Effects of (−)-2′-Deoxy-3′-Thiacytidine (3TC) 5′-Triphosphate on Human Immunodeficiency Virus Reverse Transcriptase and Mammalian DNA Polymerases Alpha, Beta, and Gamma

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(−)-2′-Deoxy-3′-thiacytidine (3TC) is a selective inhibitor of human immunodeficiency virus replication in vitro (J. A. V. Coates, N. Cammack, H. J. J. Jenkins, A. J. Jowett, M. I. Jowett, B. A. Pearson, C. R. Penn, P. L. Rouse, K. C. Viner, and J. M. Cameron, Antimicrob. Agents Chemother. 36:733-739, 1992). The effect of 3TC 5′-triphosphate on both the RNA-dependent and DNA-dependent activities of human immunodeficiency virus type 1 reverse transcriptase and DNA polymerases alpha, beta, and gamma from HeLa cells was investigated. 3TC 5′-triphosphate is a competitive inhibitor (with respect to dCTP) of the RNA-dependent DNA polymerase activity (apparent $K_i = 10.6 \pm 1.0$ to 12.4 $\pm$ 5.1 $\mu$M, depending on the template and primer used); the DNA-dependent DNA polymerase activity is 50% inhibited by a 3TC 5′-triphosphate concentration of 23.4 $\pm$ 2.5 $\mu$M when dCTP is present at a concentration equal to its $K_i$ value. Chain elongation studies show that 3TC 5′-triphosphate is incorporated into newly synthesized DNA and that transcription is terminated in a manner identical to that found for ddCTP. The 50% inhibitory concentrations of 3TC 5′-triphosphate against DNA polymerases alpha, beta, and gamma are concentrations of dCTP equal to the $K_i$ values. 3TC 5′-triphosphate is competitive with respect to dCTP. The values of $K_i$ were determined to be 18.7 $\mu$M for DNA polymerase beta and 15.8 $\pm$ 0.8 $\mu$M for DNA polymerase gamma.

The (−)-enantiomer of 2′-deoxy-3′-thiacytidine (also known as 3TC; Fig. 1) is an effective inhibitor of human immunodeficiency virus (HIV) replication (6) that has now entered clinical trials. Related 2,3′-dideoxyxynucleosides, e.g., dideoxyctidine (ddC) and 3′-azido-3′-deoxythymidine (AZT), have been shown to be effective against HIV because they can be phosphorylated to the corresponding 5′-triphosphates, which act both as inhibitors of the viral reverse transcriptase (EC 2.7.7.49) and as chain terminators (12, 15, 20), preventing further elongation of the oligonucleotide chain. Because 3TC has been shown to be phosphorylated within HIV type 1 (HIV-1)-infected cells (3), we investigated the effect of the corresponding 5′-triphosphate on HIV-1 reverse transcriptase and DNA polymerases alpha, beta, and gamma, the three mammalian DNA-dependent DNA polymerases (EC 2.7.7.7) that have been investigated in the greatest detail. The effect of 3TC 5′-triphosphate on DNA polymerase gamma is particularly relevant, because it has been suggested that inhibition of this enzyme may be linked to the peripheral neuropathy observed in some patients treated with ddC or dideoxyinosine (ddl) (4, 5).

The effect of 3TC 5′-triphosphate on these enzymes is reported and is compared, under the same conditions, with the activities of racemic 2′-deoxy-3′-thiacytidine 5′-triphosphate (also known as BCH 189 5′-triphosphate), the (+)-enantiomer of the same compound, ddATP, ddCTP, and AZT 5′-triphosphate. ddATP, ddCTP, and AZT 5′-triphosphate were chosen for comparison because they are thought to be the forms of the anti-HIV compounds ddi, ddC, and AZT that are active against the viral reverse transcriptase (1, 13). We also report on the ability of 3TC 5′-triphosphate to act as a chain terminator of DNA synthesis catalyzed by HIV-1 reverse transcriptase.

