Single-Step Kinetics of HIV-1 Reverse Transcriptase Mutants Responsible for Virus Resistance to Nucleoside Inhibitors Zidovudine and 3-TC†

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ABSTRACT: Two mutants of HIV-1 reverse transcriptase (RT) associated with high-level resistance of the virus to AZT (RT-AZT: D67N, K70R, T215Y, K219Q, and M41L) or 3-TC (RT-3TC: M184V) were expressed in *Escherichia coli* and purified. None of these mutants showed significant changes in the affinity and kinetics of binding to a DNA/DNA primer/template. RT-AZT was investigated in detail with respect to its kinetics of incorporation of nucleotides. No change in the relative rates of TMP and AZTMP incorporation could be detected for RT-AZT with respect to wild type RT. These results imply that there is no increased discrimination against AZTTP in the mutant. This was found for DNA/DNA and DNA/RNA primer/template. Additionally, rapid kinetics of incorporation of 3′-amino-3′-deoxythymidine 5′-monophosphate (a possible metabolite of AZT) were investigated and compared with TMP incorporation, but no difference in its relative rates of incorporation between wild type RT and RT-AZT was detected. In contrast, the already very slow rate of incorporation of 3-TCMP seen with wild type enzyme was drastically reduced (by a factor of 23 and 36 with DNA/DNA primer/template and DNA/RNA primer/template, respectively) for RT-3TC, showing a clear correlation between *in vitro* and *in vivo* effects. The affinity of 3-TCTP to the RT-3TC−primer/template complex was not affected by the mutation M184V. A 1.6-fold cross-resistance to ddATP, the converted form of the prodrug ddI, could also be shown for RT-3TC, but no cross-resistance to ddCTP was detected. Additionally, rapid kinetics of AZTMP incorporation by RT-3TC were investigated. There was an indication of a slightly higher rate of incorporation of AZTMP by RT-3TC than wild type RT.

Nucleoside-based inhibitors of HIV-1 reverse transcriptase (RT)[1] were the first class of substances found to have activity against HIV-1 replication in cell-culture experiments. In agreement with this, they can have a dramatic effect in infected patients, as shown by the reduction of viral load and increase in counts of CD4 T-cells (Ho et al., 1995; Wei et al., 1995). In general, these effects correlate with the ability of the triphosphate form of the nucleosides to inhibit the polymerase activity of the viral RT by chain termination. The additional requirement for phosphorylation of the nucleoside prodrugs to their triphosphates by cellular kinases means that this correlation is less direct than it might otherwise be, since not all nucleoside analogs are phosphorylated with equal efficiency.

The failure of such substances as long-term therapeutic agents arises from the fact that resistant strains of the virus emerge, or are selected, under the selective pressure of the drugs (Larder et al., 1989; Wainberg et al., 1995). As a consequence parameters such as the viral load return toward their level at the start of therapy within weeks or months (D'Aquila et al., 1995). This effect is correlated with mutations in the viral RT. Depending on which nucleosides are used there are several distinct patterns emerging with significant cross-resistance between various inhibitors (Larder & Kemp, 1989; Kellam et al., 1992; Gu et al., 1992, 1994; St Clair et al., 1991; Gao et al., 1993). Two substances which have been examined extensively are zidovudine (3′-azidothymidine, AZT) and lamivudine (3′-thiacytidine, 3-TC). Zidovudine is the drug that has been used most extensively and for the longest period in AIDS therapy. High-level zidovudine resistance in patients is associated with five mutations in the RT gene occurring in a certain order during therapy (Larder & Kemp, 1989; Kellam et al., 1992). Lamivudine has been used only recently in therapy. High-level resistance to lamivudine occurs within 4 months (Schinazi et al., 1993; Wainberg et al., 1995) and is associated with the amino acid exchange M184V in HIV-1 RT (Gu et al., 1992; Gao et al., 1993). There is particular interest in combination therapy using these two substances, since the mutation leading to high-level resistance against lamivudine (M184V) appears to resensitize zidovudine-resistant strains [i.e., those containing correspondingly mutated RTs (Larder et al., 1995)].

Detailed studies of the biochemical mechanism of those resistances together with structural data should provide a better basis for rational drug design particularly with respect to the problems of development of resistance. We report here detailed kinetic studies of the interactions of zidovudine and lamivudine with their respective resistant mutants.

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[1] Abbreviations: AZT, 3′-azidothymidine; dNMP, 5′-deoxynucleoside monophosphate; dNTP, 5′-deoxynucleoside triphosphate; HIV-1, human immunodeficiency virus type 1; HPLC, high-performance liquid chromatography; 3′-NH_2-3′TTP, 3′-amino-3′-deoxythymidine 5′-triphosphate; PCR, polymerase chain reaction; p/t, primer template hybrid; RT, reverse transcriptase; TFA, trifluoroacetic acid; 3-TC, (−)-β-L-2′,3′-dideoxy-3′-thiacytidine.

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MATERIALS AND METHODS

Expression and Purification of Proteins. Recombinant heterodimeric HIV-1 RT was expressed in *Escherichia coli* and purified as described before (Müller et al., 1989, 1991b). Enzyme concentrations were routinely determined by using an extinction coefficient of 260 450 M^{-1}.

Cloning of the HIV-1 RT Mutants. All HIV-1 RT mutants were created by site directed mutagenesis using the polymerase chain reaction (PCR). The enzyme resistant to 3-TC (RT-3TC) contains the amino acid exchange M184V (Gu et al., 1992). The enzyme resistant to AZT (RT-AZT) contains the following five amino acid changes: M41L, D67N, K70R, T215Y, and K219Q (Larder & Kemp, 1989; Kellam et al., 1992). The mutations were introduced into the plasmids pRT_{66} and pRT_{51} (Müller et al., 1989). In the case of RT-3TC two PCR primers were synthesized. One of them was complementary to the DNA sequence of amino acids 182−191 and contained the desired point mutation in the codon of Met 184. Both primers contained specific restriction sites at their 5′-ends (*Eco*RV, *Xho*II). The PCR primers were hybridized to the wild type plasmid (pRT_{66}) and PCR was carried out according to a standard protocol. The resulting fragment of 125 bases was cut with the specific restriction enzymes and purified. The corresponding wild type fragment within the vectors (pRT_{66} and pRT_{51}) was deleted in two steps. First, a 1.25 kb fragment was cut from the 6.3 kb vector with *Hin*dIII and *Eco*RV and both parts were isolated. Second, the corresponding wild type sequence of the PCR fragment lying at the end of the 1.25 kb fragment was deleted with *Xho*II and the resulting 1.125 kb fragment was purified. The isolated vector fragment (5.05 kb) was then ligated with the 1.125 kb fragment and the PCR product. This procedure was performed for both plasmids. A co-expression plasmid was cloned as described before (Müller et al., 1989). The mutations in RT-AZT were introduced by the same method.

