--The p51 and p66 subunits of wt and K65R mutant RT were overexpressed separately in \textit{E. coli} JM109. The p51/p66 heterodimeric forms of wt or K65R RT were formed by mixing aliquots of the p51 and p66 lysates prior to purification, as described under "Materials and Methods." High performance liquid chromatographic method of the ddNTP inhibitors were able to reduce RT activity, with no inhibition by ddCTP, ddATP, or ddGTP. Kinetic parameters of the reconstituted RT enzymes were calculated as described previously (\textit{Wu et al.}, 1993). Inhibition constants were calculated by fitting the data to the equation} \(K_{\text{inhib}} = K_e + [S]/K_m\) (\textit{Segel}, 1975).

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\textbf{RESULTS}

\textbf{Cloning and Expression of wt and K65R RT.}--The p51 and p66 subunits of wt and K65R mutant RT were overexpressed separately in \textit{E. coli} JM109. The p51/p66 heterodimeric forms of wt or K65R RT were formed by mixing aliquots of the p51 and p66 lysates prior to purification, as described under "Materials and Methods." High performance liquid chromatographic method of the ddNTP inhibitors were able to reduce RT activity, with no inhibition by ddCTP, ddATP, or ddGTP. Kinetic parameters of the reconstituted RT enzymes were calculated as described previously (\textit{Wu et al.}, 1993). Inhibition constants were calculated by fitting the data to the equation} \(K_{\text{inhib}} = K_e + [S]/K_m\) (\textit{Segel}, 1975).
HIV Cross-resistance to ddC, 3TC, and ddl

Table II
Inhibition of wild type and K65R reverse transcriptase by deoxyribonucleotide triphosphates

| Template/primer | Substrate | Inhibitor | IC₅₀ | K₆₅R | K₅₅R | K₆₅R/K₅₅R |
|----------------|-----------|-----------|------|-------|-------|-------------|
| poly(rA)-oligo(dT) | dTTP | ddTTP | 0.011 | 0.067 | 0.005 | 0.023 |
| | | AZTTP | 0.027 | 0.099 | 0.008 | 0.055 |
| | | >100 | >100 | >100 | >100 | >100 |
| | | ddATP | >100 | >100 | >100 | >100 |
| | | ddCTP | 0.08 | 2.8 | 0.07 | 1.5 |
| | | 3TCTP | 0.30 | 2.2 | 0.05 | 1.4 |
| | | >100 | >100 | >100 | >100 | >100 |
| | | ddATP | >50 | >50 | >50 | >50 |
| | | AZTTP | >50 | >50 | >50 | >50 |
| poly(rT)-oligo(dC) | dCTP | ddCTP | 0.08 | 0.08 | 0.02 | 0.015 |
| | | AZTTP | 0.2 | 1.6 | 0.04 | 0.75 |
| | | >100 | >100 | >100 | >100 | >100 |
| | | ddCTP | >50 | >50 | >50 | >50 |
| | | 3TCTP | >50 | >50 | >50 | >50 |
| | | ddGTP | >50 | >50 | >50 | >50 |
| | | 3TDP | >50 | >50 | >50 | >50 |
| | | ddTTP | >50 | >50 | >50 | >50 |
| | | AZTTP | >50 | >50 | >50 | >50 |
| poly(rC)-oligo(dG) | dGTP | ddGTP | 0.001 | 0.004 | 0.002 | 0.007 |
| | | AZTTP | 0.007 | 0.013 | 0.006 | 0.13 |
| | | >100 | >100 | >100 | >100 | >100 |
| Heteropolymeric | dNTPs | >100 | >100 | >100 | >100 | >100 |

* The highest concentration of inhibitor used in these inhibition studies was 50 μM.
* The concentration of each of the dNTPs was 5 μM.

II). Similar decreases in sensitivity of the K65R mutant were noted for inhibition by ddCTP (Fig. 1A), 3TCTP (Fig. 1B), and ddATP (Fig. 1C) of dCMP incorporation into the heteropolymeric P/T. The sensitivity of the K65R mutant to inhibition by AZTTP was less affected, with about 4–6-fold increases in K₆₅R relative to wt RT with either homopolymeric or heteropolymeric P/T (Table II; Fig. 1F). Interestingly, the K65R mutant and wt enzymes had essentially equal sensitivities to inhibition by ddGTP and ddCTP of dCMP incorporation into the heteropolymeric P/T (Fig. 1, D and E).