**MATERIALS AND METHODS**

**Abbreviations used.** The abbreviations and trivial names used in this report are as follows: 3TC, (−)-2′-deoxy-3′-thiacytidine; BCH 189, (+)-2′-deoxy-3′-thiacytidine; HIV, human immunodeficiency virus; dNTPs, 2′-dideoxynucleoside 5′-triphosphates; ddNTPs, 2′,3′-dideoxyxynucleoside 5′-triphosphates; AZT, 3′-deoxy-3′-azidothymidine; ddATP, 2′,3′-dideoxyadenosine 5′-triphosphate; ddC, 2′,3′-dideoxythymidine; ddCTP, 2′,3′-dideoxyxycytidine; ddGTP, 2′,3′-dideoxyguanosine 5′-triphosphate; ddTTP, 2′,3′-dideoxythymidine 5′-triphosphate; 1C_{50}, 50% inhibitory concentration; $K_i$, apparent $K_i$.

**Materials.** dNTPs, ddNTPs, poly(rA), poly(dT)$_{12-18}$, poly (rA) · poly(dT)$_{12-18}$, poly(dA) · poly(dT)$_{12-18}$, poly (dC)$_{12-18}$, and Q-Sepharose Fast Flow were from Pharmacia LiC, Milton Keynes, United Kingdom. [H]$^3$]dNTPs were from NEN Research Products, Stevenage, United Kingdom. [35S]dATP was obtained from Amersham International PLC, Buckinghamshire, England. The MS2 phage RNA was purchased from Boehringer Mannheim, East Sussex, United Kingdom. The specific synthetic oligonucleotide primer [5′-CAC-TCC-GAA-CTG-CG(T)-3′] was synthesized at Glaxo Group Research Ltd. Double-stranded DNA cellulose, calf thymus DNA, DNase I from bovine pancreas, N-ethylmaleimide, pepstatin A, and phenylmethylsulfonyl fluoride were

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from Sigma Chemical Co. Ltd., Poole, United Kingdom. Single-stranded DNA-agarose was a product of Bethesda Research Laboratories, Gaithersburg, Md., and was purchased from GIBCO Ltd., Uxbridge, United Kingdom. HeLa Ohio cells and cell culture media were from ICN Flow, High Wycombe, United Kingdom. CRL 164 cells were from the American Type Culture Collection, Rockville, Md. P11 cellulose phosphate was from Whatman Lab Sales Ltd., Maidstone, United Kingdom, and was precycled before use, according to the manufacturer’s directions. The washed cellulose phosphate was stored in the presence of 0.5 M potassium phosphate buffer (pH 7.0) and was used within 1 week of preparation. 3T C5-‘triphosphate and the 5’-triphosphates of the racemic form (BCH 189) and the (+)-enantiomer were from the Medicinal Chemistry Department, Glaxo Group Research Ltd. AZT 5’-triphosphate was provided both by the Medicinal Chemistry Department, Glaxo Group Research Ltd., and the Department of Chemistry, University of Exeter, Exeter, United Kingdom. Monoclonal antibody SJK-287-38, which is specific for DNA polymerase alpha (22), was prepared from CRL 164 cells by the Research Services Department, Glaxo Group Research Ltd. Other chemicals were from Sigma; BDH Ltd., Poole, United Kingdom; or FSA Laboratory Supplies, Loughborough, United Kingdom, and were analytical reagent grade or the highest grade available. Milli-Q-grade water was used throughout.

Preparation of poly(A) · poly(dT)12-18. Poly(A) and poly(dT)12-18 were annealed together essentially as described previously (9) by using an average of 5.5 mol of oligo(dT)12-18 per mol of poly(A). MS2 RNA and its corresponding oligonucleotide primer (see below) and poly(rI) and poly(dC)12-18 were annealed as described above, except that the temperature was 60°C and the incubation time was 15 min.

Preparation of activated calf thymus DNA. Activated calf thymus DNA was prepared essentially as described previously (2). It was then dialyzed against 0.01 M KCl and stored at −20°C until it was required.