Enzyme Activity Determination. Polymerase activity was measured by a standard assay using poly(rA)/oligo$(dT)_{15}$ as a primer/template (Restle et al., 1990). The specific activity of wild type RT was about 5000 units/mg. One unit catalyzes the incorporation of 1 nmol of TMP in 10 min at 37 °C.

Nucleotides. [γ-^{32}P]ATP was obtained from DuPont-New England Nuclear. 2′,3′-Dideoxy-3′-thiacytidine was kindly provided by Glaxo Wellcome Research and Development. All other nucleotides were from Boehringer, Mannheim.

Oligodeoxynucleotides. Oligodeoxynucleotides were synthesized on an Applied Biosystems 380 B DNA synthesizer and purified by HPLC on a reversed phase column by standard procedures (Müller et al., 1991a) or by using denaturing polyacrylamide gel electrophoresis (15% acrylamide, 7 M urea) and electroelution of the desired DNA band. All oligodeoxynucleotides contain the DNA sequence corresponding to the primer binding site of the HIV-1 RNA. An 18/36mer DNA/DNA primer/template (p/t) was used for fluorescence studies as well as incorporation studies (primer sequence, 5′-TCCCTGTTCGGGCGCCTC-3′; template sequence, 5′-TGTGGAAAATCTCATGCAGAGGCGCCCGAACAGGGA-3′). For incorporation assays the first template base after the 3′-OH end of the primer was changed depending on the dNTP used for incorporation. Incorporation assays with a DNA/RNA p/t were done with a 20/35mer (primer sequence, 5′-TTGTCCCTGTTCGGGCGCCA-3′; template sequence, 5′-GGGUUAAUCUCUGCAUGGCGCCCGAACAGGGACAA-3′). Primer and template oligodeoxynucleotides were annealed by heating equimolar amounts in 50 mM NaCl and 20 mM Tris-HCl, pH 7.5, for 10 min at 90 °C, followed by cooling to room temperature over a period of about 2 h in a water bath.

Fluorescently Labeled Oligodeoxynucleotides. During oligodeoxynucleotide synthesis an amino modifier (C2dT from Glen Research) was incorporated next to the last nucleotide at the 3′-end of the 18mer primer (see section "Oligodeoxynucleotides" above). The amino group was reacted with an excess of mansyl chloride (50 mM solution in dry acetone) in 30% dimethylformamide and 0.05 M sodium carbonate, pH 10, by shaking the solution for 2 h in darkness. The product was purified by HPLC using the standard protocol for oligodeoxynucleotide purification (Müller et al., 1991a). Fluorescence measurements shown here were done with a 18/36mer p/t (see section "Oligdeoxynucleotides" above).

3′-Termination of Primer/Template with AZT. For synthesis of 3′-terminated p/t, 500 pmol of an 18/36mer p/t was incubated with 250 pmol of RT and 10 nmol of AZTTP in 20 μL of RT-reaction buffer at 37 °C for 30 min. The reaction was stopped by phenol/chloroform extraction, and p/t was purified with a G-50 Micro Column (Pharmacia).

5′-^{32}P-Labeling of Primers. Primer were 5′-end labeled with T4 polynucleotide kinase (New England Biolabs) using 10 μCi of [γ-^{32}P]ATP (3000 Ci/mmol) per 100 pmol of DNA and the reaction buffer supplied from NEB. The reaction was stopped by phenol/chloroform extraction, and the primer was purified with a NucTrap Probe Purification Column (Stratagene). Concentrations were determined by thin-layer chromatography using a phosphorimager (Bio-Rad) for quantification.

Buffers. All experiments were carried out in a buffer containing 50 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 50 mM KCl, and 1 mM dithiothreitol (RT reaction buffer).

Fluorescence Titrations of Primer/Template Binding. Fluorescence titrations were performed using an SLM Smart 8100 spectrofluorometer equipped with a PH-PC 9635 photomultiplier. To monitor the fluorescence change upon binding of the labeled p/t to RT, the samples were excited at 340 nm and the emission intensity was measured at 427 nm (slit widths 1 and 16 nm, respectively). Data were transferred to a personal computer and evaluated using the data analysis program Grafit (Erithacus Software). A quadratic equation analogous to the one given by Müller et al., (1991a) describing the binding equilibrium was used for the fitting procedure. Values for the dissociation constant (K_d), the amplitude of the fluorescence change, and the enzyme concentration were allowed to vary during the fit procedure. All experiments were done at a temperature of 25 °C.

Displacement Titrations of Primer/Template. Affinities of terminated p/t in the absence or presence of the following correct nucleotide were determined by displacing the fluoresently labeled and terminated p/t with poly(rA)/oligo$(dT)_{15}$. 35 nM terminated p/t were preincubated with 50 nM RT without or with 100 μM dGTP. Fluorescence changes upon displacement of the complex were monitored as described above. These competitive titrations were evaluated by using the program Scientist, which allows the user to define the system under investigation as a series of parallel equations

defining (in this case) each discrete equilibrium, the relationship between the total and free concentrations of the components, and the way in which the observable signal is generated. The K_d of poly(rA)/oligo(dT)$_{15}$ was determined independently and kept constant during the fit procedure.