DISCUSSION

The K65R mutation of HIV-1 RT occurs within an IKKK motif conserved in other DNA polymerases (Hizi et al., 1989; Boyer et al., 1992). Mutations correlated with resistance to antiviral drugs have been described at sites 65, 67, 69, 70, and 74 (Larder et al., 1989; St. Clair et al., 1991; Gu et al., 1994). These residues are in the "fingers" subdomain of HIV-1 RT (Kohlstaedt et al., 1992; Jacobo-Molina et al., 1993) and may be important for enzyme-P/T interaction (Kohlstaedt et al., 1992) and for the binding of dNTP substrate complementary to the template base during formation of the RT-P/T-dNTP ternary complex (Wu et al., 1993).

It was reported previously that the RNA-dependent DNA polymerase activity of K65R RT is less than 5% of that associated with wt enzyme (Boyer et al., 1992). We did not observe any reduction in activity of recombinant K65R RT, with both wt and K65R mutant RT having similar V values for RNA-dependent DNA synthesis determined with several P/T (Table I). Fully processed HIV-1 RT is a heterodimer consisting of 66- and 51-kDa subunits, the latter derived by proteolytic cleavage of the 66-kDa subunit by the HIV-1 protease (Rey, 1984; Hoffman et al., 1985). Although both the p66-p66 homodimer and p51-p56 heterodimer are functionally active, only the heterodimer is present in mature HIV-1 virions (Veronese et al., 1986). The differences between our results and those of Boyer et al. might be attributed to the fact that our studies were performed with the more biologically relevant p51-p66 heterodimer, while Boyer et al. (1992) used a p66-p66 homodimer. However, we have found that the p66 form of our recombinant K65R RT has activity comparable to that of the wt p66 (data not shown).

We used a variety of P/T in the present studies. The apparent K₉₅ values for each of the dNTPs with both wt and mutant RT were lower when measured with heteropolymeric P/T than with homopolymeric P/T. This may be due to differences in RT conformation in the RT-P/T complex with different primer-templates. We have noted significant changes in non-nucleoside inhibitor and monoclonal antibody binding to RT when the enzyme is complexed to different P/T.² The K₉₅ and V values for our recombinant wild type RT-catalyzed incorporation of dTMP and dCMP into RNA templates were comparable to previously published values (Martin et al., 1993; Reardon et al., 1990).

No significant differences were observed in the K₉₅ for dTTP and dGTP, whereas 2–3-fold increases in K₉₅ were seen for dCTP and dATP with the mutant enzyme relative to wt RT. Nonetheless, both wt and K65R RT had similar V values no matter which dNTP was used, implying that the K65R mutation results in a selective alteration in recognition for dCTP and dATP, but not for dTTP or dGTP.

The "catalytic efficiency" of the enzyme was assessed from V/K ratios (Table I). With dCTP as substrate and poly(rT)-oligo(dC) as P/T, conditions which promote distributive DNA synthesis (Martin et al., 1990), the K65R mutant showed a 3-fold lower V/K compared to wt RT. However, no significant differences in V/K ratio was noted in processive DNA synthesis between the wt and K65R RT with dTTP as substrate and poly(rA)-oligo(dT) as P/T. Our findings differ from those reported with the ddl-resistant L74V mutant (Martin et al., 1993), which showed a lower V/K ratio than wild type RT for processive synthesis using dTTP and poly(rA)-oligo(dT), but no difference in V/K for incorporation of dCMP into poly(rA)-oligo(dC).

The K65R mutant RT showed a 20-fold increase compared to wt enzyme in the value of K₉₅ for ddCTP and 3TCTP inhibition of dCMP incorporation into poly(rT)-oligo(dC) (Table II). Similar increases in K₉₅ were noted for inhibition by each of ddCTP, 3TCTP, and ddATP of dCMP incorporation into heteropolymeric P/T (Fig. 1, A, B, and C). The ratio K₉₅/K₆₅R used to normalize for changes in substrate recognition by the enzyme (Segel, 1975) was increased approximately 10-fold with the K65R mutant. These findings are consistent with the 10-fold increases in IC₅₀ for ddC, 3TC, and ddl inhibition of replication of HIV-1 containing the K65R mutation (Gu et al., 1994). The parallel results obtained for ddc/3TC/ddI with in vivo drug sensitivity assays and for ddCTP/3TCTP/ddATP with in vitro