Determination of nucleotide concentrations. The concentrations of stock solutions of nucleotides were determined from their absorptions at λmax by using the values for molar absorption coefficients given by Dawson et al. (8). The concentrations of the 2′-deoxy-3′-thiacytidine 5′-triphosphates were determined at pH 7.0 by using an ε297 value of 9.1 × 103 liter mol−1 cm−1. This is the value given previously (8) for dCTP and is very close to the value (9.7 × 103 liter mol−1 cm−1) for thymidine.

Enzyme assays. (i) MS2 RNA and oligonucleotide primer assay. The MS2 RNA and oligonucleotide primer assays contained 100 mM Tris-HCl (pH 8.0), 2.5 mM MgCl2, 50 mM KCl, 5 mM dithiothreitol, 2.5 to 25 μM dCTP, 2.5 to 30 μM dGTP, 2.5 to 25 μM dATP, [3H]dCTP (20 to 40 μCi ml−1), 50 μg of MS2 RNA ml−1, and 100 nM primer. A total of 20 μl of the substrate mixture was preincubated at 37°C for 30 min before the addition of 10 μl (234 ng) of HIV-1 reverse transcriptase solution. After incubation at 37°C for a further 15 to 30 min, during which time the rate of incorporation of dCTP was linear with time, reactions were terminated by the addition of 25 μl of 100 mM sodium EDTA. A total of 20 μl was then spotted onto a DEAE filter mat; and the paper was washed three times for 10 min each time with 5% (wt/vol) Na2HPO4, two times for 5 min each time with water, and finally with methylated spirit. The filter mat was dried in a current of warm air, and radioactivity was counted in an LKB (Milton Keynes, United Kingdom) beta-plate scintillation counter according to the manufacturer’s directions.

(ii) Poly(rA) · poly(dT) assay. The poly(rA) · poly(dT) assays were carried out as described above, except that they contained 30 mM Tris-HCl (pH 8.0), 8 mM MgCl2, 60 mM KCl, 10 mM dithiothreitol, 2.5 to 30 μM dTTP, [3H]dTTP (20 to 40 μCi ml−1), and 50 μg of the poly(rA) · poly(dT) template and primer ml−1.

(iii) Poly(rI) · poly(dC) assay. The poly(rI) · poly(dC) assays were carried out as described above, except that they contained 30 mM Tris-HCl (pH 8.0), 1.0 mM MgCl2, 60 mM KCl, 2.5 mM dithiothreitol, 2.5 to 25 μM dCTP, [3H]dCTP (20 to 40 μCi ml−1), and 40 μg of the annealed poly(dC) · poly(rI) template and primer.

(iv) DNA-dependent DNA polymerase activity of reverse transcriptase. Assays for the DNA-dependent DNA polymerase activity of reverse transcriptase were performed at 37°C in 0.2 ml of 75 mM Tris-HCl buffer (pH 7.5) containing 6.5 mM MgCl2, 83 μM dATP, 83 μM dGTP, 83 μM dTTP, 1.67 mM 2-mercaptoethanol, 0.01 M MgCl2, 50 mM KCl, and [3H]dCTP and a potential inhibitor, as appropriate. When the effect of AZT 5′-triphosphate was investigated, dTTP was the radiolabeled nucleotide. When the effect of ddATP was investigated, ddATP was the radiolabeled nucleotide, with the other ddNTPs each present at 83 μM. Samples of 25 μl (generally six samples per assay) were removed at intervals over the first 20 to 30 min of the reaction and were spotted onto a DEAE filter mat that was previously soaked in 0.1 M tetrasodium EDTA and air dried. The filter mat was washed, and the radioactivity was counted as described above.