Rapid Kinetics of Primer/Template Binding. Experiments on the kinetics of association of RT with labeled p/t were performed using a stopped-flow apparatus (High Tech Scientific, Salisbury, England). 50 nM of p/t (final concentration) were rapidly mixed with increasing concentrations of RT. Excitation of the mansylated p/t was at 365 nm using a Hg-Xe high-pressure arc lamp and detection was through a filter with a cutoff at 398 nm. Data were collected using an analog digital converter in an IBM PC-compatible computer and analyzed using the software package provided by High Tech Scientific or using Grafit. Data were analyzed by fitting to a double-exponential equation. The rate of the first phase is dependent on the concentration of RT as described previously (Rittinger et al., 1995). The linear fit of its concentration dependence yields k_{+1} and k_{-1} of p/t binding.

Rapid Kinetics of Nucleotide Incorpoporation. Rapid quench experiments were carried out in a chemical quench−flow apparatus (KinTek Corporation, University Park, PA). The apparatus was modified for using small reaction volumes (15 μL). Reactions were started by rapidly mixing the two reactants (15 μL each) and then quenched with 0.6% trifluoroacetic acid (TFA) after defined time intervals. All concentrations reported are final concentrations after mixing in the rapid quench apparatus. For studying incorporation of different nucleotides the template sequence was varied at the first base of the template overhang (see Materials and Methods). Products were analyzed by sequencing gel electrophoresis (10% polyacrylamide) under denaturing conditions with 7 M urea and quantitated by scanning the dried gel using a phosphorimager (Bio-Rad). Data were evaluated by the fitting program Grafit (Erithacus Software).

For pre-steady state kinetics a preformed complex of RT−p/t (100 and 200 nM, respectively) was rapidly mixed with an excess of nucleotide (100 μM) and stopped after various times in the millisecond range. Data were fitted to a burst equation (single-exponential plus slope). The effective pre-steady state rate constant (k_{pol}) at the given nucleotide concentration is given by the exponential part, whereas the steady state rate, which is determined by k_{off} of p/t binding, was calculated from the slope divided by the enzyme concentration (Kati et al., 1992). Affinities of nucleotides were determined by the dependence of the pre-steady state burst rate on the nucleotide concentration. To measure the affinities of dTTP and AZTTP, the RT−p/t complex (100 and 200 nM, respectively) was rapidly mixed with various concentrations of nucleotide and quenched after $t_{1/2}$ of the maximal pre-steady state rate. The corresponding rates were then calculated from the concentration of elongated primer by converting the single exponential equation into

$$k = -\ln(1 - ([p_{+1}]_t/[p_o]))/t(\text{sec})$$

where P_o corresponds to the concentration of RT−p/t complex available for incorporation at $t = 0$ (burst amplitude) and t equals the reaction time ($t_{1/2}$ of the maximal pre-steady state rate). The observed rates were then plotted against the nucleotide concentration, and the dissociation constant (K_d) was calculated by fitting the data to a hyperbola.

Single-Turnover Kinetics of 3-TCMP Incorporation. Single-turnover kinetics of 3-TCMP incorporation were measured by chemical quenches in the range of minutes to hours. Reactions were started by mixing 5 μL of RT−p/t complex with 5 μL of nucleotide solution (final concentrations were 100 nM p/t, 250 nM RT, and the desired concentration of nucleotide). Reactions were quenched with 0.6% TFA after various times and analyzed as described above in "Rapid Kinetics of Nucleotide Incorporation". Data were fitted by a single-exponential equation which yields k_{pol}. The affinities of 3-TCTP were determined by measuring k_{pol} at increasing nucleotide concentrations. The concentration dependence of k_{pol} was fitted to a hyperbola which yields the affinity of 3-TCTP for RT−p/t.

Chain Termination Assay. Chain termination assays were performed using either single-stranded M13mp9 DNA (Boehringer, Mannheim) or a 1.2 kb RNA fragment of the HXB2 strain of HIV-1, produced by *in vitro* transcription. Primers were 5′-labeled with ^{32}P. Reaction mixtures contained 0.05 pmol of p/t, 0.7 pmol of RT, 150 μM of each nucleotide (dGTP, dATP, dCTP, dTTP), and increasing concentrations of AZTTP (0, 2, 15, 75, and 150 μM) in a total volume of 10 μL. Reactions were carried out for 15 min at 37 °C in RT reaction buffer, stopped by adding 10 μL of urea gel loading buffer, and analyzed by sequencing gel electrophoresis (10% polyarylamide, 7 M urea).

RESULTS

Mutants of Reverse Transcriptase. An RT mutant that confers a high degree of resistance to AZT at the level of virally infected cells was prepared by site-directed mutagenesis (RT-AZT). The mutant contains the five most commonly identified mutations associated with AZT resistance in both subunits (M41L, D67N, K70R, T215Y, K219Q) (Larder & Kemp, 1989; Kellam et al., 1992). In the case of 3-TC resistance, a mutant with a single amino acid exchange M184V in both subunits (Gu et al., 1992) was prepared and studied (RT-3TC). Both mutants were expressed and purified as already described for the wild type protein (Müller et al., 1989).

The influence of the mutations on the activity of RT was tested by a standard RT assay (Restle et al., 1990). The activity of RT-AZT was 100% of wild type activity. The activity of RT-3TC was only about 70%, which is in agreement with other studies (Wakefield et al., 1992; Boyer & Hughes, 1995). These authors showed that the RNA dependent polymerase activity of RT-3TC is decreased 60−75% of wild type activity whereas the DNA dependent polymerase activity is not affected by this mutation.

Interaction with Primer/Template. To investigate whether the affinity of the mutant RTs to a DNA/DNA p/t was influenced by the mutations, we performed equilibrium titrations with an 18/36mer p/t using the fluorescence of a label attached to the 3′-end of the primer molecule as signal (see Materials and Methods). This label at the 3′-end results in a very large increase in fluorescence upon interaction with RT, allowing accurate determination of affinity. Figure 1 shows the results of a typical titration of the labeled p/t molecule with the RT-3TC mutant. The equilibrium constants (K_d) of the different RT molecules are summarized in

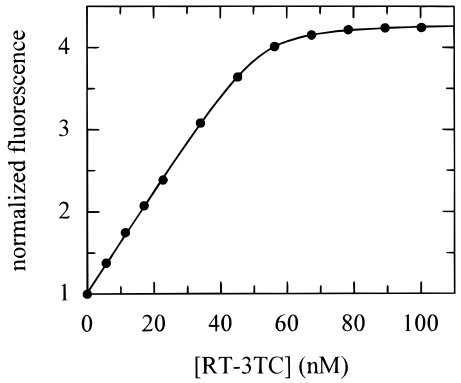

FIGURE 1: Titration of fluorescence-tagged p/t (45 nM) with RT-3TC using the increase in fluorescence of the mansyl label as a signal for binding (for conditions, see Materials and Methods). The curve shows the best fit of the data to the quadratic equation describing the binding equilibrium (Müller et al., 1991a) with a K_d of 0.9 nM.