² R. S. Fletcher and M. A. Parniak, unpublished results.
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Fig. 1. Dideoxynucleotide triphosphate inhibition of wild type and K65R mutant RT-catalyzed incorporation of dCMP into heteropolymeric template-primer. The inhibition of wt RT (○) and K65R mutant RT (■) by varying concentrations of ddCTP (A), 3TCTP (B), ddATP (C), ddGTP (D), ddTTP (E), or AZTTP (F) was assessed using a heteropolymeric primer-template and 5 μM each of dATP, dGTP, dTTP, and [α-32P]dCTP as described under "Materials and Methods." The data are the means ± S.D. for three separate experiments, each performed in duplicate.

enzyme studies suggest that the K65R mutation results in changes in substrate/inhibitor recognition by RT. This is entirely consistent with our previous observations that the binding of these compounds is competitively inhibited by a monoclonal antibody that binds to residues 65-73 of RT (Wu et al., 1993).

Interestingly, ddATP, ddTTP, and AZTTP were unable to inhibit dCMP incorporation into poly(rI)-oligo(dC) catalyzed by either the K65R mutant or wt RT, even at concentrations up to 100 μM. Similarly, when poly(rA)-oligo(dT) and dTTP were used as primer-template and substrate, neither ddCTP nor ddATP was able to inhibit wt and K65R mutant RT (Table II). However, ddCTP, ddATP, ddGTP, 3TCTP, and AZTTP were each effective inhibitors of wt RT-catalyzed dCMP incorporation into heteropolymeric P/T. The inhibitors ddCTP, 3TCTP, and ddATP were significantly less effective against the K65R mutant RT, whereas ddTTP, AZTTP, and ddGTP gave essentially similar inhibition of wt and mutant enzymes. Assays employing heteropolymeric P/T were carried out in the presence of saturating levels of the other three dNTPs. Chain termination may therefore be more important than competitive inhibition in ddC/3TC/ddI-mediated inhibition of HIV replication.

Although neither the V/K ratio nor Kᵦ differed significantly between wt and K65R RT for dTTP with both homopolymeric and heteropolymeric P/T, the Kᵦ and Kᵦ/Kᵦ values for inhibition of K65R RT by ddTTP and AZTTP were increased approximately 6-fold, using a homopolymeric P/T. This implies some decrease in ddTTP and AZTTP recognition by the K65R RT. With the heteropolymeric P/T, although the Kᵦ and Kᵦ/Kᵦ values for AZTTP were 4-6-fold higher with the K65R RT compared to wt, no differences were noted in the same parameters for ddTTP. We cannot yet explain the increased Kᵦ for both ddTTP and AZTTP with the K65R mutant in the absence of corresponding changes of Kᵦ for dTTP using homopolymeric P/T. In addition, the increased inhibition by ddTTP of the K65R mutant when assayed with the heteropolymeric P/T compared to the homopolymeric poly(rA)-oligo(dT) is puzzling but may be related to the effect of P/T structure on RT conformation. Moreover, HIV-1 variants containing the K65R mutation do not express decreased sensitivity to AZT in infected cells (Gu et al., 1994; Zhang et al., 1994). Interestingly, similar findings were obtained with the ddI-resistant L74V mutant (St. Clair et al., 1991; Martin et al., 1993). In contrast, RT in lysates of HIV strains showing high level resistance to AZT in infected cells (Wainberg et al., 1990) or recombinant RT containing mutations responsible for AZT resistance (Lacey et al., 1992) do not exhibit altered sensitivity to AZTTP in vitro. Thus, although two mutations (D67N and K70R) associated with resistance to AZT occur in the same region of RT as those associated with resistance to ddI and ddC, altered recognition of substrate and inhibitor does not appear to be a factor in AZT resistance.

In summary, our data suggest that cross-resistance to ddC, 3TC, and ddI conferred by the K65R mutation in HIV-1 RT is due to selective alterations in substrate and inhibitor recognition by the enzyme. This mutation occurs in a region of RT due to selective alterations in substrate and inhibitor recognition. More studies of the changes in RT structure resulting in decreased inhibitor recognition by the K65R mutant are in progress.

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