(v) DNA polymerase gamma. During its purification, DNA polymerase gamma was assayed in 50 mM Tris-HCl buffer (pH 8.5) containing 0.5 mg of bovine serum albumin ml−1, 50 mM KCl, 0.5 mM dithiothreitol or 1.67 mM 2-mercaptoethanol, 0.5 mM MnCl2 or 6.5 mM MgCl2, 0.1 mg of poly(rA) · poly(dT)12-18 ml−1, and 1 μM dTTP (containing 1 μCi of [3H]dCTP per ml) ml−1. Assay solutions, which contained 10 μl of enzyme solution, were incubated at 37°C for 45 min, and then 25-μl samples were removed and spotted onto a DEAE filter mat that was previously soaked in 0.1 M tetrasodium EDTA and air dried. The filter mat was washed, and the radioactivity was counted as described above.

For most of the detailed kinetic studies with DNA polymerase gamma, assays were carried out at 37°C in 75 mM
Tris-HCl buffer (pH 7.5) containing 6.5 mM MgCl₂, 83 µM dATP, 83 µM dGTP, 83 µM dTTP, 1.67 mM 2-mercaptoethanol, 0.011 mg of activated calf thymus DNA ml⁻¹, 0.42 mg of bovine serum albumin ml⁻¹, 50 mM KCl, [³H]dCTP, and potential inhibitor as appropriate; however, when the effect of ddATP radiolabeled potential activity was lost. The DNA polymerase activity was total separated on residual DNA polymerase alpha and was almost totally inhibited by 150 mM KCl. This behavior was expected for DNA polymerase alpha (23). The DNA polymerase alpha activity was also inhibited by the anti-polymerase alpha antibody SJK-287-38 obtained from CRL 164 cells in an antibody concentration-dependent manner. It has been reported (22) that this antibody is specific for DNA polymerase alpha and does not detectably interact with DNA polymerase beta or gamma.

DNA polymerase beta activity was not retained by the Q-Sepharose column under the conditions described above. Non-Q-Sepharose-bound DNA polymerase activity was further purified by chromatography on P11 cellulose phosphate, and the DNA polymerase beta activity, which is resistant to 5 mM N-ethylmaleimide (see for example, reference 17) and which was eluted from the column by approximately 0.7 M KCl, was further purified by chromatography on single-stranded DNA agarose. All DNA polymerases were stored at −70°C until use.

**Kinetic studies.** DNA polymerases alpha, beta, and gamma were initially characterized by determination of their Kₘ values for dNTPs by using activated calf thymus DNA as the template and primer and the methods described above. The initial rates of reaction were determined as the slopes of the linear portions of counts per minute-against-time plots. Kₘ values for dNTPs were determined from plots of the dNTP concentration/initial rate of reaction against the dNTP concentration.

IC₅₀s were measured at dNTP concentrations that were equal to the Kₘ values. They were normally determined from plots of inhibitor concentration/fraction inhibition against the inhibitor concentration, which were linear over the inhibitor concentration ranges used. The exception to this was the analysis of the data for 3TC 5'-triphosphate with DNA polymerase alpha. As discussed below, such plots were not appropriate for the analysis of these data. IC₅₀ for 3TC 5'-triphosphate with DNA polymerase alpha were, therefore, estimated from the apparently linear portions of plots of the reciprocal of the rate of reaction against the inhibitor concentration. For more detailed kinetic analyses, double-reciprocal plots were constructed for several inhibitor concentrations. Values of Kᵢ were determined from secondary plots of slope against inhibitor concentration. Graphs were generated by using RS1 software (BBN Software Products Corporation, Cambridge, Mass.).

**RESULTS**

MS2 RNA and oligoprimer assay. The use of the MS2 RNA assay system enabled us to undertake a direct comparison of the inhibitory effects of all 5'-triphosphates used in this study. Reaction conditions were optimized for this template from this column was almost identical to that reported previously (14). This observation, together with the preparations' ability to use poly(rA) · poly(dT)₁₂₋₁₈ as template and primer in the presence of 50 mM potassium phosphate buffer (pH 7.5) and Kₘ values for dNTPs (see below), was used as evidence that the DNA polymerase activity was that of a gamma polymerase.