Table 1: Parameters for p/t Binding

enzyme	K_d (nM)	k_{+1} (M^{-1} s^{-1})	k_{-1} (s^{-1})
RT wt	1.4 ± 0.2	$(3.2 \times 10^8) \pm (0.2 \times 10^8)$	17 ± 8
RT-3TC	0.9 ± 0.1	$(3.5 \times 10^8) \pm (0.1 \times 10^8)$	17 ± 3.4
RT-AZT	0.5 ± 0.2	$(6.4 \times 10)^8 \pm (0.1 \times 10^8)$	1 ± 19

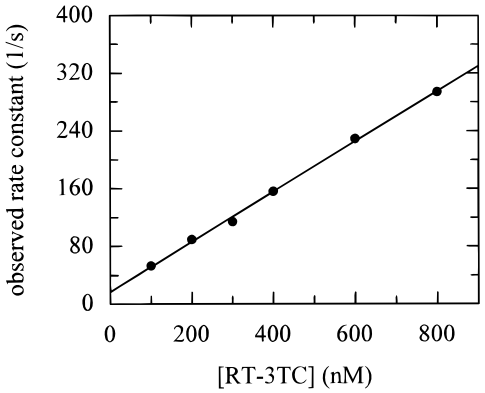

FIGURE 2: Dependence of the pseudo-first-order rate constant of p/t binding on the RT-3TC concentration (for conditions and analysis, see Materials and Methods). The slope of the linear fit yields a value of k_{+1} of 3.5×10^8 M^{-1} s^{-1} for the first step in p/t binding. The intercept with the *y*-axis yields a value of k_{-1} of 17 s^{-1} for the first step in p/t binding.

Table 1. Alternatively, labels could be attached to the 5′-end of the primer or protein fluorescence could be used as a signal for the binding of unlabeled p/t. In all cases, K_d values in the range of 1−5 nM were obtained, with no sign of an effect of the resistance-conferring mutations on p/t binding (data not shown).

The 3′-end labeled p/t was also used for rapid mixing studies in the stopped-flow instrument, and such studies led to determination of the association rate constant (k_{+1}) and the dissociation rate constant (k_{-1}) as described previously (Rittinger et al., 1995). As noted by these authors, there are two phases in the association transient: the first rapid phase reflecting initial relatively weak binding and the second reflecting a conformational change of RT upon p/t binding. This second step leads to the tight RT−p/t complex with a K_d in the nanomolar range. In Figure 2 the concentration dependence of the first phase of p/t binding (observed pseudo-first-order rate constants) with RT-3TC is shown, where the slope yields k_{+1} and the intercept with the *y*-axis yields k_{-1}. The values of k_{+1} and k_{-1} obtained with the mutants were essentially unchanged compared with wild type enzyme (Table 1). There was some indication of tighter binding of RT-AZT, but the effects are relatively small, and the low value of k_{-1} obtained was probably a result of the inaccuracy of the procedure of extrapolating the observed pseudo-first-order rate constants. It should be noted that the equilibrium constant K_d cannot be directly calculated from k_{+1} and k_{-1}, since the initial weak binding is followed by a conformational change of RT leading to the lower K_d measured in equilibrium titrations.

Table 2: Affinities of p/t Terminated with AZTMP to RT-AZT and Wild Type RT

	K_d (nM)	
enzyme	in absence of dGTP	in presence of dGTP
RT wt	2.0 ± 0.3	1.6 ± 0.3
RT-AZT	2.0 ± 0.3	0.6 ± 0.1

Interaction of RT-AZT with p/t Terminated by AZTMP. Although the stoichiometric relationships *in vivo* render it unlikely, it is possible that formation of a stable complex between RT and p/t terminated with AZTMP could contribute to the inhibitory effect of AZT *in vivo*. This should be reflected in a higher affinity of wild type RT for this complex in comparison with RT-AZT. We therefore measured the affinities of a p/t terminated with AZTMP to RT in the absence and presence of the following correct nucleotide (dGTP) by displacement of the terminated fluorescent p/t complex with poly(rA)/oligo(dT)$_{15}$. Results are summarized in Table 2. The K_d values for p/t terminated with AZTMP were similar for mutant and wild type RT and decreased by a factor of 3.3 and 1.3, respectively, in presence of the next nucleotide. Thus, there is almost no difference in the K_d values of normal and terminated p/t (compare Table 1), indicating that a possible difference in the contribution of this effect to the inhibitory power of AZT with wild type RT and RT-AZT, respectively, cannot be the explanation for AZT resistance.

Kinetics of TMP and AZTMP Incorporation with RT-AZT and Wild Type RT. Since the mechanism of inhibition of chain-terminating nucleotides involves addition to the end of the growing DNA chain, the kinetic parameters governing this reaction are of importance for a quantitative understanding of their mode of action. In the present work, the quench−flow method was used as described previously (Kati et al., 1992) to measure the pre-steady state rate constant of addition of a single nucleotide to a DNA/DNA p/t as a function of nucleotide concentration. This provides the determination of the maximal pre-steady state rate constant of nucleotide incorporation (k_{pol}) and of the affinity (K_d) of nucleotides to the RT−p/t complex.

Figure 3A compares the results obtained for the incorporation of TMP or AZTMP using wild type RT. It can be seen that the k_{pol} of TMP is about 3-fold greater than that of AZTMP. In addition, as shown in Table 3, the apparent affinity of AZTTP to the RT−p/t complex is somewhat lower than that of TTP. These results indicate that AZTTP has no competitive advantage over TTP during incorporation into DNA with wild type RT.

Figure 3B presents the results of the incorporation kinetics of TMP and AZTMP into a DNA/DNA p/t with RT-AZT. It can be seen that k_{pol} of TMP is about 3-fold greater than

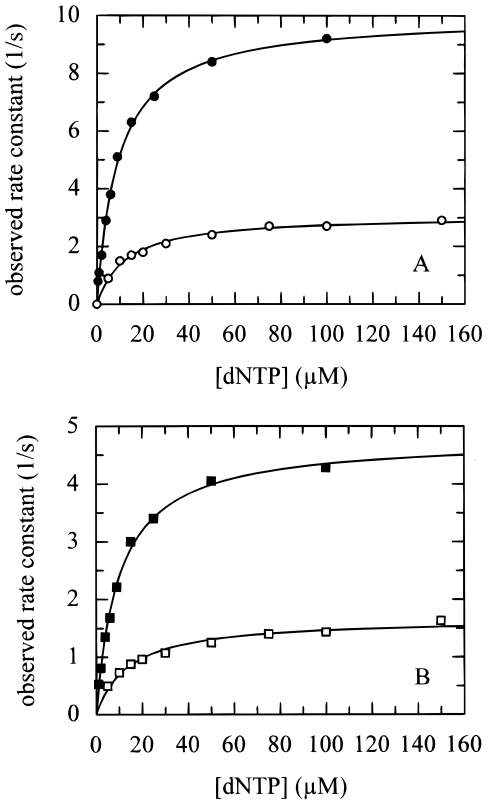

FIGURE 3: Dependence of the pre-steady-state rate constant on the nucleotide concentration. (A) Incorporation of TTP (●) or AZTTP (○) with wild type RT. (B) Incorporation of TTP (■) or AZTTP (□) with RT-AZT (for conditions, see Materials and Methods). The data were fitted to a hyperbola which yielded the K_d values for dNTP dissociation and k_{pol} (maximum first-order rate constants for incorporation) (see Table 2).

Table 3: Parameters for Incorporation of dNTP into DNA/DNA p/t

substrate	k_{pol} (s^{-1})	K_d (μM)	k_{pol}/K_d (M^{-1} s^{-1})	factor TTP/AZTTP
RT wt + TTP	10 ± 0.1	9.2 ± 0.4	1.09×10^6	4.3
RT wt + AZTTP	3.1 ± 0.1	12.3 ± 0.9	0.25×10^6	
RT azt + TTP	4.8 ± 0.1	10 ± 0.6	0.48×10^6	4.0
RT azt + AZTTP	1.7 ± 0.1	14 ± 1.6	0.12×10^6	

Table 4: Parameters for Incorporation of dNTP into DNA/RNA p/t

substrate	k_{pol} (s^{-1})	K_d (μM)	k_{pol}/K_d (M^{-1} s^{-1})
RT wt + AZTTP	22 ± 1.1	16.5 ± 2.7	1.3×10^6
RT azt + AZTTP	24 ± 0.4	14 ± 0.8	1.7×10^6

that of AZTMP, similar to wild type RT. Both k_{pol} values (for TMP and AZTMP incorporation) are reduced by a factor of 2 compared to wild type RT. Since the affinities of both nucleotides for RT-AZT are also hardly changed with respect to wild type RT, the differences in k_{pol}/K_d of the natural nucleotide and the inhibitor are the same for wild type and the mutant form of the enzyme (Table 3). Thus, from these studies there is no indication of what causes resistance to AZT.

The experiments described above on the kinetics of incorporation of AZTMP were repeated with a DNA/RNA p/t. The results (Table 4) show that here, as with DNA/DNA p/t, there is no significant discrimination against AZTTP as a substrate by the multiple mutant, indicating that with this type of p/t complex which exists during minus-strand elongation, there is, again, no resistance to AZTTP at the biochemical level.

Chain Termination by AZTTP with RT-AZT and Wild Type RT. If the effect of resistance to RT-AZT were too small (but still significant) to be measured by single-nucleotide incorporation as described here, it might be detectable by potentiation during processive polymerization with long templates (Goody et al., 1991). M13 DNA was used for chain termination assays with increasing concentrations of AZTTP as described in Materials and Methods. As shown in Figure 4, there is no significant difference in chain termination with the mutant enzyme compared to wild type RT. If anything, chain termination is more efficient with RT-AZT, in agreement with the data on single-nucleotide incorporation given in Table 2. The same assay was also performed using a 1.2 kb RNA transcript of the HXB2 strain of HIV-1 as template. In this case, no processive DNA polymerization could be achieved (data not shown), indicating that some essential components for efficient RT activity are missing in our *in vitro* system.

Kinetics of 3′-NH_2-3′TMP Incorporation with RT-AZT and Wild Type RT. Since the active form of AZT has not yet been proven to be exclusively AZTTP, but could also be a metabolized nucleotide analog, we also examined incorporation of 3′-NH_2-3′TMP, a possible metabolite of AZT. The rapid kinetics of incorporation were measured as described in Materials and Methods at a nucleotide concentration of 100 μM (data not shown). The k_{pol} value of 1.2 s^{-1} determined for RT-AZT was only a factor of 2 lower than for wild type RT ($k_{pol} = 2.4$ s^{-1}). This is the same factor as already seen for AZTTP and implies that the five mutations of RT-AZT also confer no resistance to 3′-NH_2-3′TTP at the biochemical level.

Kinetics of 3-TCMP Incorporation with RT-3TC and Wild Type RT. There is not much evidence in previously published work on the substrate properties of 3-TCTP with RT. Quench experiments on the incorporation of 3-TCMP using DNA/DNA p/t showed a very low rate of incorporation (k_{pol}) of this analog using wild type RT. Similar results were presented recently by Gray et al., (1995). As shown in Table 5, k_{pol} is 3−4 orders of magnitude slower than for the natural nucleotide dCTP with a DNA/DNA p/t and also with a DNA/RNA p/t. This is somewhat surprising in view of the relatively good inhibitory efficiency of 3-TCTP in cell-culture assays (Coates et al., 1992; Hart et al., 1992).