DNA polymerase alpha was purified in a manner similar to that described above for DNA polymerase gamma. DNA polymerase alpha-containing fractions from the Q-Sepharose column were further purified on double-stranded DNA cellulose (as above). DNA polymerase alpha activity eluted well separated from residual DNA polymerase gamma activity at a phosphate buffer concentration of about 160 mM. The polymerase alpha activity was 50% inhibited by about 80 mM KCl and was almost totally inhibited by 150 mM KCl. This behavior was expected for DNA polymerase alpha (23). The DNA polymerase alpha activity was also inhibited by the anti-polymerase alpha antibody SJK-287-38 obtained from CRL 164 cells in an antibody concentration-dependent manner. It has been reported (22) that this antibody is specific for DNA polymerase alpha and does not detectably interact with DNA polymerase beta or gamma.

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**RESULTS**

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TABLE 1. Apparent inhibition constants of HIV-1 reverse transcriptase

<table>
<thead>
<tr>
<th>Compound</th>
<th>$K_i$ (µM) for HIV-1 reverse transcriptase:</th>
<th>IC$_{50}$ (µM) for HIV-1 reverse transcriptase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Poly(rA) - poly(dT) assay</td>
<td>MS2 RNA assay</td>
</tr>
<tr>
<td>Racemic 2'-deoxy-3'-thiacytidine 5'-triphosphate</td>
<td>NT</td>
<td>7.4 ± 3.9</td>
</tr>
<tr>
<td>(-) 2'-deoxy-3'-thiacytidine 5'-triphosphate (3TC 5'-triphosphate)</td>
<td>NT</td>
<td>12.4 ± 5.1</td>
</tr>
<tr>
<td>(+) 2'-deoxy-3'-thiacytidine 5'-triphosphate</td>
<td>NT</td>
<td>1.9 ± 0.6</td>
</tr>
<tr>
<td>ddCTP</td>
<td>NT</td>
<td>0.33 ± 0.02</td>
</tr>
<tr>
<td>AZT 5'-triphosphate</td>
<td>0.017 ± 0.001</td>
<td>0.03 ± 0.02</td>
</tr>
<tr>
<td>ddATP</td>
<td>NT</td>
<td>0.09 ± 0.01</td>
</tr>
</tbody>
</table>

a In all cases, inhibition was competitive with respect to the various dNTPs. Values are means ± standard deviations.
b NT, not tested.

and primer, and kinetic constants were determined. The $K_m$ values determined for the 5'-triphosphates of 3TC, the corresponding racemate (BCH 189), and the (+)-enantiomer of 3TC are given in Table 1. In each case, the observed inhibition was competitive with respect to the varied dNTPs; and the $K_m$ values obtained for ddCTP, ddATP, and AZT 5'-triphosphate are similar to those reported previously (12, 15, 20).

Homopolymer template and primer assays. The preferred template and primer system for the analysis of 3TC and ddC 5'-triphosphates, poly(rG) · poly(dC), was not used at all effectively by HIV-1 reverse transcriptase and therefore could not be used in inhibition studies. However, a poly(rI) · poly(dC) template primer was an effective substrate for the enzyme, and $K_m$ values are given in Table 1. Studies on the effect of AZT 5'-triphosphate on HIV-1 reverse transcriptase were done by using poly(rA) · poly(dT) as template and primer (Table 1).

DNA-dependent DNA polymerase activity. The IC$_{50}$s determined for the 5'-triphosphates of 3TC and the corresponding racemate (BCH 189), ddATP, ddCTP, and AZT 5'-triphosphate, using the appropriate radiolabeled dNTP at a concentration equal to the $K_m$, the other three dNTPs at concentrations of >10 times the $K_m$, and activated calf thymus DNA as the template and primer (Table 1). With the exception of AZT 5'-triphosphate (which, by an order of magnitude, was a less effective inhibitor of the DNA-dependent DNA polymerase activity than of the RNA-dependent DNA polymerase activity), the IC$_{50}$s were not very different from the $K_m$ values determined for these compounds when inhibiting the RNA-dependent DNA polymerase activity of the same enzyme.