With the resistant mutant RT-3TC it could be demonstrated that the incorporation is even slower. In Figure 5 a comparison of the 3-TCMP incorporation into a DNA/RNA p/t by wild type RT and RT-3TC is shown. It can be seen that k_{pol} is reduced by a factor of 36 with the resistant mutant (Table 5). This result is in agreement with previous studies (Schinazi et al., 1993; Quan et al., 1996). These authors showed that the IC_{50} value of virally derived RT was increased 18−50-fold for inhibition with 3-TCTP on poly-$(I)_n$ oligo$(dC)_{12-18}$ as a p/t and that the K_i value for 3-TC is 35-fold higher with respect to wild type RT. As shown in Table 5, the incorporation rate of 3-TCMP into a DNA/DNA p/t is also reduced by a factor of 23 with respect to wild type RT.

The affinities for 3-TCTP were determined by the dependence of k_{pol} on the nucleotide concentration with DNA/RNA p/t. As shown in Figure 6, the K_d value of 3-TCTP is hardly

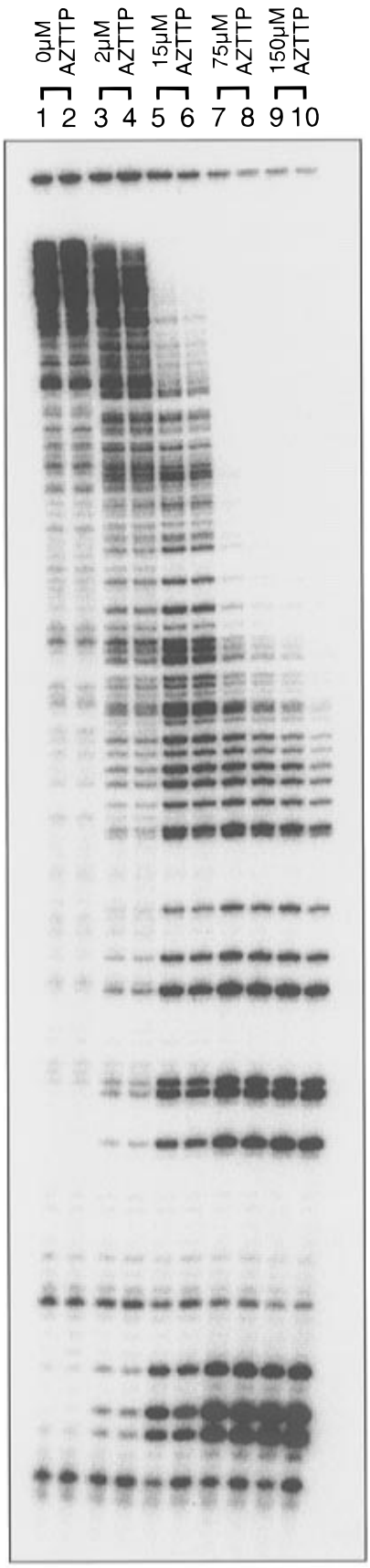

FIGURE 4: Chain termination by AZTMP incorporation during DNA polymerization with M13 ssDNA (for conditions and analysis, see Materials and Methods). Lanes 1, 3, 5, 7, and 9, wild type RT; lanes 2, 4, 6, 8, and 10, RT-AZT.

changed with RT-3TC (66 ± 30 μM and 45 ± 18 μM for RT-3TC and wild type RT, respectively).

The reduced rate of incorporation of 3-TCMP appears to be an adequate explanation for the 100−1000-fold resistance seen in cell-culture experiments and in patients treated with 3-TC.

Cross-Resistance of RT-3TC to ddATP and ddCTP. It has been reported that the mutant RT-3TC is cross-resistant to dideoxyinosine (ddI) in cell-culture assays (Gao et al., 1993; Gu et al., 1992). Since ddI is converted to ddATP in the cell (Johnson et al., 1988; Johnson & Fridland, 1989), the rapid kinetics of incorporation of ddAMP were compared with those of dAMP. As shown in Table 5, ddAMP is incorporated 2.7-fold more slowly than dAMP with wild type enzyme but 4.3-fold more slowly with the mutant RT-3TC. This modest decrease of k_{pol} (1.6-fold) is in agreement with the effect seen *in vivo* (4−8-fold resistance) (Gao et al., 1993).

In contrast, the cross-resistance to ddCTP (4−8-fold) which has been shown in cell-culture assays (Gao et al., 1993; Gu et al., 1992) could not be confirmed by quench−flow measurements of k_{pol} of ddCMP incorporation into a DNA/DNA p/t (Figure 7, Table 5).

Kinetics of AZTMP Incorporation with RT-3TC. Since it was known that the mutation M184V resensitizes zidovudine resistant strains in therapy (Larder et al., 1995) we examined whether the M184V mutation has a positive effect on the kinetics of incorporation of AZTMP (data not shown). The obtained value for k_{pol} (3.9 ± 0.6 s^{-1}) was essentially unchanged compared with wild type RT (3.1 ± 0.1 s^{-1}), indicating that there is no significant preferential incorporation of AZTMP with RT-3TC at this level.

DISCUSSION

The results presented in this paper are paradoxical in the sense that, for one inhibitor examined (3-TC), there is a clear correlation between the properties of a mutant form of RT *in vitro* and in the virus, whereas this correlation is completely lacking for the other inhibitor (AZT). As shown by the results presented here, incorporation of AZTMP into a growing DNA chain is a relatively efficient process, with the apparent affinity of AZTTP to the RT−p/t complex being quite similar to that of TTP. The rate of addition of AZTTP at saturating nucleotide concentrations is reduced compared with TTP, but only by a factor of 3. The AZT resistant mutant incorporates both TMP and AZTMP a factor of 2 more slowly than wild type. Thus there is no indication of resistance to AZTTP from these experiments.

A question that must be asked concerns the error limits of the experiments reported here and the expected nature of the dependence of the potency of chain termination (in the sense of prevention of completion of transcription) on the rate of incorporation of AZTMP at a single point in the replication process. Due to the nature of the inhibition mechanism, a low relative rate of incorporation of AZTMP compared with TMP under prevailing conditions (large excess of TTP, lower rate constant of AZTMP than TMP incorporation) can still lead to significant chain termination because a low probability of incorporation at each individual addition of TMP or AZTMP translates into a high probability of termination when several thousands of bases are added during transcription of the viral genome (Goody et al., 1991). As a consequence of this, a small change of relative efficiency of incorporation of the chain terminator and the natural nucleotide will have a large effect on the efficiency

Table 5: Comparison of k_{pol} of CTP- and ATP-Analogs with Wild Type RT and RT-3TC

enzyme-p/t	dCTP (s^{-1})	3-TCTP (s^{-1})	ddCTP (s^{-1})	dATP (s^{-1})	ddATP (s^{-1})
RT wt−DNA/DNA	6.4 ± 1.1	$(1.3 \times 10^{-3}) \pm (0.06 \times 10^{-3})$	2 ± 0.3	43 ± 9	15.7 ± 1.8
RT-3TC−DNA/DNA	4.2 ± 0.7	$(5.8 \times 10^{-5}) \pm (0.3 \times 10^{-5})$	1.7 ± 0.2	33 ± 6.6	7.7 ± 1.2
RT wt−DNA/RNA	13 ± 1.2	$(7.8 \times 10^{-3}) \pm (0.8 \times 10^{-3})$			
RT-3TC−DNA/RNA	8 ± 1.3	$(2.2 \times 10^{-4}) \pm (0.3 \times 10^{-4})$			

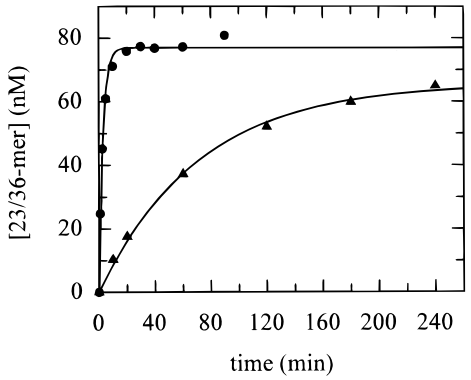

FIGURE 5: Comparison of the single-turnover kinetics of incorporation of 300 μM 3-TCMP into 100 nM DNA/RNA p/t bound to 250 nM wild type RT (●) or RT-3TC (▲), respectively. The fit to a single-exponential equation yielded k_{pol} values of 6.9×10^{-3} and 0.2×10^{-3} s^{-1} for wild type RT and RT-3TC, respectively.

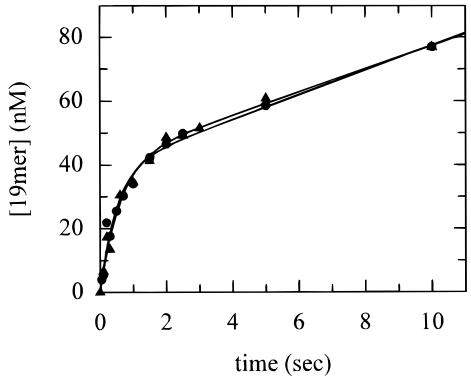

FIGURE 7: Comparison of the pre-steady state kinetics of incorporation of ddCMP (100 μM) into a DNA/DNA p/t (200 nM) bound to 100 nM wild type RT (●) or RT-3TC (▲), respectively. The pre-steady state rate (k_{pol}) was calculated by a single-exponential equation and linear components (Kati et al., 1992) (Table 5).

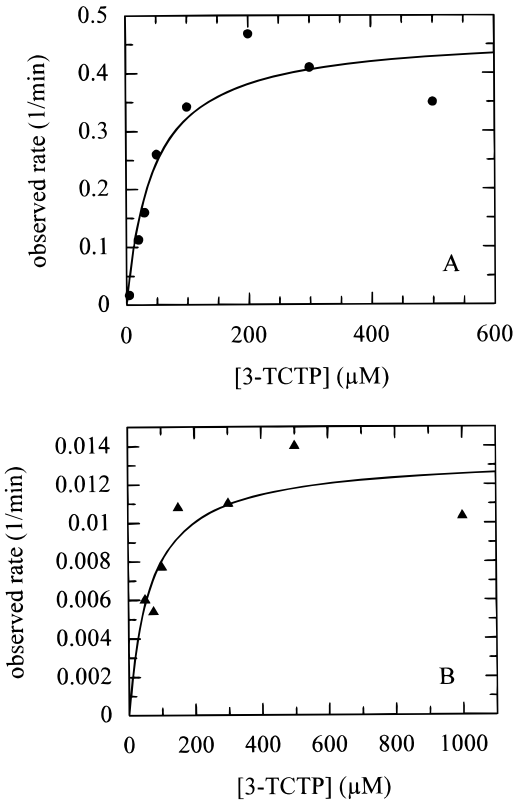

FIGURE 6: Dependence of k_{pol} of 3-TCMP incorporation on the 3-TCTP concentration. (A) Concentration dependence of 3-TCMP incorporation with wild type RT. (B) Concentration dependence of 3-TCMP incorporation with RT-3TC (for conditions and analysis, see Materials and Methods). The data were fitted to a hyperbola which yielded K_d values of 45 μM for wild type RT and 66 μM for RT-3TC.

of chain termination at some point during transcription. However, the same arguement applies quantitatively to a change of effective rate of incorporation due to changing the prevailing concentration of the chain terminator. Thus, it should be possible to compensate for such an effect (i.e., a reduction in the rate constant for AZTMP incorporation) by a corresponding increase in the AZTTP concentration, since it is the product of the rate constant and the concentration which determines the actual incorporation rate. Taking this into consideration, it becomes apparent that there is a linear relationship between the relative rate of incorporation at the single nucleotide level between two forms of RT (i.e., between wild type and mutant) and the efficiency of chain termination at some point during transcription. In other words, the concentration of chain terminator needed for 50% inhibition of completion of transcription cycle will be increased exactly in proportion to the decrease in the rate constant of chain termination at each potential position in a resistant mutant, which is the quantity measured in this work. According to these arguments, if resistance is due to the reduction of the incorporation rate of the chain terminator, high-level resistance at the viral level should be easily detectable in the experiments reported here.