Mammalian DNA polymerases. DNA polymerases alpha, beta, and gamma were first characterized by determination of their $K_m$ values for dNTPs (Table 2), which were within the ranges reported previously (10, 23, 24). IC$_{50}$s were then determined for 3TC 5'-triphosphate, its racemic form (BCH 189 5'-triphosphate), the 5'-triphosphate of the correspondingly-keyed enantiomer, ddATP, ddCTP, and AZT 5'-triphosphate by using the appropriate radiolabeled dNTP at a concentration equal to the $K_m$, the other three dNTPs at concentrations of >10 times the $K_m$, and activated calf thymus DNA as the template and primer (Table 2). More detailed kinetic studies were consistent with the inhibition of DNA polymerases gamma and beta by 3TC 5'-triphosphate being competitive with respect to dCTP. The $K_m$ values determined were 15.8 ± 0.8 µM for DNA polymerase gamma and 18.7 µM for DNA polymerase beta; they are consistent with the IC$_{50}$s given in Table 2.

Chain termination studies. The ability of 3TC 5'-triphosphate to serve as a substrate for HIV-1 reverse transcriptase was examined with primed MS2 RNA as the template and primer (Fig. 2). Comparison of the primer extension products observed in the presence of ddCTP (Fig. 2, lanes C) and 3TC 5'-triphosphate (Fig. 2, lane B(-)) shows that the two triphosphates were incorporated and that chain termination by the two triphosphates occurs in apparently identical ways.

DISCUSSION

The competitive nature (with respect to dCTP) of the inhibition of HIV-1 reverse transcriptase by 3TC 5'-triphosphate and the chain termination that it brings about when it is incorporated into the site of dCTP indicate that the mode of action of 3TC as an antiviral agent may be similar to that proposed for ddC (19). However, the observation that both 3TC 5'-triphosphate and the corresponding (+)-enantiomer inhibit HIV-1 reverse transcriptase is unusual. The (+)-enantiomer, which has a conformation typical of those of

TABLE 2. Effects of 3TC and related nucleoside 5'-triphosphates on mammalian DNA polymerases

<table>
<thead>
<tr>
<th>Enzyme$^a$</th>
<th>IC$_{50}$ (µM) at [dNTP] = $K_m$</th>
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<tbody>
<tr>
<td>(-)-Enantiomer</td>
<td>Racemate</td>
</tr>
<tr>
<td>(3TC 5'-triphosphate)</td>
<td>(BCH 189 5'-triphosphate)</td>
</tr>
<tr>
<td>DNA polymerase alpha</td>
<td>175 ± 31</td>
</tr>
<tr>
<td>DNA polymerase gamma</td>
<td>43.8 ± 16.4</td>
</tr>
<tr>
<td>DNA polymerase beta</td>
<td>24.8 ± 10.9</td>
</tr>
</tbody>
</table>