There is, however, a caveat to this conclusion, and this arises from the uncertainty of the relationship between the concentration of AZT added as a nucleoside to infected cells in culture (or present in the blood of infected individuals) and the concentration of AZTTP reached in cells. It has been suggested that most of the AZT which enters cells is present in the monophosphate form, since the nucleoside is a good substrate for thymidine kinase, but AZTMP is a poor substrate for thymidylate kinase (Furman et al., 1986). If the concentration of AZTMP reached in the cell is high in comparison with the K_m value for thymidylate kinase, increasing the concentration of AZT will not significantly increase the rate of phosphorylation of its monophosphate to the diphosphate, since the enzyme is saturated. This would mean that only a certain concentration of AZTTP can be reached in the cell, whatever the prevailing AZT concentration (above a certain minimum). If this concentration (i.e., that of AZTTP) is just enough for potent inhibition of wild type RT, a small change (reduction) of the rate of incorporation of AZTMP, which might not be detected in the

experiments reported here, could have a marked effect on the potency of inhibition (because of the potentiation effect with the long viral template) which could not easily be overcome by increasing the AZT concentration. Because of these arguments, it is neccessary to consider assays of chain termination under conditions which are nearer to those in infected cells. A first attempt at this has been made in the present work by examining the efficiency of chain termination on very long templates (Figure 4). The lack of a reduction of terminating efficiency with RT-AZT suggests that increasing the length of template toward that of the natural template alone is not enough to allow expression of resistance, at least with DNA templates.

Since the experiments reported here do not show resistance of the supposedly resistant mutant to AZTTP, we have to consider other explanations. As shown by other groups (Cretton et al., 1991; Placidi et al., 1993) AZT can be chemically reduced in human cells. Since the main reduction product found was 3′-NH_2-3′-thymidine, we tested the incorporation of 3′-NH_2-3′TMP with RT-AZT but were unable to detect resistance to this compound (as its triphosphate) at the biochemical level. Apart from reduction of AZT, other metabolic changes are possible, e.g. base exchange by nucleosidases, nucleoside phosphorylase, or nucleotidases. This could lead to the synthesis of the 3′-azido forms of adenylate, inosinate, or guanylate within the salvage pathway. We have not yet tested the properties of the triphosphates of these potential metabolites.

One of the reasons for examining both the AZT and 3-TC resistant mutants is the fact that introduction of the M184V mutation into virus variants which are resistant to AZT appears to result in resensitization to AZT *in vivo* (Larder et al., 1995). A simple explanation for this effect would be that the mutation at position 184 results in preferential incorporation of AZTMP. Using the 3-TC mutant investigated here, we could show a slight increase in k_{pol} for AZTTP (factor of 1.26), but this can only be regarded as a preliminary suggestion of good substrate properties of AZTTP with this single mutation, since the error estimates on the individual values do not allow a definite conclusion on this point. Obviously, this effect has to be examined against the background of AZT resistance mutations. A meaningful analysis of such experiments will, however, still be dependent on demonstration of AZT resistance at the level of isolated RT or in an assay including as yet not identified factors involved in expression of the resistance.

As shown in the results, 3-TCMP is incorporated very slowly with respect to natural substrates such as dCMP. However, the inhibitory efficiency of 3-TC in cell culture is comparable to that of AZT (Coates et al., 1992; Hart et al., 1992). The question of the relatively high potency of 3-TC as an anti-HIV agent is an interesting one, since one of the most important factors, indeed an essential one, determining the effectiveness of chain terminators is efficient addition to the end of the growing DNA chain. One factor that may be of importance here is higher metabolic stability of 3-TC and its phosphates, allowing a more stable concentration of the triphosphate to be maintained (Cammack et al., 1992; Gray et al., 1995). Additionally it is possible that 3-TCMP has a higher probability of being incorporated during transcription at certain pause sites of RT during transcription (e.g. during initiation or during stops at secondary structural features of the RNA-template).

As shown by Quan et al., (1996), the K_i value of 3-TC is 35-fold higher with RT-3TC compared to wild type RT. For ddATP and ddCTP a 2-fold increase was found (Quan et al., 1996). We could show that the increase in K_i for 3-TC is due to an equivalent decrease (30−40-fold) in the incorporation rate (k_{pol}) but not to a decrease in the affinity for 3-TCTP. In agreement with this, similar results were obtained for ddATP (1.6-fold decrease of k_{pol} compared to a 2-fold increase in K_i). This effect appears to be an adequat explanation for the 100−1000-fold resistance to 3TC and 4−8-fold resistance to ddI *in vivo*. The lack of quantitative agreement between these effects is presumably related to the relatively complex process of prodrug activation, which means that there is not likely to be a linear relationship between the concentration of the triphosphate form of 3-TC achieved in the cell and the concentration of 3-TC applied, as discussed above for AZT.

The fact that cross-resistance to ddCTP could not be shown is perhaps not surprising in the view of the small magnitude of resistance seen *in vivo*. An effect in k_{pol} of about 2-fold could be near to the error limit of the experiments shown here. This problem also applies to the measured change in k_{pol} for ddAMP incorporation so that the correlation reported here should be regarded as tentative.

The discrepancy between the effects seen here and the properties of different resistant RT mutants with their inhibitory chain-terminating triphosphates suggests that there may be more than one mechanism operative in resistance to chain terminators. For 3TC it has been demonstrated that the incorporated 3TCMP can be removed by cytoplasmic exonucleases from the terminated chain (Skalski et al., 1993). This possibility has not yet been examined in detail for AZT resistance. It might be more efficient when termination is catalyzed by a "resistant" mutant. This could be the case if the normally stable complex between an AZTMP-terminated p/t and RT were less stable with RT-AZT than with wild type RT. Experiments reported here suggest that this is not the case, since steady state addition of AZTMP using catalytic amounts of RT was similar with wild type RT and RT-AZT. This steady state rate is largely determined by the rate of dissociation of RT from the AZTMP-terminated p/t and thus reflects the stability of the complex. In addition to this, in experiments described in the results section, we have shown that there is no significant difference in affinity of AZT-terminated p/t to wild type RT and RT-AZT, both in the absence and in the presence of the next complementary nucleoside triphosphate.

Since it is important to understand the origin of AZT resistance, these studies must be extended in future work to situations more closely resembling those in infected cells. This should help to answer questions concerning the effect of template length, p/t structure, increased sensitivity to AZTTP at specific stages of (reverse) transcription, and the influence of other factors, including viral or cellular components.

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