$^a$ For DNA polymerase alpha, $K_m$ values for dATP, dCTP, and dTTP were 2.7, 3.5, and 3.4 µM, respectively: the respective values for DNA polymerase gamma were 0.13, 0.20, and 0.15 µM; and those for DNA polymerase beta were 2.5, 1.1, and 1.7 µM.
The level of inhibition of HIV-1 reverse transcriptase by 3TC 5'-triphosphate, although weaker than that observed for ddCPT and AZT 5'-triphosphate, could be consistent with the hypothesis that the antiviral activity of 3TC is mediated at least in part by inhibition of reverse transcriptase when observations on phosphorylation of the nucleoside analog in human peripheral blood lymphocytes and the antiviral activity of 3TC in infected peripheral blood lymphocytes (6) are considered. Levels of 3TC 5'-triphosphate in cells treated with 10 μM 3TC reach 2 to 14 pmol/10⁶ cells (3). This is comparable to the levels reported for ddCPT (19) and is approximately 10 times that reported for AZT 5'-triphosphate (11). The half-life of 3TC 5'-triphosphate (10 to 15 h [3]) is also longer than those reported for ddCPT (2.6 h) and AZT 5'-triphosphate (1 h). The higher levels and longer half-life of 3TC 5'-triphosphate could compensate for the weaker inhibition of reverse transcriptase compared with those of AZT 5'-triphosphate and ddCPT. The half-life of the (+)-enantiomer of 2'-deoxy-3'-thiacytidine 5'-triphosphate is shorter than that of 3TC 5'-triphosphate (3), which may account for the observation that the (+)-enantiomeric 5'-triphosphate is about a sixfold better inhibitor of HIV-1 reverse transcriptase than 3TC 5'-triphosphate is (Table 1), even though it is only about half as effective against the virus in vitro (7).

All of the 5'-triphosphates tested in the study described here were weak inhibitors of DNA polymerase alpha (Table 2). The behavior of 3TC 5'-triphosphate with DNA polymerase alpha needs more comment, however. Plots of inhibitor concentration/fraction inhibition against the inhibitor concentration, which were good straight lines with the racemic form and the (+)-enantiomeric form with DNA polymerase alpha and with all inhibitory compounds tested with DNA polymerases beta and gamma, showed no discernible trends in the dependence of the ratio inhibitor concentration/fraction inhibition on the inhibitor concentration. Plots of the reciprocal of the initial rate against the inhibitor concentration were normally curved, with the inhibition at high concentrations of 3TC 5'-triphosphate being greater than that expected from the degree of inhibition at low concentrations. However, at concentrations of 3TC 5'-triphosphate below 120 to 180 μM, the plots closely approximated straight lines. IC₅₀₅ were estimated from these essentially linear portions of the plots.

The inhibition of DNA polymerase alpha by 3TC 5'-triphosphate is complex. Expression of the data in the form of Hill plots shows that, for fraction inhibitions of between 0.1 and 0.9, the plots are linear, with slopes of between 1.86 and 2.17. There would therefore seem to be at least two different types of binding site for 3TC 5'-triphosphate on DNA polymerase alpha. Such complex binding was not apparent, though, for the racemate or the (+)-enantiomer.

The inhibition of DNA polymerase gamma by the 5'-triphosphates used in this study is very different from that found with DNA polymerase alpha. 3TC 5'-triphosphate is a rather weak inhibitor of this enzyme (average IC₅₀, 43.8 μM), but the racemic form is about 650 times more inhibitory and the (+)-enantiomer is even more effective. Comparison of the IC₅₀₅ (Table 2) shows that the effective inhibition of DNA polymerase gamma by the racemate triphosphate is almost entirely due to the (+)-enantiomer. Inhibition of DNA polymerase gamma such as that seen with the racemate and the (+)-enantiomer could lead to considerable cellular toxicity. Cellular toxicity was, in fact, seen when CEM cells were incubated with the corresponding nucleosides (6), although this toxicity need not necessarily be due...
or be solely due to inhibition of DNA polymerase gamma. The (−)-enantiomer was significantly less toxic to the cells than was either the racemate or the (+)-enantiomer. The approximately 1,000 times more effective inhibition of DNA polymerase gamma by the (+)-enantiomer compared with that by the (−)-enantiomer is perhaps not surprising since the configuration of the (+)-enantiomer (21) is the same as that seen in the natural substrates, the dNTPs. This result is in marked contrast, however, to the results observed with HIV-1 reverse transcriptase (see above), in which the inhibition by the (+)-enantiomer was only about six times greater than that by the (−)-enantiomer, and the (−)-enantiomeric nucleoside, like the (+)-enantiomeric nucleoside, is a very effective antiviral compound in cultured HIV-1-infected cells (6).

There are important differences between the effects of 3TC 5′-triphosphate and ddATP and ddCTP on DNA polymerase gamma. The dNTPs are nearly 1,000 times more effective than 3TC 5′-triphosphate as inhibitors of this enzyme. If inhibition of DNA polymerase gamma is an event that results in cellular toxicity, then 3TC might be expected to be significantly less toxic than ddC or ddi. ddi has been shown to result in intracellular conversion to ddATP in order to be active against HIV-1 (1, 13).

The results obtained with DNA polymerase beta were qualitatively very similar to those obtained with DNA polymerase gamma. 3TC 5′-triphosphate is not a potent inhibitor of DNA polymerase beta (average IC50, 24.8 μM), whereas the racemic form is significantly more inhibitory. ddATP and ddCTP are also significantly more inhibitory to DNA polymerase beta than is 3TC 5′-triphosphate, although AZT 5′-triphosphate is a poor inhibitor of this enzyme.

The results presented above (Tables 1 and 2) seem to be unable to explain two important aspects of the inhibition of HIV-1 by 3TC in cells cultured in vitro. First, the Ks values for 3TC 5′-triphosphate are several orders of magnitude greater than the 50% inhibitory doses of 3TC for the virus; i.e., the nucleoside is more effective against the virus than the 5′-triphosphate appears to be against the viral reverse transcriptase. However, an absolute correlation between enzyme inhibition in vitro and the effects in cells need not be expected, since there are many conditions that differ between the assays described here and the conditions in which HIV-1 reverse transcriptase functions in infected cells. The templates and primers used in the study of the effect of 3TC 5′-triphosphate on the DNA polymerase activity of reverse transcriptase, i.e., primed MS2 RNA, activated DNA, and poly[rI]· poly[dC], are, by necessity, artificial and different from those that the enzyme will encounter in HIV-1-infected cells. Different templates and primers will give rise to binary enzyme-template and primer complexes whose interactions with a particular ligand might result in significantly different Ks values. Such an explanation for the observed results with 3TC and its 5′-triphosphate cannot be ruled out, although there is little difference in the inhibition of HIV-1 reverse transcriptase by 3TC 5′-triphosphate in the presence of these different templates.

The second consideration concerns the specificity of 3TC and its 5′-triphosphate. In vitro studies (6) showed that 3TC is a very effective anti-HIV-1 compound, but it shows little toxicity against mammalian cells. Comparison of the Ks values for 3TC 5′-triphosphate against HIV-1 reverse transcriptase with the values obtained for DNA polymerases beta and gamma suggests that 3TC might be as toxic to mammalian cells as it is to HIV-1. This clearly is not the case (6). The two apparent difficulties are probably reconcilable when the results of the chain termination studies (Fig. 2) are considered. These results show that 3TC 5′-monophosphate units are effectively incorporated into the growing oligodeoxyribonucleotide chain in place of dCMP, and the chain terminates. HIV-1 reverse transcriptase has not been shown to have exonuclease activity against deoxyribonucleotides, and once chain termination has occurred, that polynucleotide molecule is of no further use for viral replication. The rate of incorporation of 3TC 5′-monophosphate units into oligodeoxyribonucleotide chains has not yet been determined, but because only one such event is needed within a particular polynucleotide chain, the effectiveness of 3TC 5′-triphosphate can be much more pronounced than the Ks values determined from initial rate kinetics suggest.

Chain termination studies with 3TC 5′-triphosphate and DNA polymerases beta and gamma have not yet been carried out, but even if both of these enzymes are able to incorporate 3TC 5′-monophosphate into DNA, DNA proofreading and repair enzymes may remove the unusual nucleotide. Efficient proofreading of the HIV-1 genome has not yet been demonstrated. Thus, the potency of 3TC against HIV-1 is probably more due to the chain-terminating property of the 5′-triphosphate than to competitive inhibition, and the selectivity is probably due to the lack of exonuclease activity within or associated with HIV-1 reverse transcriptase. Work is in progress to try to obtain evidence for these hypotheses.

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